Investigation into the Novel Application & Utilisation of Algae to Aid in Developing Sustainable Irish Freshwater Aquaculture

By

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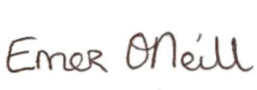
DECLARATION

I hereby declare that the work contained within this thesis, submitted to Athlone Institute of Technology for the degree of Doctorate of Philosophy, has not been accepted for the award of any other degree, in any other higher education institute, and is entirely my own work and to the best of my knowledge contains no work previously written or published by another party, except in the case of referenced material.



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Confidentiality Statement

All of the information in this thesis is confidential and shall not be disclosed to any further parties without the permission of the first author due to intellectual property constraints. Details of the information presented shall be decided upon with the members of the projects prior to public dissemination.

DEDICATION

This thesis is dedicated to the memory of my beautiful mother Dympna, who passed away during my postgraduate journey to PhD, and never got to share in the joy of this, the highlight thus far of my academic career. Thank you for always believing in me Mammy. Without you, none of this would have been possible. I know you have been by my side these past couple of years. All of this was done for you.

"*All that I am, or ever hope to be, I owe to my angel mother*." – Abraham Lincoln

"*Those we love don't go away. They walk with us every day. Unseen, unheard, but always near. Still loved, still missed and very clear*." – Author Unknown

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ABSTRACT

Aims and Rationale: Aquaculture is the fastest growing food producing industry in the world producing >50% of the world's seafood. Food Wise 2025, an initiative set by the Irish government to develop the Irish Agri-Food industry, predicted that aquaculture production has the potential to increase to 81,700 tonnes per annum by 2023. However, several issues have hampered the growth of freshwater aquaculture including licensing issues, environmental concerns and spatial limitations. With the destruction of the surrounding marine environments becoming a real threat, land-based aquaculture and freshwater aquaculture is predicted to become a more common practice across Ireland; however, increasing issues with uncertainties associated with global warming and climate variances will also play a significant factor in the future sustainable intensification of this industry. Although research is still significantly lacking, interest in algae as natural means of supporting and enabling development of aquaculture is increasing for water quality and waste mitigation. This timely and novel study investigate the role of algae in conventional pond-based aquaculture systems and is the first to report on the transitioning to a fully recirculated aquaculture process using the peatlands. Thus, the overarching aim of this timely study is to investigate algae as a means to assist in addressing current and future issues within the Irish freshwater aquaculture industry with a global orientation.

Methodology and Findings: A novel ecotoxicological toolbox, representative of Irish freshwaters, was developed to help address aquaculture licensing issues and inform the aforementioned environmental concerns. Native algae and daphnid species were compared to standardised species where this toolbox was first piloted using a traditional flow-through aquaculture system between April 2018 and October 2018. The toolbox consisted of measuring conventional physicochemical parameters currently used to assess water quality along with using two ISO standardised bioassay (*Pseudokirchneriella subcapitata* algal bioassay and *Daphnia* crustacean bioassay); thereafter, the toolbox was applied to monitoring freshwater aquaculture between March 2019 and August 2019. Findings revealed that reliance upon physicochemical analysis alone would only provide a snap shot in time of the water quality. Supplementation with algal, from perspective of real-time ecotoxicological toolkit, enables a broader determination of overall aquaculture effluent and re-circulated water quality that includes capacity for potential use as novel early warning intervention for the industry.

This study constitutes the first to investigate development of integrated multi-trophic aquaculture system (IMTA) on the peatlands as a next-generation approach to sustainable production of farmed fish along with mitigating against environmental discharge of effluent to receiving waters in the Irish Midlands. However, as this was an entirely novel IMTA concept built in protected areas of the peatlands that are conserved ecologically and for their biodiversity, this important research evaluated the efficacy of this novel process using both conventional physiochemical parameter monitoring in tandem with using this ecotoxicological toolbox of algal communities that was first applied between May 2019 and August 2019. Research findings revealed that using algae as one of its primary means of wastewater assimilation, was unlikely to cause adverse effects on the surrounding peatland. Physicochemical parameters provided a baseline of conditions best suited for algal growth. Algae and cyanobacteria communities were enumerated using microscopy and real-time flow cytometry, and were identified using microscopy and Illumina DNA sequencing. Characterisation found a vast variety of algal species present in the system with 1864 species across 210 genus identified. The majority of species present were considered beneficial or neutral; whereas, some algal/cyanobacteria species were considered as potentially hazardous where their appearance coincided with fish mortalities in the IMTA process. Fluctuations in physicochemical parameters due to increased rainfall attributed to two successive storms also coincided in fluctuations in algal numbers in this IMTA process and with increases in fish mortalities. During such instances of low nitrate (algae's preferred nutrient source),

low levels of algae were also observed. Findings revealed that this environmental flux provided an opportunity for cyanobacteria (whose preferred nutrient source is ammonium) to outcompete beneficial algae for its nitrogen nutrient source; increased cyanobacteria levels resulted in increased fish mortalities. Use of the toolkit, along with characterising and monitoring the physicochemical parameters, enabled real-time monitoring of the IMTA that included substantial variances in the system caused by uncertainty with climate (storms); thus, highlighting the potential utility of this toolbox for supporting predicting, modelling and management decision marking. This timely study also showed that despite advances to remove end-of-time solutions for treating effluent, and smart use of algae/bacteria for recirculation of waste water – climate variance can significantly impact upon next-generation sustainable approaches for freshwater aquaculture. This uncertainty due to climate change was not considered at the outset of the project, nor was the simultaneous occurrence of a COVID-19 pandemic that affected ability to take samples on site to support a battling industry, and for trouble shooting; yet this was achieved.

This timely project also addressed pressing unprecedented challenges for the freshwater aquaculture industry during periods affected by global warming or climate change. During May 2018, Ireland experienced its highest ever recorded temperature (>30^{o}C) with absence of rainfall for 16 weeks leading to drought. During this time, the algal bioassay component of this novel ecotoxicological toolbox was shown to be capable of monitoring environmental flux and can be potentially used as an early warning indicator for climate variance for the aquaculture industry. During February 2020, Ireland experienced two extratropical cyclone storms less than a week apart, increasing rainfall levels from a monthly average of 70.3mm to 197.5mm. This erratic weather was the main cause of algae and physicochemical fluctuations that subsequently led to aforementioned instances of fish mortalities within the novel peatland IMTA system. This highlighted the need for developing real-time monitoring tools that respond to rapid variances in the environment, which is a limiting factor in the gap associated with conventional physicochemical methods that are traditionally used by the industry.

Conclusions and Implications: The application and utilisation of algae has demonstrated a range of potential benefits and implications for the Irish freshwater aquaculture industry. This research has led to the potential development of a sustainable water quality control tool for fish farms, which will support and enable real-time monitoring catering for holistic environmental situations. Findings from this novel research will potentially support future proofing of the industry by providing smart tools for informing development of the industry that is pivoting towards the use of IMTA processes. The information generated and openly shared will assist in harmonising traditional and novel processing applications within the industry. This is particularly timely, as the Irish EPA are currently investigation the regulation of wastewater in aquaculture. This study also highlights potential solutions for complex challenges, including use of specific beneficial algal to offset disturbances to balanced aquaculture processes. It is envisaged that data generated through this novel project will inform future digitalisation for intensification of the industry. The findings of this project are strongly aligned with the refreshed national priority for research areas, including food, health and wellbeing.

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Chlamydomonas sp. – Green Algae
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Chlorella reinhardtii – Green Algae
Chlorella sp. – Green Algae
Chlorella vulagris – Green Algae
Chloroccum sp. – Green Algae
Closterium sp. – Green Algae
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Cyclotella sp. – Diatom
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Parietochloris sp. – Green Algae
Pediastrum sp. – Green Algae
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Oreochromis niloticus – Nile Tilapia

Penaeus merguiensis – Banana Shrimp

Perca fluviatilis – European Perch

Salmo salar – Atlantic Salmon

Salmo trutta – Brown Trout

LIST OF ABBREVIATIONS

3N-BBM+V Triple Nitrate Bold's Basal Medium with Vitamins
AE Alkyl Ethoxylates
AIT Athlone Institute of Technology
APHA American Public Health Association
ASV Amplicon Sequence Variant
B Billion
BIM Bord Iascaigh Mhara
BLAST Basic Local Alignment Search Tool
BOD Biochemical Oxygen Demand
bp Base Pairs
CCAP Culture Collection of Algae and Protozoa
CFP Common Fisheries Policy
COD Chemical Oxygen Demand
DAFM Department of Agriculture, Food and the Marine
ddNTP Dideoxynucleotide Triphosphate
DM Diatom Medium
DNA Deoxyribonucleic Acid
dNTP Deoxynucleotide Triphosphate
DO Dissolved Oxygen
DS Dissolved Solids
dsDNA Double Stranded Deoxyribonucleic Acid
EAS Extensive Aquaculture System
EC European Commission
EDTA Ethylene Diamine Tetra-acetic Acid
EEC European Economic Community
EIA Environmental Impact Assessment
EIAR Environmental Impact Assessment Report
EIS Environmental Impact Statement
EPA Environmental Protection Agency
EU European Union
FACS Fluorescence Activated Cell Sorting
FAO Food and Agriculture Organisation of the United Nations
FCM Flow Cytometry
FCR Food Conversion Ratio
FGS First Generation Sequencing
FSC Forward Scatter
FTS Flow Through System
HAB Harmful Algal Bloom
HDPE High Density Polyethylene
HGP Human Genome Project
HIAC High Accuracy
IAS Intensive Aquaculture System
IMTA Integrated Multitrophic Aquaculture
ISE Ion Selective Electrode
ISO International Organisation for Standardisation
JM Jarworski's Medium
LAS Alkyl Benzene Sulfonates
LCF Life Cycle Assessment
LPS Lipopolysaccharide
M Million
MAC Maximum Allowable Concentration
N North
NBDC National Biodiversity Data Centre
NCBI National Centre for Biotechnology Information
NEPA National Environment Policy Act
NGS Next Generation Sequencing
NHA National Heritage Area
NIS Natura Impact Statement

NOAA National Oceanic and Atmospheric Administration

NPWS National Parks and Wildlife Services

NUIG National University of Ireland, Galway

PBS Phosphate Saline Buffer

PCR Polymerase Chain Reaction

PUFA Polyunsaturated Fatty Acid

RAS Recirculating Aquaculture System

SAC Special Area of Conservation

SAS Semi-intensive Aquaculture System

SCI Site of Community Importance

SEA Strategic Environmental Assessment

SI Statutory Instrument

SPA Special Protection Area

SS Suspended Solids

SSC Side Scatter

ssDNA Single Stranded Deoxyribonucleic Acid

SWOT Strength, Weakness, Opportunity, Threat

UK United Kingdom

USD United States Dollar

UV Ultraviolet

W West

WFD Water Framework Directive

WHO World Health Organisation

UNITS OF MEASUREMENT

%	percent
µL	microlitre
µm	micrometre
µS cm^{-1}	microsiemens per centimetre
CI	confidence interval
C_n	carbon chain length
E_rC_{50}	effective concentration the reduces 50% of the growth rate
ft	foot
G	gravitational force
g	grams
g L^{-1}	grams per litre
h	hour
ha	hectare
IC_{50}	concentration that causes inhibition in 50% of the population
K	thousand
kg	kilogram
kg/m^3	kilogram per metre cubed
L	litre
lb	pound
lux	illuminance
M	molar
mM	millimolar
mg L^{-1}	milligrams per litre
min	minute
mL	millilitre
mL^{-1}	per millilitre
mm	millimetre
mm^2	millimetre squared
mV	millivolt
N	normality
n	number of replicates
nm	wavelength of maximum absorbance
^oC	degrees Celsius
p	calculated probability
psi	pounds per square inch
r	correlation coefficient
R^2	coefficient of determination
s	second
SD	standard deviation
v/v	volume per volume
X	magnification

$(NH_4)_6Mo_7O_{24}$	Ammonium Molybdate
3,5-DCP	3,5-Dichlorophenol
Ag_2SO_4	Silver Sulphate
$C_{12}H_{16}Cl_2N_2$	N-(1-Naphthyl) Ethylenediamine Dihydrochloride
$C_2H_3NaO_2$	Sodium Acetate
$C_6H_{12}O_6$	Glucose
C_6H_5OH	Phenol
$Ca(NO_3)_2$	Calcium Nitrate
$CaCl_2$	Calcium Chloride
$CaCO_3$	Calcium Carbonate
CO_2	Carbon Dioxide
$CoCl_2$	Cobalt Chloride
dH_2O	Distilled Water
EDTA	Ethylenediaminetetraacetic Acid
$FeCl_2$	Iron Chloride
$H_2NSO_2NH_2$	Sulphanilamide
H_2SO_4	Sulphuric Acid
H_3BO_3	Boric Acid
HCl	Hydrochloric Acid
$HgSO_4$	Mercuric Sulphate
$K_2Cr_2O_7$	Potassium Dichromate
K_2HPO_4	Dipotassium Phosphate
KH_2PO_4	Monopotassium Phosphate
$MgSO_4$	Magnesium Sulphate
$MnCl_2$	Manganese Chloride
N	Nitrogen
Na Fe EDTA	Ferric Sodium Ethylenediaminetetraacetic Acid
Na_2 EDTA	Disodium Ethylenediaminetetraacetic Acid
$Na_2[Fe(CN)_5NO]$	Sodium Nitroprusside
$Na_2B_4O_7$	Sodium Tetraborate
Na_2HPO_4	Disodium Phosphate
Na_2MoO_4	Sodium Molybdate
$Na_2S_2O_3$	Sodium Thiosulphate
Na_2SiO_3	Sodium Silicate
Na_2SO_4	Sodium Sulphate
$Na_3C_6H_5O_7$	Tri Sodium Citrate
$NaCl_2$	Sodium Chloride
$NaHCO_3$	Sodium Hydrogen Carbonate
NaN_3	Sodium Azide
$NaNO_3$	Sodium Nitrate
NaOCl	Sodium Hypochloride
NaOH	Sodium Hydroxide
NH_4^+	Ammonium
NH_4Cl	Ammonium Chloride
NH_4VO_3	Ammonium Metavanadate
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2	Oxygen
P	Phosphorus
PO_4^{3-}	Orthophosphate
$ZnCl_2$	Zinc Chloride
$ZnSO_4$	Zinc Sulphate

PROJECT BACKGROUND

This research was conducted in collaboration with two complimentary research groups aimed at addressing critical issues in the Irish aquaculture industry as highlighted by aquaculture industry stakeholders, commercial operators and policy makers.

EcoAqua was a multi-disciplinary aquaculture project led by Athlone Institute of Technology (AIT) and the National University of Ireland, Galway (NUIG) in conjunction with Bord Iascaigh Mhara (BIM) and the Department of Agriculture, Food and the Marine (DAFM) that ran from 2017 to 2019. The project was set up to improve management and production efficiency of farmed fish. The project outputs included providing new information, new methods and increased research awareness focused on fish health and the environment. **Within the EcoAqua project, my novel contribution included the analysis of the physicochemical and ecotoxicological characteristics of the water entering and exiting the fish farm in order to development an ecotoxicological toolbox specifically aligned with Irish freshwater ecosystems. This included investigating the relationship between the use of naturally occurring microalgae and climate variance from a monitoring and impact perspective.**

After the successful application of the toolbox to determine the potential use of peatland bogs for aquaculture and subsequent publication of the first ever pilot study on the same, further research into aquaculture and the role of microalgae in freshwater aquaculture on peatland bogs was conducted. AquaAlgaePlus was a multi-disciplinary aquaculture project led by AIT, in conjunction with BIM and Bord na Mona that ran from 2019 to 2021. The project was designed to improve management and production efficiency of farmed fish in a first of its kind, peatland based integrated multi trophic

aquaculture (IMTA) process. The main aim of this research project was to develop a comprehensive understanding of the role of algae in freshwater aquaculture so as to specifically inform and guide the development of the novel innovative peatland IMTA process. **Within the AquaAlgaePlus project, my novel and timely work included the analysis of physicochemical characteristics of the novel farm process alongside the enumeration and identification of algae and cyanobacteria communities in order to assist in better understanding the role of the algae within the novel peatland IMTA process.**

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND TO RESEARCH

Aquaculture is one of the fastest growing food producing industries in the world (Fečkaninová *et al.*, 2017; Liu *et al.*, 2017). Food security is becoming more and more essential (FSAI, 2019). Aquaculture provides an important means of food security both directly as a source of food production and indirectly by providing employment opportunities (Lehane, 2013). According to the United Nations (UN) aquaculture now provides fish availability to countries and regions that would have previously been limited or non-existent, often at cheap prices, thus providing improved nutrition and food security (Fish Farming Expert, 2020). The Irish aquaculture industry was worth €173.5M (Million), producing just over 38,238 tonnes of fish in 2019 (Dennis *et al*., 2020); this equated to a decrease of 19% volume and 13.5% value since 2017 (BIM, 2018a; Dennis *et al.*, 2020). Food Wise 2025 is a strategy developed by the Department of Agriculture, Food and the Marine (DAFM) for the Irish agri-food sector. This ten-year plan sets out and underlines the sectors position in the Irish economy. It also illustrates the potential for expansion within the sector (DAFM, 2015a). Food Wise 2025 predicts that the Irish agri-food sector has the potential to increase exports to €19B (Billion), per annum by 2025. As part of this prediction, it proposes that Irish aquaculture industry, or more specifically, aquaculture production should be increased to 81,700 tonnes by 2023 in order to assist in meeting this goal (DAFM, 2015a). However, issues with the Irish aquaculture licensing process, associated with the adoption of European Union (EU) environmental protection directives resulting in space limitations have hampered the growth and the development of the industry (Moylan *et al.*, 2017).

There is an increasing interest in exploiting low-cost environmental-friendly 'natural' processes in aquaculture (Han *et al*., 2019). For example, the aforementioned aquaculture issues have led to an increased research focus on developing integrated multitrophic aquaculture systems or IMTA (Granada *et al*., 2016) along with eco-innovation and monitoring of traditional processes (Tahar *et al.*, 2018a, 2018b; Rowan, 2019). Advances in aquaculture must also be balanced with the need to meet commitments as set out by the Water Framework Directive (WFD), which aims to achieve good water status in all waters across all EU member countries (Voulvoulis *et al*., 2017; WFD Ireland, 2018a). As part of Ireland's Strategic Plan for Sustainable Aquaculture Development, and in addition to their research into further sustainable development of traditional aquaculture processes such as flow-through systems (FTS), Bord Iascaigh Mhara (BIM) undertook a feasibility study to assess the novel use

of peatlands for aquaculture diversification (DAFM, 2015b). Bord Na Móna, a state company that was originally developed to establish Irish peat resources for economic benefit, owns or controls approximately 80,000 ha of bog. The urgent threat of climate change, in addition to some of these peatlands now being listed as important habitats under the EU's Birds and Habitats Directives due to their scarcity, have resulted in dramatic changes in the peat industry including conversion of peatland-usage to wind energy, forestry, biodiversity, amenity and waste management (Bord na Móna, 2019; Irish Peatland Conservation Council, 2019; O'Neill *et al*., 2019; Toner, 2018; Ward *et al*., 2019). Recently Bord Na Mona, in conjunction with BIM, has further expanded use of these cutaway bogs to develop Ireland's first IMTA system adhering to organic principles. This IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*Lemna minor – L. minor*) and gibbous duckweed (*Lemna gibba – L. gibba*) and exploits use of microalgae for waste removal (Bord na Móna, 2019). This IMTA process differs from traditional aquaculture practices that use water from rivers and lakes where the latter traditional systems must consider potential pollutants from agricultural runoff, industry, waste-water treatments plants, *etc*. (Rowan, 2011; Tahar *et al.*, 2017; Tiedeken *et al.*, 2017; Tahar *et al.*, 2018).

It is only in recent years that studies have been conducted, confirming the potential beneficial roles of microalgae in aquaculture (Ansari *et al.*, 2017; Gao *et al*., 2016; Han *et al*., 2019). Microalgae could efficiently assimilate nutrients providing a good method for wastewater remediation (Han *et al.*, 2019; Leng *et al*., 2018; J. Wang *et al.*, 2015) in aquaculture, having already demonstrated promising performances in the food and agriculture industries, and in municipal wastewater treatment (De-Bashan *et al.*, 2004; Han *et al.*, 2019; Lu *et al*., 2017, 2015). Microalgae synthesise high value compounds *e.g.*, proteins, lipids and pigments (Han *et al*., 2019). Studies conducted by Ansari *et al.* (2017), Lu *et al.* (2017) and Sirakov *et al.* (2015) have also demonstrated the application of various microalgal species for the production of biomass which could be exploited as a partial feed replacement and to enhance aquatic animal immunity. Due to the potential of these benefits, the use of microalgae in aquaculture has recently emerged into the forefront. However, the role of algae in aquaculture is still lacking, where there are clear gaps in knowledge to be informed by research (Han *et al*., 2019).

1.2. Structure of Thesis

This, **chapter one**, will highlight the main topics and background information for this research. The research aims, scope and research objectives have been indicated. Additionally, the justification of the

research and the study areas involved have been articulated. A list of the contributions to the existing state of the art knowledge have also been addressed. **Chapter two** critiques the available literature reviewed for this research, which includes an in-depth insight into the historical context of aquaculture and algae, and algae's current application to aquaculture. Additionally, the chapter highlights the strengths, weaknesses, opportunities and threats (SWOT) arising from knowledge gaps and how addressing the same will contribute to advancing Irish freshwater aquaculture. **Chapter three** focuses on addressing current environmental concerns and licensing issues in the Irish freshwater aquaculture industry. Research includes the development of an ecotoxicological toolbox to assess the impact aquaculture output water may have on the receiving freshwater ecosystem, thus informing on the numerous issues associated with the environmental concerns linked to aquaculture. **Chapter four** focuses on addressing space and resource limitation issues in the Irish freshwater aquaculture industry by investigating 1) the use of peatlands as viable locations for aquaculture practices in the future and 2) the novel aquaculture process applied to the first of its kind, peatland aquaculture facility. **Chapter five** aims at addressing future issues in the Irish aquaculture industry highlighted by this research, most notable 1) issues potentially associated with climate change variances and 2) the need to ensure *in-situ* technology is providing reliable information when access to wet labs may be limited due to unavoidable circumstances (*e.g.,* COVID-19). **Chapter six** provides an overall discussion and conclusion to the research carried out and reflects on the implication of this research, indicating any recommendations for its application to the Irish freshwater aquaculture industry. For the development of aquaculture, future implications arising from conducting this research are articulated from a technological and sustainable perspective.

1.3. Research Aims

The main overall aim of this research was to determine the potential beneficial uses and roles of algae to aid in addressing current and future issues in the Irish freshwater aquaculture industry. In summary, the primary aims of this research were;

1. To develop a comprehensive understanding of traditional and novel aquaculture processes in Ireland.
2. To develop a comprehensive understanding of the role algae and their current uses in aquaculture, specifically as it is related to the first novel IMTA process in Irish peatlands.
3. To investigate the beneficial applications of algae in aquaculture to help combat issues within the Irish aquaculture industry, such as environmental concerns, limited space and resources, the threat

of climate change variances, and the uncertainty and flux caused by the global pandemic (COVID-19) that included several extended periods of lockdown.

1.4. Scope & Research Objectives

The Irish agri-food industry, which includes the aquaculture sector, is the oldest indigenous industry in Ireland, playing a vital part in the country's economy. However, issues within the sector (*e.g.*, environmental concerns and climate change variances) require research and development to allow the industry to expand to its potential in a sustainable manner, thus meeting with governmental commitments to expand the industry whilst meeting and adhering to European environmental protection directives. To assist in meeting this, this research focused on two main aspects that addressed the application of algae to both traditional and novel aquaculture processes. The first aspect focused on current issues facing the industry such as environmental concerns and limitations in space and resources. The second aspect focused on future issues facing the industry that were highlighted throughout this research. Figure 1.1 provides a breakdown of the objectives for each of the inter-related core themes of the research.

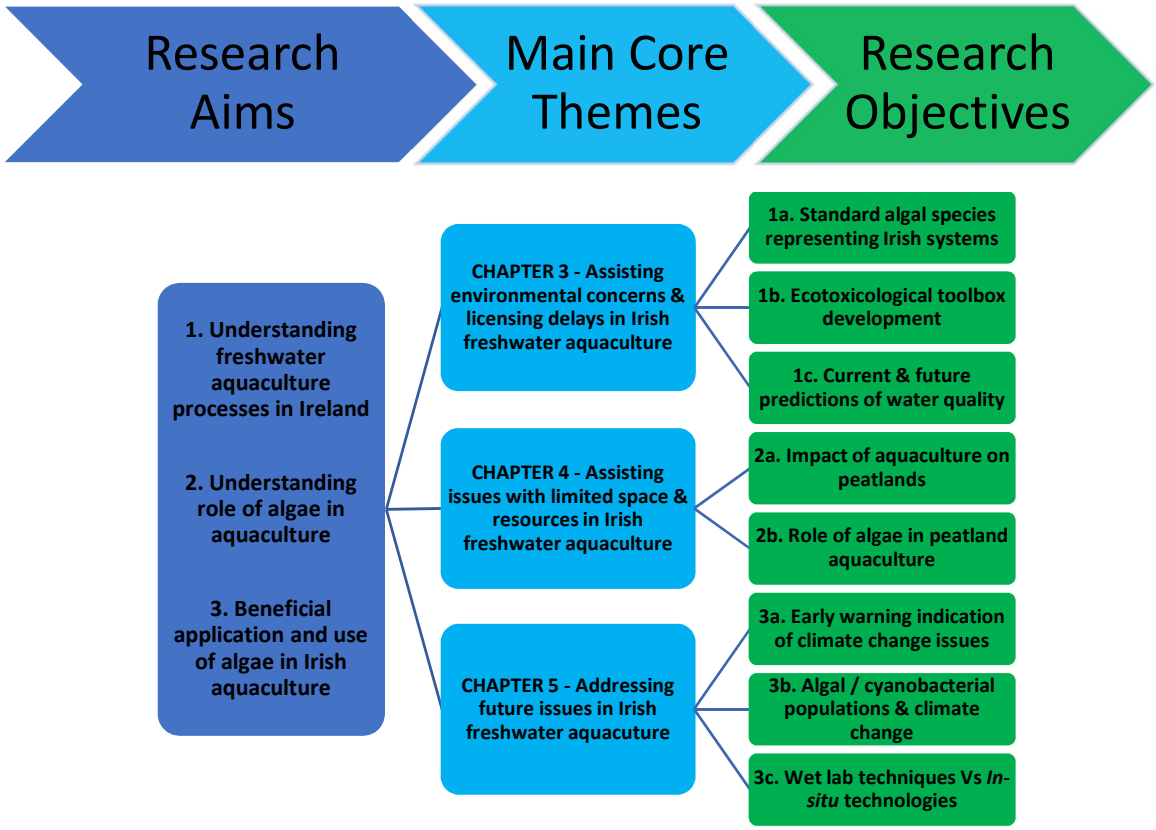


Figure 1.1: Connections between the **research aims** (dark blue), research **objectives** (green) and the **main core** research **themes** (light blue).

The primary objectives of the research were;

1. To analyse standardised algal species against the most common species found within Irish waters to determine whether the standardised species, which are not commonly found in Ireland, were representative of the systems they would be applied to. Then develop an ecotoxicological toolbox, which included the selected algal species to provide a means to analyse and assess the water quality entering and exiting the aquaculture facility. And finally analyse the physicochemical parameters most commonly used to monitor the quality of aquaculture water, thus giving a baseline, in parallel with the ecotoxicological analysis in order to provide a more in-depth indication for both the current and future predictions of water quality.
2. To employ the developed ecotoxicological toolbox to determine the impact aquaculture may have on peatland environments and determine the role of algae in a novel, first of its kind, IMTA aquaculture process in order to inform its management for sustainable production efficiency.
3. To monitor what effect weather variances have on water quality and algal growth in order to determine the potential application of algae as an early warning indicator for unforeseen issues associated with climate change variances, to monitor the effects changes in climate has on algal and cyanobacterial populations in order to provide a means to indicate any potential issues associated with negative algae and cyanobacterial blooms. Finally, to compare wet laboratory techniques with *in-situ* technologies for monitoring algal/cyanobacteria populations in order to ensure unforeseen issues are indicated early enough so that sustainable mitigation can be applied before major problems occur.

1.5. JUSTIFICATION OF RESEARCH

Food Wise 2025 predicted that, as part of the expansion of the Irish agri-food sector, the Irish aquaculture industry had the potential to increase production to 81,700 tonnes, by 2023. However, as of the end of 2019 38,238 tonnes were produced. Issues with environmental concerns have hampered the growth and development of the industry. Advances in aquaculture must also be balanced by the need to meet commitments as set out by the WFD which aims to achieve good water status in all waters across all EU member countries. Additionally, the ever-increasing indication of climate change variances may also be impeding growth.

The adoption of environmental EU directives has had a knock-on effect on the Irish aquaculture industry. Environmental concerns associated with industries, which includes aquaculture processes,

located in areas that are now protected under the birds and habitats directives have led to issues and delays in the Irish aquaculture licensing process as well as limiting the space and resources available for expansion of the industry. A mandatory environmental impact assessment (EIA) now needs to be conducted as part of the Irish aquaculture licensing process. Freshwater aquaculture waste-water discharge is currently regulated by the Environmental Protection Agency (EPA) and monitoring is conducted by local authorities, and in some cases Irish Water (EPA, 2018). However, current regulations may not be specifically applicable to aquaculture and the EPA are now actively investigating. Hence, there is an urgent need to develop an ecotoxicological toolbox consisting of tests representative of the receiving Irish freshwater aquatic ecosystem downstream of fish farms. This toolbox aims to assist in ensuring the aquaculture industry complies with the adopted EU directives (Birds Directive, Habitats Directive, WFD, *etc.*) thus assisting in the improvement of the licensing process and therefore, the sustainable growth and development of the industry.

Climate change variance is becoming an ever-increasing concern to all industries and walks of life, including Irish aquaculture. For example, March 2018 saw the greatest level of snow fall Ireland have observed in recent memory, with snow drifts >9 ft. experienced and many rural communities were left snowed in for days. Then just a few short weeks later in May 2018, Ireland experienced a heat-wave where the highest temperatures ever recorded in the country were observed, leading to 16 weeks unbroken drought conditions nationwide and hose-pipe bans being enforced. February 2020 was one of the wettest on record. The Irish midlands traditionally get an average of 70.3mm of rainfall for that period. However, according to Met Éireann metadata, 197.7mm of rainfall fell for that month (Met Éireann, 2021). This high level of rainfall was as a result of two extratropical cyclone storms hitting Ireland in that month and in close proximity to one another. Storm Ciara (formed 7th February 2020, dissipated 16th February 2020) and Storm Dennis (formed 11th February 2020, dissipated 18th February) affected Ireland less than a week apart. All of these unforeseen changes in weather conditions have had indirect effects on the aquaculture industry *e.g.*, the aquaculture facilities involved in this research reported increases in mortalities following some of these events. The use of algae may provide a natural means to predict potential issues in the event of uncharacteristic weather conditions, thus allowing for sustainable mitigation processes to be applied before highly problematic implications are observed. Not only will this assist in the sustainable growth and development of the industry, the natural basis of using algae will also ensure that the environmental EU directives will not be impacted.

1.6. STUDY AREA

The research within this project was applied to a traditional and a novel freshwater aquaculture facility. These two facilities were focused on to 1) ensure the research conducted was applicable to traditional aquaculture practices already established in freshwater Irish systems and, 2) ensure the research conducted was equally applicable to more novel aquaculture practices being developed in Ireland. These two facilities were also chosen as the traditional system provides hatchery and nursery facilities to the novel peatland IMTA aquaculture facility.

TRADITIONAL FLOW-THROUGH PRODUCTION SYSTEM:

Keywater Fisheries is a traditional freshwater perch (*Perca fluviatilis*) farm located in Boyle, Co. Sligo (53°58'13" N - 8°24'46" W) 8 km outside Boyle town, Co. Roscommon, that employs a flow through system (FTS) and a recirculating aquaculture system (RAS) for culture. Water is taken in from a small freshwater river running adjacent to the farm before it is released back into the river after treatment. Keywater is a low production farm making it ideal for pilot studies and is often used for research purposes. An indoor RAS system contains brood-stock tanks, hatchery tanks for eggs and juveniles and nursery tanks for juveniles. The larger fish are cultured in three 'grow out' ponds outside. These are earthen pill ponds that are divided into two where fish are cultured in one section and water is treated in the other section. Air lifts are used for oxygenation purposes. There is low flow within the individual ponds so a paddle wheel is used for circulation and additional aeration. Water from both systems is passed into a wetland pond for final treatments before being released back into the river.

NOVEL PEATLAND IMTA PRODUCTION SYSTEM:

Oasis fish farm is an innovative peatland cut-away integrated multi-trophic aquaculture (IMTA) system process set in the middle of Mount Lucas Wind Farm, Co. Offaly (53°17'3" N - 7°11'45" W). This IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*L. minor*) and gibbous duckweed (*L. gibba*) and exploits the use of microalgae for waste removal. The aquaculture system consists of four split (pill) ponds connected to an algae and duckweed lagoon with 16 channels serving as a treatment system. Fish are kept at a density that does not exceed the organic farming standard (*e.g.,* < 20 kg/m^{-3} for perch), using screens at the D-ends of each split pond. The space between two D-end fish culture areas is also used to treat waste with free living algae in suspension. Flow in each split pond is generated and water is circulated using an airlift. Each D-end fish culture area is equipped with oxygen and temperature probes connected to paddlewheels to provide extra oxygen when necessary. The farm is designed to hold a maximum of 32,000 Kg of fish.

The conducted research was specifically formulated around the FTS and the novel IMTA system unique to Ireland, with the knowledge acquired from the traditional aquaculture methods (FTS) being transferred to inform the development of the novel peatland IMTA system. The inflow, outflow and treatment points were focused on in the FTS as the facility was directly linked to the surrounding aquatic ecosystem. The intake and output points were focused on in the IMTA system as this facility was directly linked to the peatlands, which are now protected sites. As the IMTA is effectively a novel closed / semi-closed system, analysis within the farm was also conducted. A range of monitoring programs were applied to both facilities. A pilot study was conducted in Keywater Fisheries between April 2018 and October 2018 in order to establish a baseline for freshwater aquaculture intake and output water quality. Application of the developing ecotoxicological toolbox was conducted in Keywater Fisheries between March 2019 and August 2019. A pilot study and application of the toolbox was applied to Oasis Fish Farm between May 2019 and August 2019. Analysis of the physicochemical parameters and algal communities present in the novel system were applied to Oasis Fish Farm between December 2019 and October 2020.

1.7. CONTRIBUTION TO EXISTING KNOWLEDGE

All dissemination of research and knowledge contribution can be found in appendix 1. A summary of the contribution this research has made to existing knowledge is as follows;

Journal Publications

O'Neill, E.A., Rowan, N.J. (2022). Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland. **Sci. Total Environ**. 802, 149800. (Impact Factor 7.963)

O'Neill, E.A., Stejskal, V., Clifford, E., Rowan, N.J. (2020). Novel use of peatlands as future locations for the sustainable intensification of freshwater aquaculture production – A case study from the Republic of Ireland. **Sci. Total Environ**. 706, 136044. (Impact Factor 7.963)

O'Neill, E.A., Rowan, N.J., Fogarty, A.M. (2019). Novel use of the alga *Pseudokirchneriella subcapitata*, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish farming as a case study. **Sci. Total Environ**. 692, 209–218. (Impact Factor 7.963)

Presentations

O'Neill, E., Murphy, E., Lynch, M., Rowan, R. (2019). Sustainable Intensification of Freshwater Aquaculture using Peatlands – Role of Algae. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 22nd November.

O'Neill, E., Fogarty, A., Donohoe, O., Rowan, N. (2018). Development of Ecotoxicological Toolbox for Assessing Freshwater Finfish Aquaculture Effluent. 28th Irish Environmental Researchers Colloquium (ENVIRON 2018). Cork Institute of Technology, Cork. 26th – 28th March.

Posters

O'Neill, E., Fehrenbach, G., Murphy, E., Pogue, R., Lynch, M., Rowan, N. (2020). Developing Sustainable Freshwater Aquaculture using Irish Peatlands during COVID-19 Crisis. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 19th June.

O'Neill, E., Murphy, E., Lynch, M., Rowan, R. (2019). Sustainable Intensification of Freshwater Aquaculture using Peatlands – Role of Algae. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 22nd November. **Winner – Best Poster**

O'Neill, E., Rowan, N., Fogarty, A. (2019). Development of an Ecotoxicological Toolbox for Assessing Irish Freshwater Finfish Aquaculture Effluent. 29th Irish Environmental Researchers Colloquium (ENVIRON 2019). Carlow Institute of Technology, Carlow. 15th – 17th April

O'Neill, E., Fogarty, A., Donohoe, O., Rowan, N. (2018). Development of Ecotoxicological Toolbox for Assessing Freshwater Finfish Aquaculture Effluent. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 21st April.

O'Neill, E., Fogarty, A., Donohoe, O., Rowan, N. (2018). Development of Ecotoxicological Toolbox for Assessing Freshwater Finfish Aquaculture Effluent. 28th Irish Environmental Researchers Colloquium (ENVIRON 2018). Cork Institute of Technology, Cork. 26th – 28th March.

Technical Reports

O'Neill. E., Fehrenbach, G., Murphy, E., Pogue, R., Lynch, M., Rowan, N. (2021). Investigation to elucidate role and relationship between algal and microbial communities in freshwater aquaculture. AquaAlgaePlus Final Report, June.

Kennedy, A., Tahar, A., Cooney, R., Naughton, S., **O'Neill, E.**, Fogarty, A., Kavanagh, S., Rowan, N., Clifford, E. (2019). Supporting the sustainable development of the Irish freshwater aquaculture industry. EcoAqua Final Report, October.

CHAPTER 2

LITERATURE REVIEW

2.1 AQUACULTURE

Aquaculture is the rearing, breeding and harvesting of aquatic animals and plants in all forms of water environments (*e.g.,* freshwater, marine, brackish) where fish are grown to market size in raceways, cages, tanks or ponds (Figure 2.1) under controlled conditions (Kutty, 1987; NOAA, 2018; O'Neill *et al*., 2019, 2020).

Figure 2.1: Freshwater aquaculture pond in Ireland (Source: BIM).

2.1.1 HISTORICAL CONTEXT OF AQUACULTURE

Although agricultural farming was invented during the New Stone / Neolithic Age (*ca*. 8000 – 4000 BC), aquaculture was not developed until 1000's of years later. Aquaculture originated in Asia with common carp (*Cyprinus carpio*) being farmed in China as early as 2000 – 1000 BC. However, aquaculture was not practiced in other continents until more recent centuries. See Figure 2.2 for a summary of the development of the aquaculture industry. The delay in aquaculture development compared to agriculture is thought to be partly due to the fact that humans are terrestrial inhabitants who therefore cannot fully or readily appreciate aquatic environmental parameters. There are some environmental factors that are thought to profoundly affect aquatic organisms, including; water oxygen (O_2) content, carbon dioxide (CO_2) solubility, dissolved nutrient content, toxic nitrogenous waste, pH, buffering capacity, salinity, turbidity, the presence of heavy metals or other toxic

compounds and molecules in solution, phytoplankton and zooplankton concentrations and the current velocity (Lucas, 2019).

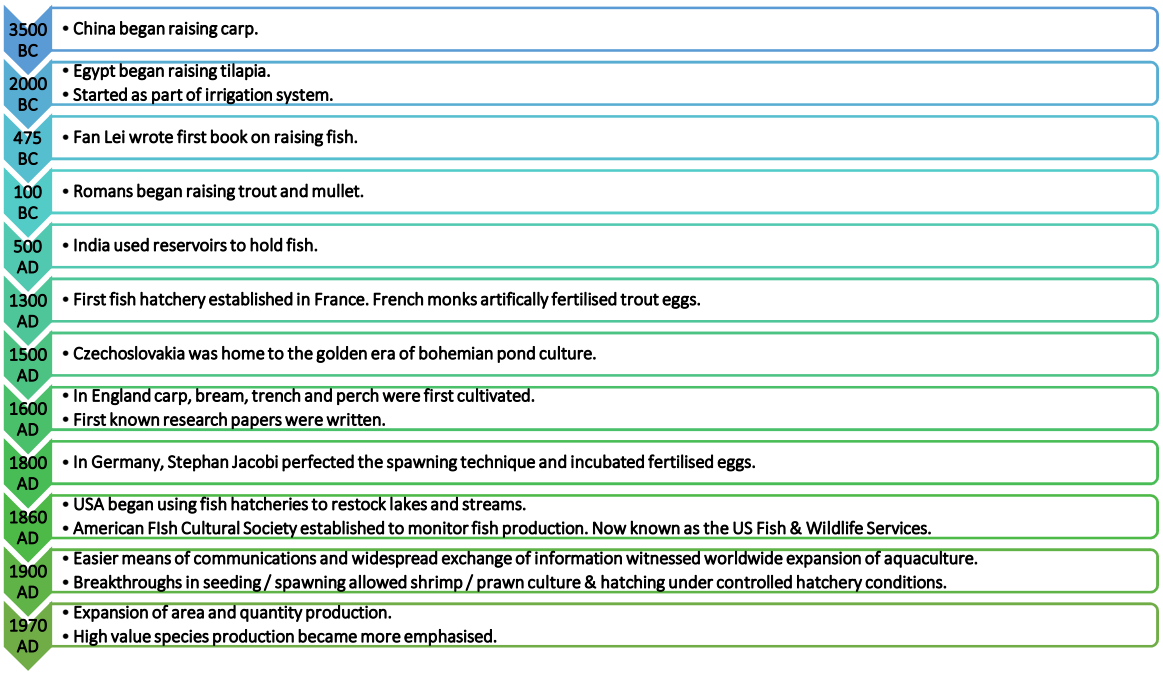


Figure 2.2: Summarised **timeline** of the **global aquaculture development** industry ranging from 3500 BC up to the late 1970's (Laux, 2015; FAO, 2018a; Lucas, 2019).

2.1.2 AQUACULTURE VS CAPTURE FISHERIES

There has been widespread recognition that capture fishery production is at its peak and has hit its maximum sustainable production yields indicating that aquaculture will become an increasingly important and main source of seafood (Lucas, 2019). The depletion of wild capture fishery practices has subsequently resulted in the rapid development of aquaculture (Han *et al.*, 2019) making it the fastest growing food producing industry worldwide (Ottinger *et al.*, 2016; O'Neill *et al.*, 2019, 2020). Between 1989 and 2016, global capture fishery production has remained relatively static with an increase of only 2.58M tonnes (88.22M tonnes in 1989 to 90.91M tonnes in 2016) (Lucas, 2019; Ottinger *et al.*, 2016). Aquaculture, on the other hand, has risen significantly with an increase of 67.71M tonnes over the same time period (12.32M tonnes in 1989 to 80.03M tonnes in 2016), with this increase continuing to rise (Huynh *et al.*, 2017; Lucas, 2019). According to the Food and Agriculture Organisation of the United Nations or FAO (2016) and their latest statistics on global fisheries and aquaculture in 2016 global aquaculture produced 110.2M tonnes valued at $243.5B USD or €222.2B. Of that 110.2M tonnes produced; 80M tonnes were food fish specifically for human consumption. In the same year capture fisheries produced 90.19M tonnes in total. In 2017, global aquaculture production exceeded capture fishery production by >18M tonnes (Tacon, 2020). Aquaculture now

accounts for ~50% of fish produced for human consumption (FAO, 2018b, 2018c). This figure is expected to rise to ~62% by 2030 (Fredricks *et al.*, 2015; Liu *et al*., 2017). The FAO have predicted that by 2030 151.2M tonnes of fish will be produced via aquaculture, with 109M tonnes for human consumption. However, only 92M has been predicted for capture fisheries, and of that 74M tonnes will be for food fish (FAO, 2018b, 2018c). The stagnated growth of the capture fishery sector is due to the fact that the majority of wild fish stocks have reached their maximum sustainable yields and, in some cases, have surpassed it. In 2015, the FAO indicated that 59.77% of the world's marine stocks have reached maximum biologically sustainable yields. Only 7.09% are considered under fished. However, 33.14% are overfished, with the Mediterranean and black sea at the highest level of >60% overfishing (Cao *et al*., 2015). The dramatic increase in aquaculture production is also attributed to increased consumer demand for fish (Seoane *et al.*, 2014; Tahar *et al*., 2018b, 2018a) as a result of the dramatic growth in global population (Seoane *et al*., 2014). Farmed fish is rich in protein and is also considered to be a more efficient protein utilisation and feed conversion source than other animals destined for protein production (Tschirner and Kloas, 2017).

2.1.3 GLOBAL & EUROPEAN AQUACULTURE

Asia provides 89.4% of the world's total aquaculture production, followed by the America's (4.2%), Europe (3.7%), Africa (2.5%) and Oceania (0.3%). Globally, China is the largest aquaculture producer accounting for 61.5% of the world total, followed by India (7.1%) and Indonesia (6.2%). Inland and freshwater aquaculture produces the greatest tonnage at 51.4M tonnes, compared to 28.7M from marine sources. Of that, inland finfish produces 47.2M tonnes. Marine and coastal finfish accounts for 6.57M tonnes, all molluscs' accounts for 17.11M tonnes whilst inland and marine crustaceans are at 3.03M and 4.83M tonnes, respectively. Salmonids such as salmon and trout account for 18.1% of total production, followed by cod, hake and haddock at 9.6%, with tuna and billfishes at 8.6%. In total, finfish production accounts for 65.4%, crustaceans at 23% and molluscs at 11% (Cao *et al.*, 2015; FAO, 2018c, 2018a, 2016).

The FAO and the European Commission (EC) Eurostat's most recent European fisheries and aquaculture figures have indicated that in 2015-2016, Europe produced 2.95M tonnes of fish via aquaculture equating to €3.89B which accounts for 3.7% of the total world production. Norway is the biggest European aquaculture producer at 1.38M tonnes of fish which is the equivalent to 39.2% of the total European production. Norway is followed by Spain (293,510 tonnes) and the United Kingdom or UK (211,568 tonnes). Unlike the global trend previously mentioned, aquaculture in Europe only accounts for 19.9% of production, whilst capture fisheries accounts for 80.1%. In Norway <40% of fish

is produced via aquaculture. Within the EU it is less again, at <30%. Also, unlike global trends, 47% of production is molluscs, 29.7% diadromous fish (*e.g.*, sturgeon), 14.3% marine fish and 7.2% freshwater fish. Mediterranean mussels account for 25.1% of production with culture occurring off the bottom of the Mediterranean Sea. Atlantic salmon account for 15.1% of production with 99.8% of culture occurring in offshore cages in the Atlantic Ocean. Rainbow trout account for 13.3% of production with 64.5% of culture occurring in inland tanks. Although molluscs produce the greatest tonnage it only accounts for approximately 4.5% (€175M) of total aquaculture production value. Atlantic salmon and rainbow trout produce the greatest production value at 24.4% (€949M) and 13.2% (€513M) respectively (Eurostat, 2018; FAO, 2018a).

2.1.4 AQUACULTURE ENVIRONMENTS

The term "seafood" is regularly used inclusively for all animals and plants from aquatic environments (Lucas, 2019). However, the environments or habitats of aquaculture are more traditionally categorised into three main groups of water types; marine, brackish and freshwater (Callaway *et al*., 2012; Kutty, 1987). These categories are based on water salinity where marine water has a salt content of greater than 35% (g L^{-1}), brackish water has an average content of between 0.5% to 30-35% and freshwater contains less than 0.5% (Rath, 2011).

Marine aquaculture, or mariculture, is a form of aquaculture where farming of marine organisms for foodstuff and other products (cosmetics, pharmaceuticals, jewellery) occurs in natural marine habitats or in land / sea based enclosures (Phillips, 2009), where water salinity is greater than 35% (Rath, 2011). A wide range of organisms are farmed around the world's coastlines, including; shrimps, molluscs, marine finfish and seaweeds (BIM, 2018a; Kutty, 1987; Phillips, 2009). Mariculture has provided vast possibilities for sustainable fish production as well as economic development. However, with an increase in demand for fish, large scale farming of natural marine habitats may pose several threats to coastal environments, such as waste and nutrient discharge, degradation of the natural habitats, introduction and / or transmission of disease to wild fish stocks and introduction and / or accidental release of invasive species (Lucas, 2019; Phillips, 2009; Southgate and Lucas, 2019).

Brackish water aquaculture normally occurs in estuaries, river deltas, lagoon and backwaters, which are under tidal regime (Kutty, 1987). Depending on the tidal phase and the volume of fresh water discharging through the river into the sea, the salinity in these habitats can fluctuate from 0.5% to 35% (Krishnan *et al*., 2014; Kutty, 1987). Euryhaline fish are primarily farmed in brackish water *i.e.*, fish that can withstand or tolerate large fluctuations in the salinity of the surrounding water (Kutty,

1987; Southgate and Lucas, 2019). Examples of such fish include mud-skippers, mullets and several species of crap (Kutty, 1987). Brackish water is currently only marginally used for aquaculture practices (Gjedrem *et al.*, 2012).

Freshwater aquaculture is a form of aquaculture that occurs in fresh water systems such as rivers, lakes, streams, ponds, *etc*. (Kutty, 1987; Southgate and Lucas, 2019). It is the cultivation of any aquatic organism or plant in water conditions where the salinity does not exceed 0.5% (European Commission, 2018; Kutty, 1987). Examples of freshwater species include; trout, perch, pike, tench, carp and roach, with trout and perch being the most common freshwater species cultivated through aquaculture in Ireland (Inland Fisheries Ireland, 2018). Despite that fact the planet Earth is covered in approximately 70% marine / brackish water, in 2017 more that 83.6% of fish production via aquaculture were freshwater fish (Tacon, 2020). Freshwater aquaculture will be the focus of this research.

2.1.5 AQUACULTURE CHARACTERISATION

One of the most basic principles of characterising aquaculture is based on the intensity of the production process. According to Southgate and Lucas (2019), the intensity of aquaculture defines the various densities of organisms, be they animal or plant, per unit area or per unit volume. The farming intensity / density along with the system design and economic feasibility are some of the many factors that contribute to and influence the commercial success of aquaculture facilities (Bunting, 2013; Good and Davidson, 2016; Kumar *et al*., 2018; Southgate and Lucas, 2019). Culture intensity considers the inputs into the systems in order to maintain the most optimum growth conditions for the cultured organisms *i.e*., the greater the intensity / density of the organisms, the greater the required inputs (Troell *et al*., 2004). All systems, be they natural or artificial, require some form of energy input for sustainability (Southgate and Lucas, 2019; Troell *et al*., 2004). Even if there is perfect recycling of organic matter, there will still be energy loss through metabolism and that energy must be replaced (Southgate and Lucas, 2019). Natural aquatic ecosystems usually consist of primary producers, various levels of consumers (primary, secondary, tertiary) and decomposers (Jiang and Pu, 2015), see Figure 2.3. These systems are self-sustaining with the recycling of nutrients and input of energy from the sun. They are classified by long, complex food chains (Edwards, 2015). The energy transfer from one level of the chain to the next is classically in the order of 10% (Figure 2.3). These significant declines in energy with every step of the food chain (-90%) have major implications for aquaculture (Southgate and Lucas, 2019). It is more proficient to produce primary producers or animals at lower levels of the food chain (Kumar *et al*., 2018). This is very important in areas such as developing countries where aquaculture products are valued as a major source of animal protein and are farmed for food security

rather than for export trade (Edwards, 2015; Southgate and Lucas, 2019). As a results of this, culturing intensities are artificially manipulated in order to meet demands and are divided into three main categories; intensive, semi-intensive and extensive (Southgate and Lucas, 2019).

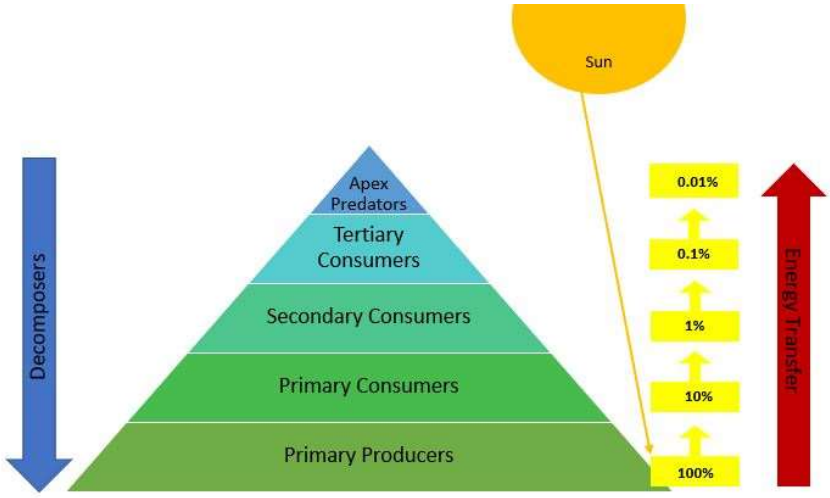


Figure 2.3: Multitrophic pyramid indicating each level of the aquatic food chain along with the energy transfer percentage as it travels through each level.

Intensive aquaculture systems (IAS) are a complete contrast to natural systems (Southgate and Lucas, 2019). All required nutrients are introduced from artificial feed inputs. Production can occur in ponds, cages, raceways or tanks (Sultana *et al*., 2017). However, in order to achieve peak stocking densities, maintenance of water quality parameters is essential (Datta, 2012). IAS are characterised by very simple food chains, low energy losses from feed inputs with high food conversion ratios (FCR's) from specialised feeds, no recycling of energy, completely non self-sustaining, requirement of high energy inputs and high yields per unit volume / unit area (Southgate and Lucas, 2019). The water quality is usually sustained by high exchange rates of water and in some cases, by the additional use of mechanical means (Datta, 2012; Southgate and Lucas, 2019). Indoor systems traditionally require mechanical resources for gaseous exchange, to remove particulate waste and to produce O_2 (Southgate and Lucas, 2019; Suantika *et al*., 2020). Outdoor systems, on the other hand, usually have a soil substrate and phytoplankton present, particulate waste settles out, bacteria conduct decomposition and gaseous exchange is enhanced by mechanical aeration (Datta, 2012; Southgate and Lucas, 2019). Stocking densities fluctuate significantly with the type of system in use and cultured organisms present however, it is always relatively high (Southgate and Lucas, 2019).

Extensive aquaculture systems (EAS) are widely used, especially in developing countries. It is the chief source of aquaculture production worldwide (Southgate and Lucas, 2019). An EAS is part of a natural aquatic ecosystem and greatly depends on it for conservation of water quality, as well as for much of

the cultured organism's food and other requirements (Buck *et al*., 2008; Southgate and Lucas, 2019). EAS have limited inputs to sustain animal growth and survival *e.g*., they have some basic organic fertilisers (plant and animal waste) but no aeration (Southgate and Lucas, 2019). These systems traditionally have a low stocking density. Additionally, the natural gaseous exchange and natural production of feed within the system is adequate to support the cultured organisms (Billard and Dabbadie, 2017). With the exception of seaweeds and bivalves, a considerable amount of EAS produce low value fish (*e.g.*, carp and tilapias). This is possible because of the low costs of production associated with extensive culturing (Southgate and Lucas, 2019).

Although there is no immediate cut-off point between IAS and EAS, semi-intensive aquaculture systems (SAS) are used as an estimation to define the middle ground between the two (Southgate and Lucas, 2019). An SAS does rely more on natural productivity however, supplementation is required (Rocha *et al*., 2019). Supplementation may take many different forms and includes; the additional facilitation of mechanical aeration to sustain dissolved oxygen (DO) levels, the use of organic and inorganic fertilisers to improve natural productivity and the practiced need of prepared feeds for supplemental feeding (Dato-Cajegas and Yakupitiyage, 1996; Southgate and Lucas, 2019). An SAS is almost exclusive to ponds and allows for an increase in stocking densities within the ponds (Southgate and Lucas, 2019).

2.1.6 AQUACULTURE PROCESSING SYSTEMS

The majority of the world's aquaculture production employs traditional farming methods in static systems such as ponds. These static ponds tend to have no water exchange during the farming period. The only addition of fresh water occurs by "topping up" to offset evaporation (Southgate and Lucas, 2019). Production is usually extensive due to major issues in sustaining water quality parameters should intensive measures be employed under these static conditions (Buck *et al*., 2008). A limited increase in stocking densities and biomass can be performed. This necessitates increasing the use of fertilisers and supplementary feeds to sustain productivity (Datta, 2012). However, this in turn requires more stringent management processes for such water quality problems as intolerable concentrations of nitrogenous waste compounds and low DO levels which occur at night. The addition of mechanical aeration can provide sustainable DO levels in the presence of higher stocking densities subsequently accomplishing greater productivity (Datta, 2012; Southgate and Lucas, 2019). However, the use of mechanical aerators are often not available or feasible due to limited electrical supplies in rural regions where static systems and pond farming are generally employed (Southgate and Lucas, 2019).

OPEN AQUACULTURE SYSTEMS

Open system aquaculture occurs in the natural environment of the water body being used *i.e.*, the cultured organisms are confined and protected in a large amount of water (*e.g.*, large water bodies such as an ocean or a lake) so that the water quality is sustained by natural water flows and processes (Edwards, 2015; Radford and Slater, 2019; Southgate and Lucas, 2019). There is no unnatural circulation of water through or within the open system. These systems tend to have low operating costs as water pumping is not needed. Capital costs diverge significantly depending on the type of culture *e.g.*, bivalve culture is typically low cost whilst intensive fish culture is much higher (Pahri *et al.*, 2015; Popp *et al*., 2018; Southgate and Lucas, 2019). According to Southgate and Lucas (2019), most open water farming systems must be leased from appropriate government agencies and are not usually available for free hold purchase. Compared to other farming systems, open systems are susceptible to issues that either are not applicable to or are more problematic to alleviate (Valenti *et al*., 2018). The lack of control over water quality is a major issue connected with the site selection for open systems. The quality of the water depends on local factors and cannot be altered. It is therefore essential that the producer is aware of all water quality parameters occurring in the system (*e.g.*, salinity, water temperature, pH, algal blooms, *etc.*) before developing the aquaculture facility. Large dissimilarities in growth rates can be caused by seasonal variations in the environmental factors (Southgate and Lucas, 2019). Key variances in growth and survival rates can by caused by local changes in the environment. Open systems are also more susceptible to predation (Valenti *et al.*, 2018). Protective devices can be added to control predation. Most countries require these predator control measures to be non-destructive *i.e.*, the predator animal is not harmed. However, these methods are typically expensive to run and uphold. Predation is not exclusive to wild animals. Poaching and human interference can also cause major problems. Despite the fact that any interference with aquaculture stocks and equipment is an offence in most countries, the responsibility of protection tends to fall to the aquaculture producer due to complications in enforcement (Southgate and Lucas, 2019). Cage, net and pond aquaculture are classified as open systems.

SEMI-CLOSED AQUACULTURE SYSTEMS

Semi-closed systems water supplies are usually restricted in isolated units with some water flow-through (Figure 2.4). These systems fall specifically between open and static systems in terms of water replacement with adjacent water sources (Southgate and Lucas, 2019). Semi-closed systems have a level of water exchange which is significantly larger than that of static systems but considerably less than in open systems. In these systems water is frequently and continuously brought to the facility (Soltan, 2016). The source may be marine, brackish or freshwater. Water is extracted from a

dependable source which flows to, through or near the facility. This is most commonly conducted by pumping, but also may be driven by tidal exchange or gravity. The water is replaced to sustain water quality (Southgate and Lucas, 2019). As these farms are not situated within a natural aquatic ecosystem there is a degree of water quality control but only to the amount the water flow can be manipulated *i.e.*, stopped, decreased or increased (Pedersen and Wik, 2020). If the water source is of an undesirable quality or if the source becomes contaminated, the water flow within the system can usually be stopped to prevent issues within the farm. However, the culture in the farm may then be left in stagnant water of worsening quality (Soltan, 2016). Semi-closed systems have many advantages and benefits which range from boosting production from ponds by replacing some water while sustaining some dependence on the natural processes of the aquatic ecosystem to complete reliance of water quality on water replacement leading to large rises in production. The greater the increase in production, the greater the water use per unit of production (Southgate and Lucas, 2019). In these systems water can pass once or several times through the facility. Water replacement and exchange conducted by pumps can lead to high costs. The volume of water exchanged or the height the water needs to be pumped usually determines the extent of these costs (Pedersen and Wik, 2020; Soltan, 2016; Southgate and Lucas, 2019). In large semi-closed systems with semi-intensive to intensive production water flow is usually high to very high, respectively (Southgate and Lucas, 2019). Semi-closed systems include ponds, tanks, FTS (Figure 2.4), raceways or some IMTA (Figure 2.5) processes. This research will include a traditional FTS aquaculture system and a novel IMTA system.

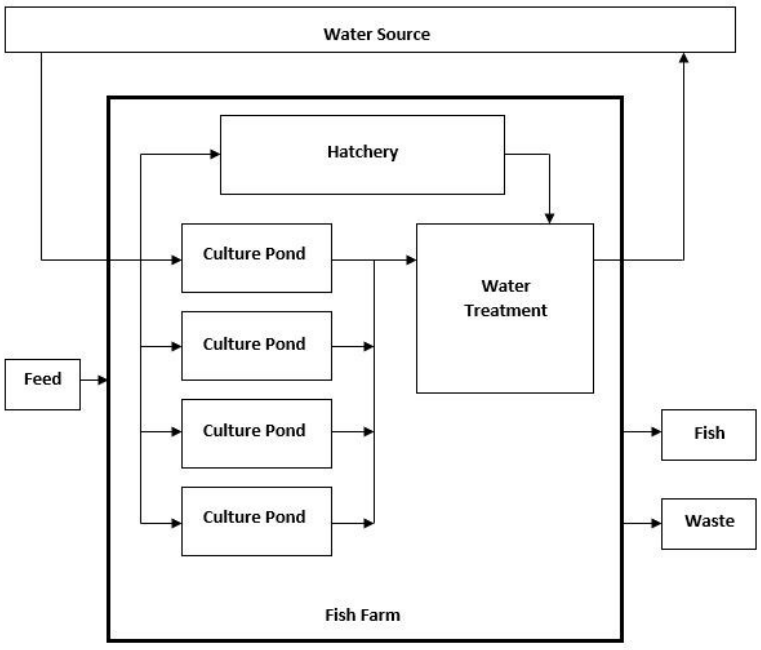


***Figure 2.4*:** Block diagram schematic of a traditional flow-through aquaculture system.

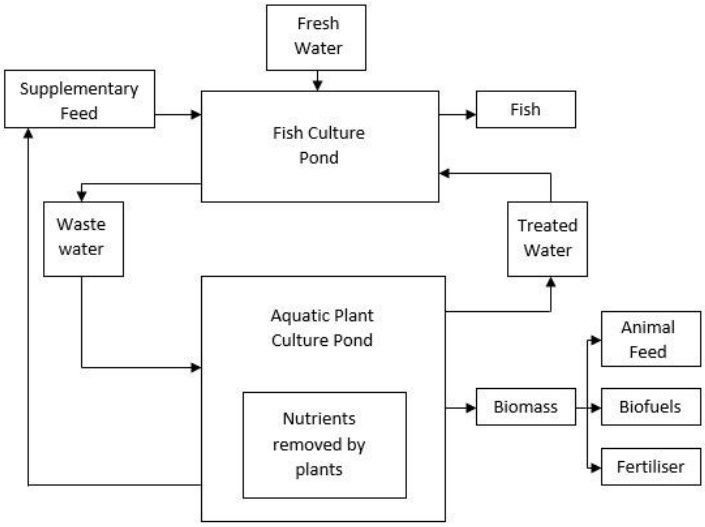


Figure 2.5: Block diagram schematic of an integrated multi-trophic aquaculture system.

CLOSED AQUACULTURE SYSTEMS

Closed systems generally have negligible association with the natural surrounding environment and the initial water source (Southgate and Lucas, 2019). These systems have practically no water exchange or replacement during production, hence the term "closed" systems (Feucht and Zander, 2015). Any addition of water is usually to counteract incidental losses or evaporation. However, it is more frequently required to sustain water quality (Southgate and Lucas, 2019). In most of these closed systems, some of the water is released and exchanged every day. This occurs as a result of features of the standard maintenance system *e.g.*, the removal of accumulated solids from filters (Soltan, 2016; Southgate and Lucas, 2019). It is much more difficult to sustain water quality in closed system than in semi-closed systems. Water quality in closed systems tend to only be sustainable by artificial means, even if there is a limited amount of water exchange every day (Soltan, 2016; Southgate and Lucas, 2019; Warren-Hansen, 2015). The cost of construction and production in intensive closed systems is usually very high. This has subsequently limited the marketable expansion of these systems to the final market size farming phase / grow-out production phase (Warren-Hansen, 2015). However, the potential for high yields with continuous production year-round close to markets powers their expansion and development (Southgate and Lucas, 2019). An example of a closed system is a recirculating aquaculture system or RAS (Figure 2.6). This research will include some RAS technology.

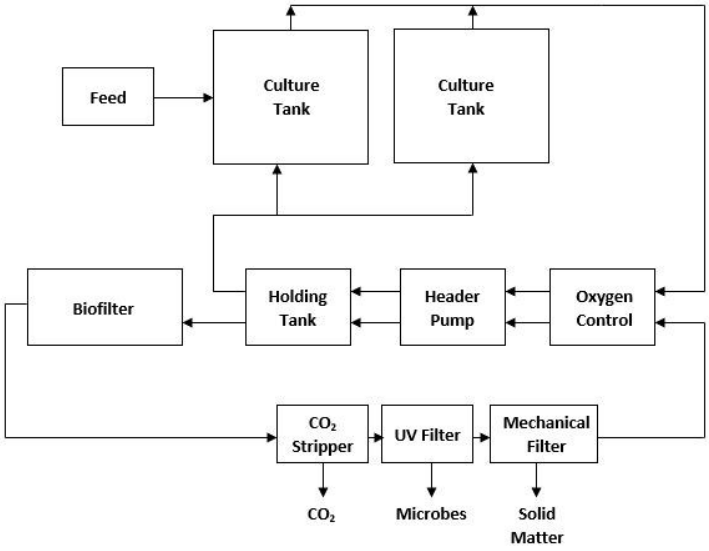


***Figure 2.6*:** Block diagram schematic of a standard recirculating aquaculture system.

2.2 AQUACULTURE IN IRELAND

Production (in tonnage), declined from 44,785 (€127M) in 2011 to 30,882 (€115M) in 2014 (BIM, 2018a). This drop was as a result of a diseases hampering the industry. The salmon sector, which is the key finfish species produced in Ireland, was fraught with a series of biological issues, primarily sea lice (BIM, 2014). From 2014 to 2017, the sectors production value has increased by almost €100M. Production peaked in 2017 at 47,147 tonnes worth €208.4M (BIM, 2018a). However, the overall output dropped by 19% volume (to 37,206 tonnes) and 13.5% value (to €179M) in 2018 (BIM, 2019). Despite an increase in production in 2019 (38,238 tonnes) the overall value output further declined to €173.5M (Dennis *et al*., 2020). The decline in production was due to the decrease in salmon production as a result of the cyclical production trends (BIM, 2019; Dennis *et al*., 2020). According to BIM (2019) and Dennis *et al.* (2020) a cyclical trend of production is required where heavy and light smolt input is alternated due to a lack of / limited capacity. Employment in the sector has also increased steadily, rising from 1748 in 2011 to 1977 in 2019 (BIM, 2019, 2018a, 2014; Dennis *et al.*, 2020). Within the country, Donegal is the leading county in employment, tonnage, value and production units, followed closely by Galway, Cork and Kerry (BIM, 2019, 2018a; Dennis *et al*., 2020).

The Irish aquaculture sector is primarily based in coastal areas but land-based RAS and more traditional freshwater and land based systems are also used, as shown in Figure 2.7 (DAFM, 2015b), and are projected to potentially grow rapidly over the next ten years. The sector provides valuable employment on a year round basis and aids in the preservation of viable local and rural communities

(BIM, 2018a; DAFM, 2015b). The Irish aquaculture sector can be divided into shellfish and finfish culture (BIM, 2018a; DAFM, 2015b).

- Rope mussels are cultured off the coasts of Donegal, Mayo and Galway but the majority of production occurs in the sheltered bays of Cork, as shown in Figure 2.7A. The mussels are grown on long ropes, between 6 to 10 m long, suspended from a long line and are held up with purpose built or specialist floats (DAFM, 2015b).
- Bottom grown mussels are cultured all around the island of Ireland (Figure 2.7B). The five main areas of production are; Lough Foyle, Co. Donegal; Carlingford Lough, Co. Louth; Waterford Estuary; Wexford Harbour and Castlemaine Harbour, Co. Kerry. Culturing is done directly off the seabed. Dredges and shallow draught vessels work the ground, allowing for the wild young mussels or mussel seed to be laid or re-laid. Once the seed has been re-laid, very little handling occurs until the mussels are ready for collection (DAFM, 2015b).
- Pacific or gigas oysters are farmed all around the island of Ireland also (Figure 2.7C). Culturing occurs in the inter-tidal zone in sheltered bays. This zone is located between the average high-water spring mark and the average low water spring mark. Some oyster larvae or spat are locally available however, it is primarily purchased from France. The stocks are reared in mesh plastic bags attached to steel trestles. These bags must be turned, shaken and re-positioned as often as possible. Ideally once every set of spring tides *i.e.,* once a month (DAFM, 2015b).
- Salmon culturing usually consists of smolt being cultured firstly in freshwater systems which are then farmed at sea in cages or netted containers. Production primarily occurs off the coasts of Donegal, Galway, Kerry and Cork (Figure 2.7D). Despite being set back by several biological issues, as previously explained, the salmon sector is still the highest value sector of the industry (BIM, 2018a; DAFM, 2015b).
- Trout farming in Ireland is divided into sea trout and freshwater rainbow/brown trout (DAFM, 2015b). Sea trout is primarily produced off the coast of Mayo (BIM, 2018a; DAFM, 2015b), whilst freshwater rainbow trout is mainly farmed in the southern half of the island, with the majority of the production concentrated in Wicklow and Kilkenny (Figure 2.7E), and is mainly sold on the domestic Irish market (DAFM, 2015b).

Irish aquaculture also produces a wide range of novel species, both in marine and freshwater, such as abalone, sea urchins, seaweed and perch (BIM, 2018a; DAFM, 2015b).

- Perch farming occurs in either traditional freshwater pond culture systems or in RAS. Perch farming is small, compared to other species, with only five main aquaculture sites on the island (Figure 2.7F). Most of the perch is exported, with Switzerland being one of the main markets (DAFM, 2015b).

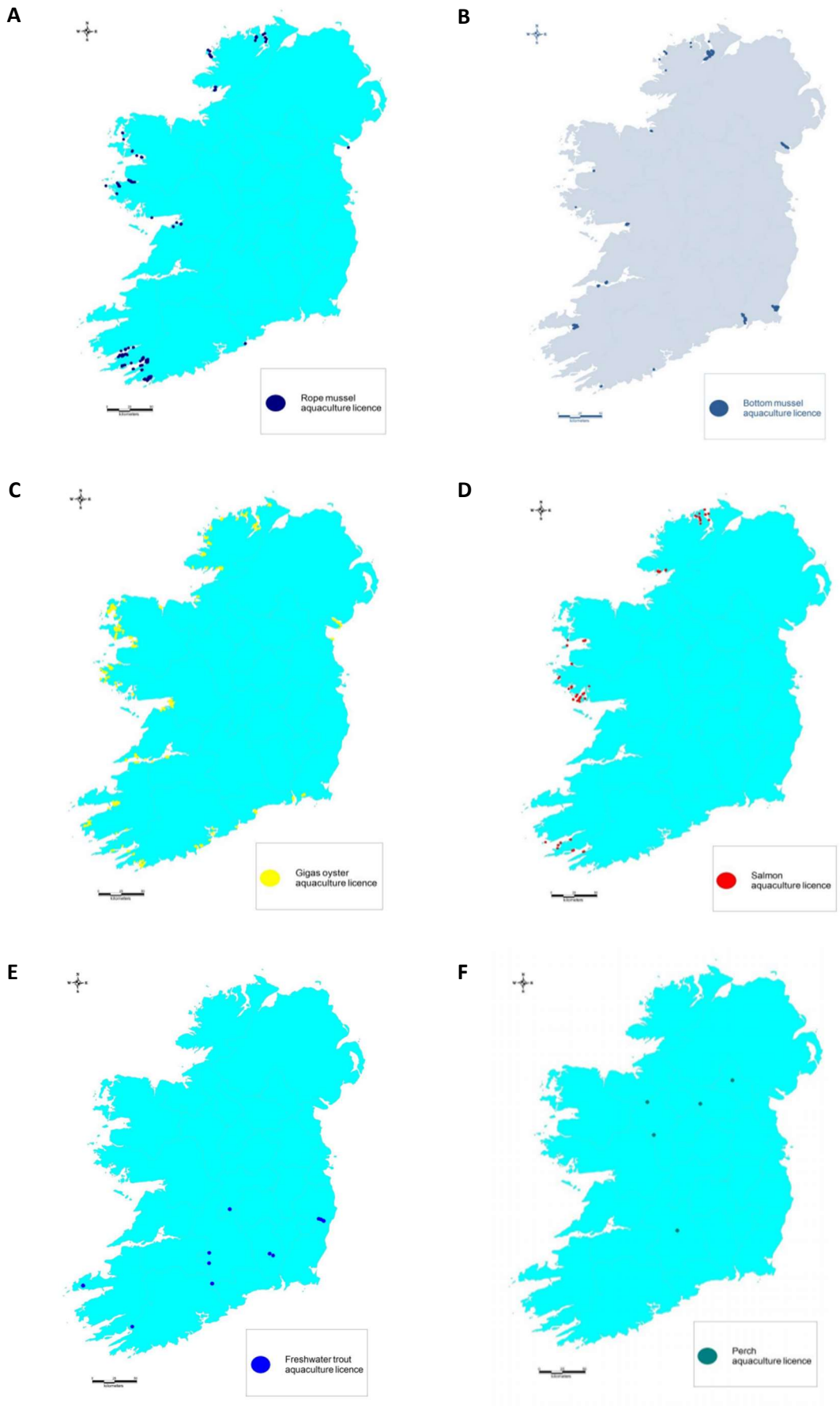


Figure 2.7: The main location of aquaculture farms across Ireland *(Source: DAFM)*.

2.2.1 *IRISH AGRI-FOOD INDUSTRY & AQUACULTURE*

The Agri-Food industry is Ireland's largest and oldest indigenous industry (DAFM, 2015a, 2015b). It has been described as an industry that is deeply entrenched in the backdrop, history and character of the country (DAFM, 2015a). The Irish Agri-Food industry encompasses a wide variety and range of sectors from primary agriculture to food and beverage production, and from forestry and forestry outputs to fisheries and fish processing (DAFM, 2015b, 2015a). It is an industry like no other due to its strategic importance to the national economy, its strong roots within local and rural communities and its providing an ever strengthening global reach (DAFM, 2015b, 2015a). Ireland now provides food produce of the highest quality to more than 175 countries (DAFM, 2015a).

Over the past decade or so, a renewed focus on growth in exports and commitments to providing continued quality excellence has created a range of new opportunities to further develop the industry (DAFM, 2015a, 2010). The Irish government has developed and introduced several plans and strategies to assist in the growth of the Agri-Food industry with particular reference to the aquaculture and seafood sector (DAFM, 2015b, 2015a, 2010).

2.2.2 *IRISH AQUACULTURE DEVELOPMENT*

In accordance with Article 34 of the Common Fisheries Policy Regulation, the EC requires all of its member states to prepare national strategic plans for aquaculture. Ireland's national strategic plan for sustainable aquaculture development is intended to inform investment priorities for aquaculture. The strategic guidelines aim to assist in identifying Irelands national targets, considering their relative starting position, national circumstances and institutional arrangements. Strength, Weakness, Opportunities, Threats (SWOT), analysis of the Irish aquaculture industry has been conducted as part of this strategic development plan (Table A2.1 of appendix 2), to highlight the main issues concerned with the Irish Agri-Food sector and the main over-arching needs of the country's aquaculture industry have been identified (DAFM, 2015b). See Table A2.2 of appendix 2. The national strategic plan has subsequently led to and assisted in the adoption and introduction of different governmental policies and strategies to assist in the development and growth of the Irish aquaculture sector *e.g.*, Food Harvest 2020 and Food Wise 2025.

In 2010, the DAFM put forward its national strategy for the sustainable development of the Irish Agri-Food industry, including the seafood sector *i.e.*, Food Harvest 2020 (DAFM, 2010). This was a ten-year plan providing a framework for development of the industry. Table A2.3 (appendix 2) lays out the overall "Smart, Green, Growth" vision for Food Harvest 2020 (DAFM, 2015b, 2010). The strategy sets

out smarter and greener ways to deliver sustainable growth, recommending a suite of actions, on a sub-sectorial basis, to support the Agri-Food industry's development (DAFM, 2010).

In 2015, five years on from Food Harvest 2020, the DAFM revised the strategy and launched a new updated strategy, Food Wise 2025 (DAFM, 2015a). This updated version was introduced to ensure the course of growth in the Irish Agri-Food industry, as a result of the Food Harvest 2020 strategy, would continue (DAFM, 2015a, 2010). Food Wise 2025 has projected that the Agri-Food industry, or more specifically exports, have the potential to grow to €19B per annum by 2025 (DAFM, 2015a). This is an increase of €7B from the predicted figures of Food Harvest 2020 (Table A2.3 of appendix 2). This growth will be achieved by expansion in the dairy, beef, consumer foods and drinks, and in the seafood sectors (DAFM, 2015a). As part of the Food Wise 2025 strategy and in order to assist in meeting this goal, it has been predicted that the Irish aquaculture industry has the potential to increase export production to 81,700 tonnes by 2023 (DAFM, 2015a). Currently, approximately 38,000 tonnes are produced in Ireland per annum, having suffered a decline in production since 2017 primarily due to limitations in space and capacity (Dennis *et al.*, 2020). As part of the new strategy, SWOT analysis was also revised (Table A2.4 of appendix 2). This highlighted that despite the predictions mentioned above, several issues have continued to hamper the industry, constraining it from reaching thee afore mentioned goals. Examples of these issues include issues with the Irish aquaculture licensing process and the adoption of several environmental EU directives.

2.2.3 ISSUES IN IRISH AQUACULTURE

Despite its numerous advantages, the rapid increase in aquaculture production has resulted in the development of several issues within the industry which include limitations in water and space, increased incidences of disease and increased environmental concerns (Ngo *et al.*, 2016; Troell *et al.*, 2017; Han *et al.*, 2019; O'Neill *et al.*, 2019). Stenevik and Sundby (2007) have also indicated that variations in climatic conditions have demonstrated substantial effects on increases as well as decreases in stocking densities. Therefore, the success of fish stock assessment depends to a large extent on the ability to predict impacts climate change has on the dynamics of aquatic ecosystems. These treats have hindered the sustainable development and expansion of the industry (Han *et al.*, 2019).

In Ireland, after the SWOT analysis conducted via the National Strategic Plan for Sustainable Aquaculture Development and the two government initiatives set out to assist in the growth and development of the aquaculture industry in Ireland (Food Harvest 2020 and Food Wise 2025), the

main issues of concern within the Irish aquaculture system were associated with environmental concerns resulting in problems with the aquaculture licensing process and space limitations.

2.2.3.1 AQUACULTURE LICENSING

Unlike other European countries (Alexander *et al*., 2015), the Irish licensing process is very complex, requiring consultation and determination from various state bodies and the general public, resulting in the balancing of many different interests (Moylan *et al.*, 2017). Irish aquaculture licensing is regulated under Section Six of the Fisheries (Amendment) Act, 1997 and states that any person wishing to engage in aquaculture on the land or sea of Ireland are obliged to be licensed with an appropriate aquaculture licence. Additionally, those wishing to partake in mariculture also require an additional foreshore licence. If a land-based aquaculture licence is sought, planning permission from local authorities and a discharge licence from the EPA, is also required. Regulations associated with the Fisheries (Amendment) Act 1997, have been also been amended, giving effect to various EU directives associated with environmental protection which has had a knock on effect on the licensing process (Moylan *et al.*, 2017). Natura Impact Statements (NIS) and Environmental Impact Assessments (EIA) / Statements (EIS) may need to be incorporated into applications, and land-based aquaculture applications additionally require water quality assessment reports. Applications may be subjected to environmental assessments under natural habitats regulations if the aquaculture facility is located within or close to Natura 2000 conservation sites. Applications for facilities in Natura 2000 conservation areas require appropriate assessment to ensure environmental compliance with the EU Habitats and Birds Directives (DAFM, 2018a, 2018b). Facilities outside of Natura 2000 sites still may also require environmental assessment (DAFM, 2018a, 2018b; Office of the Attorney General, 1997). See Table A1.5 in appendix for a break down all the criteria considered by licensing authorities.

In December 2016, the Minister for Agriculture, Food and the Marine tasked an independent group to review the Irish aquaculture licensing process (Moylan *et al.*, 2017). The requirement for this review arose from the commitments made in the Food Wise 2025 strategy and the National Strategic Plan for Sustainable Aquaculture Development (DAFM, 2015a, 2015b; Moylan *et al.*, 2017). The main objectives of the review group were to identify the changes required for the licensing process and its legal framework. The review group submitted their report to Minister Michael Creed on 31st May 2017. Issues surrounding the licensing process highlighted during the review included;

i. The length of time it takes for decisions on the licence applications to be decided.
ii. The conditions and period of the licence.
iii. The lack of transparency surrounding the licensing process.

iv. The wide variety of public bodies that play a role in the process.

It was also suggested that the current legislation may require consolidation and / or that the current act may be outdated and unworkable (Moylan *et al*., 2017). Ultimately, the review group determined that a complete overhaul of the licensing process would be required in order to meet both the terms and conditions of the range of EU directives for environmental protection adopted by Ireland, and the commitments made by the government in the Food Wise 2025 strategy and the National Strategic Plan for Sustainable Aquaculture Development (Moylan *et al.*, 2017).

2.2.3.2 Environmental Concerns

As previously mentioned, the Irish aquaculture licensing process is now also subjected to a number of EU directives, primarily in relation to EIA, *i.e.* Environmental Assessment Directives 85/337/EEC, 97/11/EC and 2003/35/EC and Strategic Environmental Assessment (SEA) 2001/42/EC, and protection of birds *i.e.,* Birds Directive 79/409/EEC & 2009/147/EC, and habitats *i.e.,* Habitats Directive 92/43/EC (Moylan *et al.*, 2017; DAFM, 2018c). There are also overlapping and similarities between these directives and the WFD, 2000/60/EC. Adoption of some of these directives has had major implications on the licensing process. For example, in 2007 the European Union Court of Justice gave an adverse ruling against Ireland in relation to Natura 2000 resulting in the implementation of the Birds and Habitats directives. This effectively stalled the licensing process until appropriate assessments could be carried out on aquaculture licence applications located in Natura 2000 sites (Moylan *et al.*, 2017). The most notable adopted directives associated with this research are the EU directives brought in under the Natura 2000 strategy and the WFD.

2.2.3.3 Natura 2000

The rapid loss of biodiversity as a result of deterioration in habitats, over-exploitation, invasive species and pollution are some of the continuing environmental challenges faced in Europe (Orlikowska *et al*., 2016). In 1992, the EU adopted legislation designed to protect habitats and species across Europe that were considered to be under serious threat (European Commission, 2000). Natura 2000 is a networking program designed to build on the Natura Network Initiative 2004 – 2006 (European Commission, 2000), aiming to ensure the long-term survival of Europe's most valuable and threatened species and habitats (European Commission, 2017a). According to Blicharska *et al.* (2016), it is thought to be considerably different from previous conservation initiatives adopted by the EU as it goes beyond out-right bans on unnecessary killings of animals and damaging of plants by focusing on sustaining conservation from a social point of view, resulting in harmonising the maintenance of habitats and species with the social, cultural and economic needs of humans. The adopted legislation

includes the Habitats Directive 92/43/EEC which complements the Birds Directive 79/409/EEC adopted in 1979 (European Commission, 2017a; Kati *et al.*, 2015). The Natura 2000 network has resulted in the protection of core breeding and resting sites of rare, vulnerable and threatened species and habitats (European Commission, 2017a). The network is the largest collection of protection areas across the world, stretching over 6% of the EUs marine territory and 18% of its land area (European Commission, 2017a; Kati *et al.*, 2015). In Ireland there are over 400 bird species, 28 land mammal species, over 12,000 insect species and over 4,000 species of plants protected (National Parks & Wildlife Service, 2018). Rare and vulnerable species such as the Blue Cornflower and Corncrake were found in abundance in Ireland over 50 years ago but have now almost disappeared. This decrease has been considered to be linked to changes in agriculture processes and practices (National Parks & Wildlife Service, 2018). The National Parks and Wildlife Services (NPWS) is responsible for designating sites of conservation in Ireland. They work with landowners, farmers, local authorities and national authorities to ensure a balance between land use and conserving nature is achieved (National Parks & Wildlife Service, 2018; Visser *et al.*, 2007). Figure 2.8 displays the location of all the current Natura 2000 sites in Ireland.

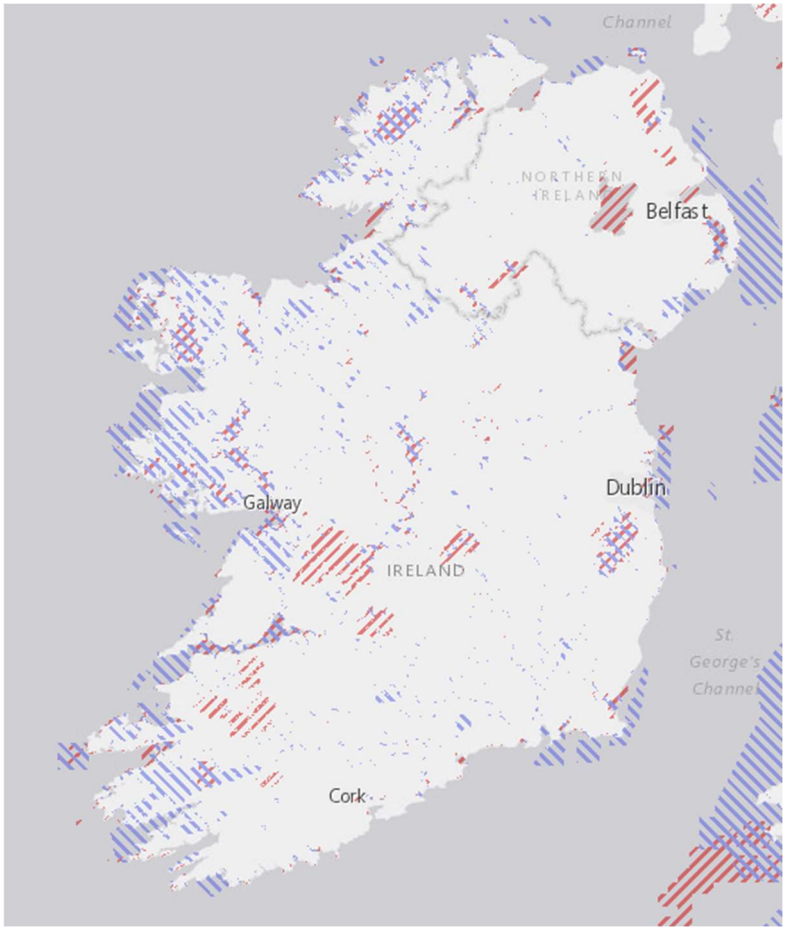


Figure 2.8: **Location of all Natura 2000 Network Sites in Ireland.** Blue areas highlighted indicate areas protected under the Habitat Directive and red areas highlighted indicate areas protected under the Birds Directive (Source: European Environmental Agency).

2.2.3.4 THE BIRDS DIRECTIVE

There are more than 500 wild bird species across Europe but approximately 32% are not in a good conservation status (European Commission, 2016a). The Birds Directives 79/409/EEC & 2009/147/EC, main aim is to protect all of these naturally occurring species (Donald *et al*., 2007; European Commission, 2016a; European Parliament and European Council, 2010). According to the European Commission (2016) expansion of urban areas and transport networks have disjointed and reduced the birds habitats; intensive fishing, agriculture and forestry processes, and the use of chemical pesticides have weakened their supply of food; and hunting for sport required regulation to prevent major damage to their populations. These are some of the main reasons why the directive was adopted in 1979 (Donald *et al*., 2007; European Commission, 2016a). The directive was amended in 2009 (2009/147/EC) and is the oldest piece of EU legislation associated with the environment (European Commission, 2016a). Birds are protected in various ways under the Birds Directive 79/409/EEC and 2009/147/EC;

i. The areas where the species and sub-species that are particularly vulnerable and under threat have been designated as Special Protection Areas (SPAs), see Figure 2.9.
ii. Species that can be hunted (82 species), can only be done so during specific times of the year and may not be done so in areas where these birds are at their most vulnerable *i.e.*, areas of nesting, reproduction and raising of chicks.
iii. Activities that directly threaten any of the birds protected under the directive are completely banned including destruction of nests and deliberate killing, capture or trade. It should be noted that some of these activities are allowed for approximately 26 species however are under very strict restrictions.
iv. Sustainable hunting management is provided by the directive however, all forms of large-scale and non-selective killing is illegal. Particularly *via* methods not listed in the directive.
v. The directive promotes research to reinforce management, protection and use of all listed species.

(Donald *et al*., 2007; European Parliament and European Council, 2010)

The Ornis Committee assists in the implementation of the directive (European Commission, 2016a; Ornis Committee, 2016).

2.2.3.5 THE HABITATS DIRECTIVE

The Habitats Directive 92/43/EEC was designed to assist in the conservation and preservation of wild areas of rare, vulnerable / threatened or prevalent plant and animal species (European Commission, 2016b; Evans, 2006). The directives main objective is the conservation of wild habitats (including

peatlands), flora and fauna, and aims to provide and promote biodiversity maintenance, taking social cultural, economic and regional requirements into consideration (European Commission, 2016b; European Council, 1992; Evans, 2006; Wätzold and Schwerdtner, 2005). It is considered the corner stone of the Birds Directive 79/409/EEC & 2009/147/EC, and the Natura 2000 initiative (European Commission, 2016b). The Habitats Directive 92/43/EEC was originally adopted in 1992 (European Commission, 2016b; European Council, 1992). Two hundred habitat types and over 1000 plant and animal species are protected under the directive (European Commission, 2016b; Evans, 2006), and are done so in a number of ways;

i. The habitats of approximately 900 species have been designated as Sites of Community Importance (SCIs), which must be managed in accordance with the ecological needs of these species. These SCIs are also included in the Natura 2000 network.
ii. Strict protection regimes are required and must be applied across the natural range or habitat of approximately 400 species. This applies to sites both inside and outside of the Natura 2000 network.
iii. Exploitation of approximately 90 habitats in particular must be compatible with maintaining a favourable conservation status.

(European Council, 1992; Evans, 2006; O'Keeffe and Dromey, 2004).

The directive also requires the conservation status of Special Areas of Conservation (SACs), see Figure 2.9, to be regularly reported on and provide information of compensation measures proposed or in place by projects that may have negative impacts on Natura 2000 sites (European Commission, 2016b; O'Keeffe and Dromey, 2004).

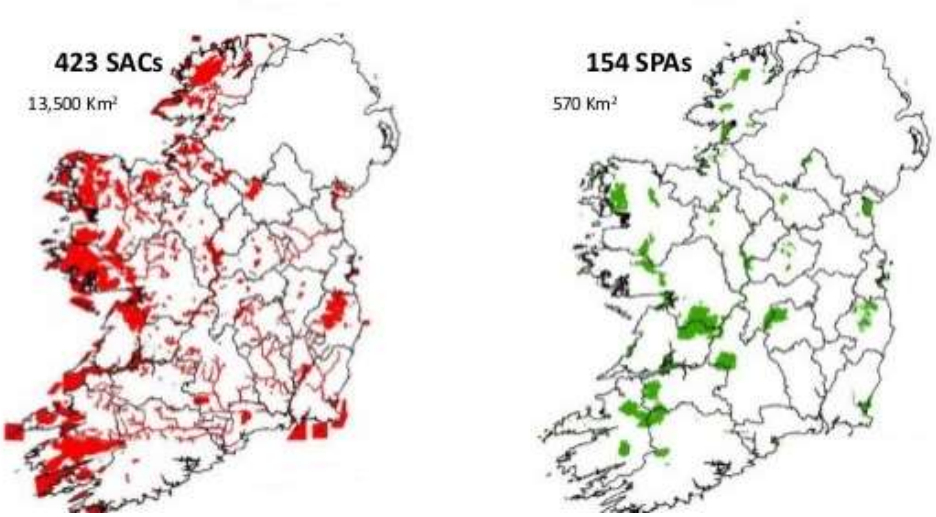


Figure 2.9: Red indicates the locations of 423 **Special Areas of Conservation (SACs),** under the Habitats Directive across 13,500 Km^2 of Ireland. Green indicates the locations of 154 **Special Protected Areas (SPAs),** under the Birds Directive across 570 Km^2 of Ireland (Source: EPA)**.**

2.2.3.6 *WATER FRAMEWORK DIRECTIVE*

Water is vital for generating and sustaining economic growth, humanity and prosperity *i.e.*, water critically supports life and should therefore be protected (European Commission, 2010). The EUs WFD 2000/60/EC was adopted in 2000 as a result of an increasing demand from environmental organisations and the general public for cleaner lakes, rivers, oceans and groundwater (European Commission, 2016c). According to Bourblanc *et al.* (2013) and Voulvoulis *et al.* (2017), the WFD may be widely accepted as one of the most ambitious and substantially important pieces of European legislation associated with the environment to date. The main aim of the directive is to provide a structure for water protection to assist EU member states to achieve good water status in all waters and ensure this status does not depreciate (Voulvoulis *et al*., 2017; WFD Ireland, 2018a). The objectives of the directive, along with the achievement of good water status include; expansion of the range of protection to all waters; management of all waters based on river basins or catchments; restructuring of legislation; enactment of a combined approach to improve standard water quality and limit emissions; and to get the general public more closely involved where possible (Cabezas, 2012; WFD Ireland, 2018b). Member states were to achieve good water status in all waters during what is now known as the first cycle, running from 2009 to 2015 (European Commission, 2016c; Voulvoulis *et al*., 2017; WFD Ireland, 2018b). However, this has not yet been achieved (Voulvoulis *et al*., 2017). The implementation of, what might be considered by some as an overly ambitious directive (Bourblanc *et al.*, 2013; Voulvoulis *et al*., 2017) has been found to be a difficult and lengthy process for most EU member states, including Ireland (Bourblanc *et al*., 2013). Extensions have subsequently been granted resulting in the introduction of a second cycle running from 2015 to 2021 and third cycle running from 2021 to 2027 (WFD Ireland, 2018b), at the end of which is the final deadline for member states to meet thee afore mentioned objectives (European Commission, 2016d). The WFD also has the potential to supplement the Habitats Directive 92/43/EEC and the Birds Directive 79/409/EEC & 2009/147/EC in improving the environmental status of aquatic Natura 2000 sites (Bennett and Sheate, 2000; Boeuf and Fritsch, 2016; Ioana-Toroimac, 2018).

2.2.4 *AQUACULTURE WASTEWATER*

The dramatic increase in the growth of global aquaculture production has displayed its augmented importance in modern day food supply (Jegatheesan *et al*., 2011) by providing a means to meet the growth in global demand (Seoane *et al*., 2014). However, despite this and many other advantages (Jegatheesan *et al.*, 2011; Martinez-Porchas *et al.*, 2014), there are many environmental concerns thought to be associated with aquaculture (Martinez-Porchas *et al*., 2014; Ngo *et al*., 2016; Troell *et*

al., 2017), and in particular to the impacts aquaculture output water is thought to have on the receiving aquatic ecosystem (Jegatheesan *et al.*, 2011).

Aquaculture output is loaded with nutrient rich waste products (Jegatheesan *et al.*, 2011; Martinez-Porchas *et al.*, 2014; Ngo *et al.*, 2016) which if released untreated into water bodies can lead to water pollution (Jegatheesan *et al.*, 2011) and potentially cause issues with meeting the objectives of the WFD. The water pollution could result in indirect negative effects such as damage or loss of habitats (Troell *et al.*, 2017) which in turn could affect the objectives of the Natura 2000 initiative. It may also cause direct negative effects such as eutrophication (Jegatheesan *et al.*, 2011; Martinez-Porchas *et al.*, 2014; Ngo *et al.*, 2016; Troell *et al.*, 2017) which is one of the greatest concerns in relation to aquaculture output discharge (Ngo *et al.*, 2016). Eutrophication (Figure 2.10) is a process by which a water body receives large levels of nutrients and organic matter that can be taken in and biologically processed (Martinez-Porchas *et al.*, 2014). This in turn results in increased levels of algal blooms and decreased levels of oxygen which can suffocate aquatic life in the water body (Jegatheesan *et al.*, 2011; Chislock *et al.*, 2013; Ngo *et al.*, 2016).

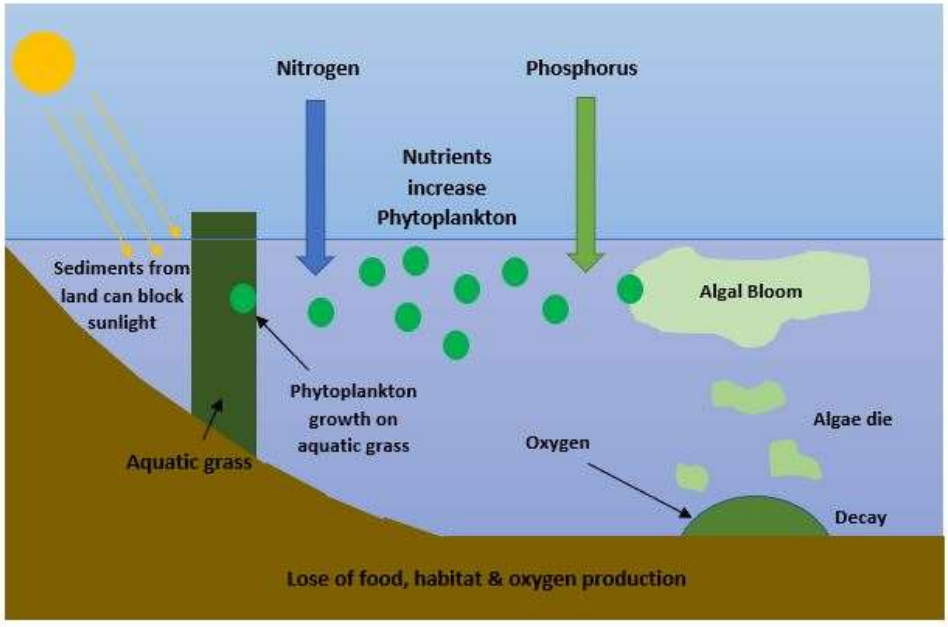


Figure 2.10: **Process of eutrophication** as a result of high levels of nitrogen and phosphorus leading to algal blooms and loss of food, habitats and oxygen production.

Aquaculture output is commonly characterised by rich levels of nutrients, such as nitrogen (N) and phosphorus (P), and organic matter (Jegatheesan *et al.*, 2011; Ngo *et al.*, 2016). These characteristics are as a result of uneaten or left over food and metabolic waste products such as faeces and urea (Jegatheesan *et al.*, 2011; Martinez-Porchas *et al.*, 2014; Ngo *et al.*, 2016; Troell *et al.*, 2017). The

primary source of N, P and organic matter in the water is derived from the application of feed (Jegatheesan *et al.*, 2011) which is necessary to maintain high production yields in order to meet demands (Feucht and Zander, 2015; Kolarevic *et al.*, 2014). Another source of organic matter is the intake water used to fill ponds or raceways and maintain the farms water levels (Jegatheesan *et al.*, 2011). The level of organic matter and nutrients is thought to be highly dependent on the scale of production, type of culturing system used, the species of fish being cultured, the feeding patterns, the farm management practices and the treatment processes used (Ngo *et al.*, 2016). For example, FTS have a much higher flow rate when compared to traditional pond and RAS systems. However the much higher flow rate usually results in a much lower concentration of pollutants due to the high level of dilution, whereas ponds and RAS usually have much lower flow rates and in turn, higher nutrient concentrations in their output water (Ngo *et al.*, 2016).

2.2.5 WASTEWATER TREATMENT

Excretions from fish being raised on aquaculture facilities *i.e.*, N, P, organic matter, *etc.*, can accumulate in the water and increase toxicity (Turcios and Papenbrock, 2014). The discharge of output water without pre-treatment has the potential to cause major issues in the receiving water system *e.g.,* eutrophication (Jegatheesan *et al.*, 2011). This can result in severe effects which can reverberate from a local and regional level to a global scale *e.g.*, it has been estimated that human induced eutrophication induces a total economic loss of $2.2B in the United States per annum (Sharrer *et al.*, 2016). As a result the capacity to habitually and assuredly decrease waste production could contribute to a range of environmental, social and economic benefits (Siddiqui, 2003; Tsukuda *et al.*, 2015). According to Jegatheesan *et al.* (2011), freshwater resources are weakening at an alarming rate and water quality regulatory bodies are imposing increasingly stringent standards for output water discharge. Aquaculture wastewater treatment systems are therefore necessary to meet discharge requirements (Bergheim and Brinker, 2003).

A range of physical, chemical and biological techniques utilised in traditional wastewater treatment have been applied to aquaculture wastewater treatment (Turcios and Papenbrock, 2014). The choice of treatment employed should take a range of conditions into consideration such as; flow rate, concentration of solids, the culture tank / pond design, the land availability, costs and current environmental regulations (Bergheim and Brinker, 2003). Solids can be removed by mechanical filtration or sedimentation (Bergheim and Brinker, 2003; Siddiqui, 2003; Turcios and Papenbrock, 2014). Sedimentation is one of the simplest forms of waste removal whereby it allows the solid particles to settle out of the output water before it is released, however sedimentation systems

normally require large areas of land (Siddiqui, 2003). Mechanical filtration such as screening is thought to be the most common solid waste removal system used whereby wastewater is passed through large filter screens *e.g.,* drum filters, which remove the particles (Bergheim and Brinker, 2003; Turcios and Papenbrock, 2014). However, post-mechanical methods also need to be employed to remove nutrients and suspended solids from the output water (Bergheim and Brinker, 2003). Aeration devices such as paddle wheels, air lifts or surface aerators can be used for the nitrification of nitrogenous waste nutrients however not denitrification (Jescovitch *et al.*, 2017) resulting in additional treatment requirements. See Figure 2.11 for the nitrification / denitrification process.

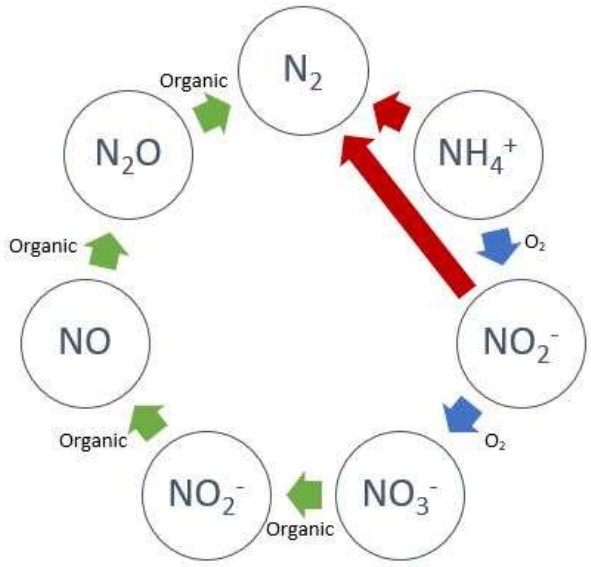


***Figure 2.11*: The nitrogen cycle displaying the nitrification and denitrification process**. Blue indicates nitrification. Green indicates denitrification. Red indicates the anammox process which is anaerobic ammonium oxidation.

2.2.6 IRISH AQUACULTURE WASTEWATER

Irish wastewater is regulated by the Environmental Protection Agency (EPA), under the Waste Water Discharge (Authorisation), Regulations, 2007 (EPA, 2018a, 2018b) which gives effect to the WFD, the Birds Directive and the Habitats Directive (Department of Environment Heritage and Local Government, 2007; EPA, 2018a). As part of these regulations, anyone who wishes to release any form of industrial effluent, including aquaculture output water, into the ecosystem must have a wastewater discharge licence (Department of Environment Heritage and Local Government, 2007; EPA, 2018a, 2018b).

The main purpose of the licence is to make necessities for environmental protection as well as for human, animal and flora protection against the release of hazardous and / or priority substances from wastewater works into aquatic environments (Department of Environment Heritage and Local

Government, 2007; EPA, 2018a, 2018b). Licensing encourages the use of wastewater treatment techniques, the regularisation of wastewater discharges, the enhanced efficiency and effectiveness in pollution control, and allows for a regulatory system that is open and transparent (EPA, 2018b). The licensing process regulates wastewater discharge from industrial wastewater works, such as aquaculture facilities, with the exception of unpolluted storm-water discharges from designated storm-water collection systems and wastewater treatment plants including; wastewater, odours, sludge disposal and noise from the treatment plants (Department of Environment Heritage and Local Government, 2007; EPA, 2018a, 2018b). The wastewater discharge authorisation process allows the EPA to put into place stringent discharge operations to ensure the limitation and control of potentially adverse effects on the receiving water bodies (EPA, 2018a). As a result, authorisation for a wastewater discharge licence can be refused if the EPA deems; the proposed works effluent will deteriorate the ecological or chemical status of the receiving water body (surface or ground), if there is a failure to include prevention and limitation measures for hazardous substances or substances present in so low a concentration that they are not deemed hazardous, or if the achievement of the objectives of any adopted environmental directives have been inconsistent, compromised or excluded (Department of Environment Heritage and Local Government, 2007; EPA, 2018b).

Although aquaculture is proving to be more and more important for food security, the aforementioned issues will continue to hamper the growth of the industry unless sustainable "green processes" are investigated in order to alleviate these pressure points. One such area that holds great potential in addressing these concerns is the application and utilisation of algae by providing a natural biological approach to alleviate pressure points and improve processes for industry development.

2.3 ALGAE

Algae are a diverse group of eukaryotic organisms (Pepper and Gentry, 2015). They are thallophytes (Krienitz, 2009). These are plants that lack leaves, stems and roots (Sambamurty, 2017). Algae are autotrophic organisms with chlorophyll *a* as their primary photosynthetic pigment (Nautiyal *et al.*, 2014). They range from single-celled organisms to multicellular organisms, like seaweed (García-Garibay *et al*., 2014). In fact algae represent one of the largest ranges in size of any group in the plant kingdom (Sambamurty, 2017). Some can grow to more than 100 ft *e.g.*, giant kelp. These are known as macroalgae. The smallest algae (microalgae) can be up $6x10^{12}$ times smaller than giant kelp (Nautiyal *et al*., 2014). Algae also have one of the largest ranges of habitation (Sambamurty, 2017). They can be found in almost any environment on earth (Khan and Rao, 2019; López-Gómez and Pérez-

Rivero, 2019). They inhibit both marine and fresh waters including; ponds, streams, warm springs, lakes and oceans (Sambamurty, 2017). However, they can also be found in the snow of some mountains, in hot springs, in desert soils and in lichens growing on rocks (Khan and Rao, 2019; López-Gómez and Pérez-Rivero, 2019; Sambamurty, 2017).

It is thought that no other plant group exhibits so many different pigmentations. These pigmentations include; chlorophylls which are green, xanthophylls which are yellow, carotenes which are orange and phycobilins which consist of blue phycocyanin and red phycoerythrin (Sambamurty, 2017). Algae can be green, blue-green, brown, red, yellow-green or golden brown depending on the predominant pigment (Khan and Rao, 2019; Sambamurty, 2017) Blue-green algae exhibit the widest range of colours; red, orange, yellow, green, blue, purple, violet, rose, blue-green and brown (Kaštovský *et al*., 2019; Sambamurty, 2017).

Algae function as the primary producer in the food chain of most habitats (Lembi, 2003; Minhas *et al*., 2020; Sambamurty, 2017; Stevenson and Smol, 2003), producing organic matter from CO_2, water (H_2O) and sunlight (Lembi, 2003; López-Gómez and Pérez-Rivero, 2019; Nautiyal *et al*., 2014). In addition to providing the base food source in these habitats, they also produce O_2 necessary for metabolising the consumed organisms (Sambamurty, 2017). Some algae are harvested and eaten as vegetables, particularly brown and red macroalgae (Barberi *et al.*, 2020; Denis *et al.*, 2010; Gadberry *et al*., 2018; Kawai and Murata, 2016; Sambamurty, 2017). For example, mucilage can be extracted from the thallus of seaweeds to be used as thickening and gelling agents (Sambamurty, 2017) such as agar (Martínez-Sanz *et al*., 2019; Michalak and Chojnacka, 2015, 2014).

Algal reproduction is highly versatile. They can reproduce both sexually and asexually (Raven and Giordano, 2014; Wetzel, 2001). Sexual reproduction involves the formation of eggs within the oogonia and sperm within the antheridia (Raven and Giordano, 2014). The egg and sperm fuse forming a diploid zygote resulting in a vegetative algal cell (John and Rindi, 2015; Raven and Giordano, 2014). Algae asexually reproduce through binary fission or fragmentation where fragments of filamentous algae break off and continue to grow (John and Rindi, 2015). Binary fission is particularly prevalent among the single-cell algae (Wetzel, 2001). Some algae can produce spores that can germinate into fully functioning vegetative cells (John and Rindi, 2015; Raven and Giordano, 2014).

2.3.1. ALGAL CLASSIFICATION

The classification of algae is highly complex, with several different variations based on many different factors such as; reproduction, pigmentation, morphology, development, biochemistry and phylogenetic relationships (Sambamurty, 2017). From a historical context, the foundation of algal classification was first set out by Carolus Linnaeus in 1753 (Baweja and Sahoo, 2015; Sambamurty, 2017). Variations of classification have been developed from then up to the late 1960's, as shown in Table A2.6 of appendix 2. The classification set out by F.E. Fritsch will be focused on as it has been considered by many to be one of the most practical classifications (Sambamurty, 2017). In 1935, F.E. Fritsch classified algae into eleven classifications based on five main criteria; pigmentation (which will be the main focus in this research), flagellation, reserve food nature, cell structure details and reproductive mode (Baweja and Sahoo, 2015; Fritsch, 1944; Sambamurty, 2017).

- Chlorophyceae is the first class of algae. The chloroplasts contain chlorophyll *a*, chlorophyll *b*, β-carotene and xanthophyll. Chlorophyceae are traditionally green algae as the dominant pigments are chlorophyll *a* and *b*. They are most commonly found in freshwater systems than marine systems and include some of the most common species *e.g.*, *Chlorella vulgaris*.
- Xanthophyceae are a smaller group than chlorophyceae. These classes of algae contain β-carotene, xanthophylls and a small amount of chlorophyll *e*. Xanthophyceae are yellow-green algae. Much like chlorophyceae, they too are more widely distributed in fresh waters.
- Chrysophyceae are a large group of algae mostly in freshwater. They contain carotenoids, fucoxanthin, diadinoxanthin and chlorophyll *a*. These are golden algae as the chlorophyll *a* is considerably less dominant than the other pigments.
- Bacillariophyceae inhibit both marine and fresh waters. They are yellow algae that are most commonly referred to as diatoms. They are a major group of algae. They contain the pigments β-carotene, fucoxanthin and diatoxanthin, as well as chlorophyll *a* and *c*, which are less dominant than the previous pigments mentioned.
- Cryptophyceae are a smaller group of algae when compared to the previous classes mentioned. They are found in all water environments but more commonly in freshwater than marine and brackish waters. Cryptophyceae are brown or 'nearly' brown algae and contain chlorophyll *a*, chlorophyll *b*, carotene, diatoxanthin, phycocyanin and phycoerythrin.
- Dinophyceae are dark yellow-brown flagellated algae. They contain chlorophyll *a*, chlorophyll *c*, carotene and dinoxanthin. They are plankton organisms and are more commonly found in marine waters than freshwater.

- Chloromonadineae are a small class of algae that are now more commonly known as Raphidophyceae. They are unicellular with large cells. They contain excess levels of xanthophyll and are considered as bright green algae. They are found in both freshwater and marine environments.
- Euglenineae are a small group of algae that are considered as 'naked' as they have no cell wall. The main photosynthetic pigments include chlorophyll *a*, chlorophyll *b*, β-carotene, neoxanthin and astaxanthin. Euglenineae are found in both marine and fresh waters.
- Phaeophyceae are a large group of multicellular brown algae that include many seaweeds and as such, the majority are found in marine waters. They have the greatest morphological complexity than any other class. The pigments present in Phaeophyceae are chlorophyll *a*, chlorophyll *c*, carotenes, fucoxanthin and diatoxanthin.
- Rhodophyceae are a large group of multicellular red algae that, similarly with Phaeophyceae, include many seaweeds. They too are primarily found in marine waters. Rhodophyceae contains r-phycoerythrin, r-phycocyanin, chlorophyll *a*, chlorophyll *b* and carotenoid tetraxanthin.
- Myxophyceae which is also known as cyanophyceae, are blue-green algae. They are found in abundance in freshwaters and a limited number are found in marine waters. Unlike all of the other classes, myxophyceae are prokaryotic. The main photosynthetic pigments present are chlorophyll *a*, c-phycocyanin, c-phycoerythrin, myxoxanthin and myxoxanthophyll (Baweja and Sahoo, 2015; Fritsch, 1944; Sambamurty, 2017).

2.3.2. POSITIVES & NEGATIVES OF ALGAE

Like most things in this world, there are both advantages and disadvantages to algae in water systems. This sub-section will focus on the main advantages of algae to aquatic ecosystems (the brighter side of algae) and the one main disadvantage of algae (the darker side of algae) which is the development of harmful algal blooms (HAB's) and their main instigator (cyanobacteria).

2.3.2.1. BRIGHTER SIDE OF ALGAE

Algae have displayed many advantages which have demonstrated their vast importance to a range of ecosystems (Borowitzka and Hallegraeff, 2007) including; oxygen generation, symbiotic relationships with their surrounding environment, pollution monitoring and their position in the food chain. In fact, they are often considered as one of, it not the most important "plants" in the world (Chapman, 2013). Algae release oxygen as part of their metabolism (Homann, 2003). Photosynthesis is a process used by plants and some organisms to convert light energy into chemical energy that is later released to fuel other organism's activities (Cardona *et al*., 2018). Photosynthesis in plants generally involves the

green pigment chlorophyll (Emerson, 1929) and as a result, green algae are often more favoured given their higher levels of chlorophyll compared to other types of algae (Håkanson *et al*., 2003). The chlorophyll captures light energy from the sun which catalyses a redox reaction and converts CO_2, H_2O and minerals into O_2 and energy-rich organic compounds, as shown in the equation in Figure 2.12 (Foyer and Noctor, 2009). The oxygen is released back into the water which is then used by fish and other aquatic organisms (Falkowski and Knoll, 2007; Lutz *et al*., 2018).

$$6\ CO_2\ \&\ 6\ H_2O \xrightarrow[Sunlight]{Chlorophyl} 6\ O_2\ \&\ C_6H_{12}O_6$$

Figure 2.12: **Equation** for the **photosynthesis reaction** conducted in algae.

In addition to producing O_2, algae can also aid in monitoring water pollution (Gokce, 2016). Algae can help control nutrient levels in water bodies. They consume N and P nutrients present in water systems for growth (Ren *et al*., 2017; Wurtsbaugh *et al.*, 2019). However, excessive levels of nutrients can lead to eutrophication and algal blooms (Pal *et al*., 2020), and HAB's also, if cyanobacteria are present at sufficient concentrations (Brookfield *et al*., 2021; Pal *et al.*, 2020; Patel *et al*., 2020). As such, alterations in the species composition, growth and productivity can provide a means of monitoring water quality (Gokce, 2016) *i.e.*, algae are an example of very useful bioindicators (Bellinger and Sigee, 2015a). A bioindicator is a living organism that can provide an impression of the health of an ecosystem (Parmar *et al*., 2016). Some organisms, including algae, are very sensitive to pollution in their environment therefore if pollutants are present the organisms may be altered chemically, physically or behaviourally, or possibly even die (Bellinger and Sigee, 2015a; Burger, 2010; Glazier, 2014; Mothersill and Seymour, 2016; Parmar *et al.*, 2016). As such, monitoring these changes can provide an early indication to the health of the environment / ecosystem / water quality (Burger, 2010; Glazier, 2014; Mothersill and Seymour, 2016; Nikinmaa, 2014).

Biological life cannot live completely isolated from other organisms. Living organisms co-exist through vast and complex interactions that sustain ecosystems (Sharman, 2006). Algae makes itself useful by maintaining symbiotic relationships with other aquatic organisms (Ramanan *et al*., 2016; Wooldridge, 2010). Symbiosis is a relationship between two organisms in which at least one organism benefits (Douglas, 2008). For example; some green algae have a mutual symbiotic relationship (both organisms benefit from the relationship) with coral. The algae live near the exterior of the coral where they metabolise and produce the O_2 and glucose the coral requires for growth. In return, the coral protects the algae from predators (Wooldridge, 2010).

Algae are important primary producers in aquatic ecosystems (Chapman, 2013; Kaštovský *et al*., 2019). Primary producers are the foundation of all ecosystems (Falkowski and Knoll, 2007). They provide the basis of the food chain by creating food through chemosynthesis or photosynthesis *i.e.*, they are autotrophic, and as such are vital to the survival of the ecosystem (Chapman, 2013; Falkowski and Knoll, 2007). They provide / produce the carbohydrate sugars necessary for organisms higher up in the food chain to grow, reproduce and ultimately survive (Chapman, 2013; Kaštovský *et al*., 2019). Primary producers reproduce rapidly which is necessary to sustain life as populations get much smaller, the further up the food chain *e.g.*, one pound (lb) of apex predators requires roughly 100,000 lb of algae at the primary producer level to survive (Falkowski and Knoll, 2007).

2.3.2.2. Darker Side of Algae

The one main disadvantage, and possibly the most common feature when discussing algae, is their ability to develop blooms (Brookfield *et al*., 2021; McGowan, 2016; Pal *et al*., 2020). Algae are highly beneficial to aquatic ecosystems (Lembi, 2003) however, when levels get too high there can be major issues (Brookfield *et al*., 2021). An algal bloom is a rapid augmentation or accumulation in the algal population of an aquatic ecosystem (Patel *et al*., 2020) and is most recognisable by the discolouration of the water due to algal pigments (McGowan, 2016). The large and often excessive growth of algae occurs on or just below the surface of the water (Brookfield *et al.*, 2021; Pal *et al.*, 2020). These blooms can be induced as a result of organic pollution *e.g*., eutrophication (Pal *et al.*, 2020) or by a naturally occurring phenomenon (Sarkar *et al.*, 2019). They can develop in waters that are rich in the nutrient's algae require for growth *e.g.*, P and N (Brookfield *et al*., 2021). Changes in temperature *i.e.*, warmer waters, can also lead to increased growth resulting in blooms (Diaz and Yeh, 2014). Hence why this occurrence most often happens between late spring and autumn.

There are a range of terms used to describe these occurrences; microalgal blooms (Oyeku and Mandal, 2020), phytoplankton blooms (Hu *et al*., 2021), red tides (Guy, 2014; Patel *et al*., 2020), toxic algae (Nawaz and Sengupta, 2018) and / or HAB's (Park *et al.*, 2017). The major types of algal blooms that are of most concern is cyanobacterial / blue-green algal blooms and red tides / red algal blooms, both of which are considered HAB's (Guy, 2014; Li *et al*., 2019; Patel *et al*., 2020). These HAB's can have severe consequences on aquatic ecosystems, human health and the economy (McGowan, 2016). Algal blooms in fact can affect entire ecosystems (Paerl *et al*., 2016). Consequences range from benign feeding of higher trophic levels to much more adverse effects (Brookfield *et al*., 2021; McGowan, 2016; Pal *et al.*, 2020) *e.g.*, blocking sunlight from reaching aquatic organisms (Brodie *et al*., 2019),

depleting oxygen levels in the water (Wilhelm, 2009) and secreting toxins into the water (Brookfield *et al*., 2021; Pal *et al*., 2020).

HAB's can produce and release toxic compounds that can cause illness in humans, domestic pets, livestock and wildlife (Grattan *et al.*, 2016; Pal *et al*., 2020). Human illnesses caused by HAB's, although rare, can be debilitating or even fatal (Daguer *et al.*, 2018; Grattan *et al.*, 2016; Naik *et al.*, 2019). Although some algae can release these compounds, the most common cause of fish kills associated with algae are as a result of oxygen depletion (Wilhelm, 2009). As the algal bloom grows, they deplete the oxygen in the water. The bloom blocks sunlight reaching the fish and aquatic plants preventing photosynthesis from occurring thus limiting the further generation of oxygen (Brodie *et al.*, 2019; Wilhelm, 2009). At this point it is the extraction of oxygen for respiration in water at night that causes the most fish kills. When these algae eventually die off, the microorganisms used to decompose the dead algae further deplete the oxygen levels (Wilhelm, 2009). This in turn can lead to more fish dying or cause them to leave the area, if possible.

Cyanobacteria were formally known as blue-green algae (Sivonen, 2009; Vachard, 2021; Vincent, 2009). They are not technically algae but are aquatic photosynthetic prokaryotic microorganisms, unlike algae which are eukaryotic (Vachard, 2021; Zahra *et al.*, 2020). Cyanobacteria are considered to be the first oxygenic photosynthetic microorganism on earth contributing to the planet's atmospheric oxygen production for the past 3B years (Zahra *et al*., 2020). Many believe plastids *e.g., chloroplasts,* evolved from cyanobacteria (Sivonen, 2009; Zahra *et al*., 2020). This ancient group of bacteria occur in most waters and can have major effects on the water quality and the aquatic ecosystem's functionality (Garcia-Pichel, 2009; Vincent, 2009). Cyanobacteria are important primary producers in environments and form part of the phytoplankton (Sivonen, 2009; Vachard, 2021). Cyanobacteria possess cellular mechanisms that make them adaptable to environmental changes and grow both easily and with remarkable speed. Their growth rate and any potential issues associated with it depends on biotic factors and variations in nutrient levels (Zahra *et al*., 2020). Cyanobacterial growth is limited by the availability of P, where increased P concentrations in water bodies due to human activity has resulted in issues with cyanobacterial blooms or HAB's. Additionally, cyanobacteria in fresh waters tend to be much more abundant and diverse at higher pH levels (Whitton and Potts, 2012). More recently changes in growth rates has also been due to global warming as a result of climate change (Zahra *et al*., 2020).

There are more than 2000 species of cyanobacteria across roughly 150 genus that display a wide range of sizes and shapes (Vincent, 2009). Cyanobacteria also range in colour including; green, blue, black and red (Li and Liu, 2019). Some cyanobacteria have been used for human consumption for centuries for their beneficial properties (Frigaard, 2018). Natural products present in cyanobacteria have displayed a range of beneficial effects including; antimicrobial activity, antibacterial activity, antialgal activity, antifungal activity, antiviral activity, antiprotozoal activity and potential anticancer activity (Demay *et al.*, 2019). Other cyanobacteria are known for their adverse effects as a result of their toxicity (Frigaard, 2018). At least five main types of cyanobacteria have been identified as toxin producers; *Microcystis aeruginosa, Nodularia* species*,* two sub-species of *Anabaena flosaquae* and *Aphanizomenon flosaquae.* They can be broken down into three main toxin types; hepatotoxins, neurotoxins and lipopolysaccharide (LPS) endotoxins (Percival and Williams, 2014).

2.3.3. INDUSTRY APPLICATION

There has been a greater focus on algae research due to their potential application to a range of industries including; energy production (Adeniyi *et al.*, 2018; Gnansounou and Kenthorai Raman, 2016; Li *et al.*, 2018; Mhatre *et al.*, 2019; Sambusiti *et al.*, 2015; Shanmugam *et al.*, 2021; Sulfahri *et al.*, 2020), agriculture (Kusmayadi *et al.*, 2021; Li *et al.*, 2018; J. S. Singh *et al.*, 2019), nutraceuticals (Jimenez-Lopez *et al.*, 2021; Sharma and Sharma, 2017), pharmaceuticals (Sharma and Sharma, 2017) cosmetics (Ariede *et al.*, 2017; Aslam *et al.*, 2021; de Oliveira *et al.*, 2021; Wang *et al.*, 2017) and municipal wastewater (Li *et al.*, 2011; Wang *et al.*, 2010; Zhao *et al.*, 2018), as shown in Figure 2.13.

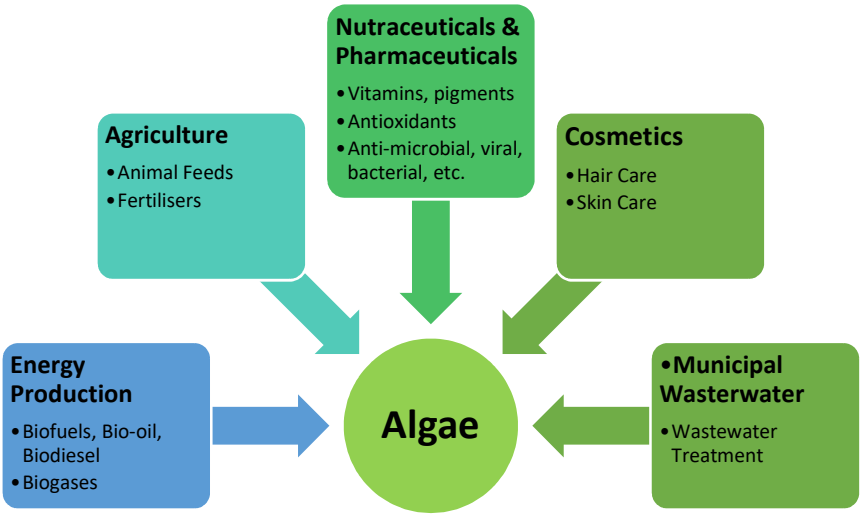


Figure 2.13: Overview of **algae application to** a range of **industries**.

2.3.3.1. ENERGY PRODUCTION

Fossil fuels *i.e.*, gas, oil, coal, *etc.*, have been the dominant global energy source since the industrial revolution (1760-1840). However, due to their continued use, especially from the 1950's onwards when consumption increased significantly (Richie and Roster, 2017), fossil fuel stocks have depleted dramatically (Sharma and Sharma, 2017), but more importantly several negative impacts have become more prominent (Richie and Roster, 2017). For example; the CO_2 produced when fossil fuels are burned is the largest driver of global warming (greenhouse gases) and climate change (Höök and Tang, 2013; Johnsson *et al.*, 2018; Richie and Roster, 2017; Sharma and Sharma, 2017). They are also major contributors to air pollution which have resulted in millions dying prematurely on an annual basis due to adverse respiratory effects (Kampa and Castanas, 2008; Perera *et al.*, 2008; Richie and Roster, 2017; Sharma and Sharma, 2017). As a result, development of sustainable energy from low carbon sources has become more eminent (Sharma and Sharma, 2017).

The main alternative to replace fossil fuel dependency and consumption is the production of biofuels (Adeniyi *et al.*, 2018; Sharma and Sharma, 2017). Biofuels are derived from renewable raw materials and, much like fossil fuels, can be gaseous, liquid or solid (Sharma and Sharma, 2017). First-generation biofuels, also known as conventional biofuels, are derived from crops such as sugar, corn, wheat, beets, *etc.* (Adeniyi *et al.*, 2018; Mohr and Raman, 2013; Naqvi and Yan, 2015). Second-generation biofuels, also known as advanced biofuels, are derived from non-food crops such as food crop waste, agricultural residues, wood chips, cooking oil waste, *etc.* (Adeniyi *et al.*, 2018; Antizar-Ladislao and Turrion-Gomez, 2008; Mohr and Raman, 2013). However, first- and second-generation biofuels cannot meet the global demand required to eliminate the necessity for fossil fuels in a sustainable manner (Adeniyi *et al.*, 2018). Algae is the main contributor to third- and fourth-generation (current) biofuels (Moravvej *et al.*, 2019). Algal biomass contains a very high oil content making them ideal for the production of sustainable biofuels (Adeniyi *et al.*, 2018). A range of algae and cyanobacteria have already proven successful for the production of biofuels. For example; *Chlorella vulgaris* (Nematian *et al.*, 2020) and *Spirogyra* sp. (Aravind *et al.*, 2021) have been utilised to produce bio-oils / biodiesel. Additionally, a range of species have also produced a range of biogases including; *Schizochytrium* sp. for biomethane (Wu *et al.*, 2020), *Chlorella reinhardtii* (Mona *et al.*, 2020) and *Anabaena* sp. (Wu *et al.*, 2020) for biohydrogen, *Ulva* sp. for bioethanol (Polikovsky *et al.*, 2020) and *Nannochloropsis* sp. for biobutanol (Shanmugam *et al.*, 2021), to name a few.

2.3.3.2. AGRICULTURE

The exponential rate of growth in the global population (Kusmayadi *et al*., 2021), which is expected to reach 8.5B by 2030 (Roser *et al.*, 2019), has led to food security deterioration (Kusmayadi *et al.*, 2021). In addition to contributing to ~25% of global greenhouse gas emissions, the expansion of agricultural intensification and food production has resulted in arduous consequences to the environment (Camacho-Rodríguez *et al*., 2016; Kusmayadi *et al*., 2021; Timmer, 2017). This has resulted in considerable changes in the composition and biodiversity of the environment as well as the production of acid rain, issues with eutrophication, soil erosion and climate change (Kusmayadi *et al.*, 2021). The use of algae can assist in mitigating these environmental concerns and issues caused by the expansion of land-based food production due to the increasing global population (Milledge, 2010). Microalgae are often used for animal feed and supplementation. Microalgae production used for animal feed application represents over 30% of the global market (Yaakob *et al*., 2014). The vast array of nutritional compounds for animal feed also present in microalgae provide better growth and health benefits to traditional feeds (Kusmayadi *et al*., 2021).

Research on algae as an animal feed has been conducted since the 1950's (Kusmayadi *et al.*, 2021). There are several advantages in utilising microalgae as feed including; resilience towards illness by providing antiviral and antibacterial actions, and enhancing immune responses (Ekmay *et al.*, 2014; Ginzberg *et al.*, 2000; Kusmayadi *et al.*, 2021; Madhumathi and Rengasamy, 2011). The use of algae as an animal feed greatly depends on the species of algae and their nutrient composition (Fradique *et al.*, 2013; Kusmayadi *et al.*, 2021). For example; up to 50% of *Arthrospira* sp.'s global population is used for animal feed supplementation due to its high protein content as well as opportune digestibility due to the small carbohydrate content (Yaakob *et al*., 2014). In the last two decades, studies have demonstrated the benefits of nutritional compounds from algae in a range of animal feeds for cattle, sheep, goats, pigs, chickens and even pets (Kusmayadi *et al*., 2021; Wells *et al*., 2017). Species of algae that have been successfully utilised in sustainable animal feed includes (but are not limited to); *Chlorella pyrenoidosa* and *Spirulina platensis* in cattle feed (Costa *et al*., 2016), *Desmodesmus* sp. (Ekmay *et al.*, 2014) and *Porphyridium* sp. (Ginzberg *et al*., 2000) in chicken feed, *Spirulina* sp. in sheep feed (Holman *et al.*, 2012), *Schizochytrium* sp. for lamb feed (Urrutia *et al*., 2016), *Haematococcus pluvialis* (Ju *et al.*, 2012)*, Cryptonemia crenulata, Hypnea cervicornis* (Da Silva and Barbosa, 2009) and *Dunaliella salina* (Madhumathi and Rengasamy, 2011) in shrimp fishmeal, and *Aurantiochytrium limacinum* (Moran *et al*., 2018) *and Desmodesmus* sp. (Ekmay *et al.*, 2014) in pig feed.

Biostimulants are substances, when applied in minute quantities, promote plant growth. They enhance crop quality, nutrition efficacy and / or abiotic stress tolerance (du Jardin, 2015). Biofertilisers, which are natural fertilisers (Ginni *et al.*, 2020), are considered to be a sub-category of Biostimulants (du Jardin, 2015). Biofertilisers are traditionally an inexpensive (Singh *et al*., 2019; Singh *et al*., 2018) means of enhancing plant growth (Saeid and Chojnacka, 2019). They provide a promising instrument in agriculture by supplying a renewable, supplementary and environmentally friendly source of plant nutrition (Mącik *et al*., 2020; Singh *et al*., 2019; Singh *et al*., 2018). They have demonstrated greater growth rates and yield than traditional chemical fertilisers (Thompson *et al*., 2020). Biofertilisers have the capacity and capability to convert nutritionally valuable elements from non-accessible and / or non-usable to highly digestible forms without producing adverse effects on the surrounding ecosystems (Mącik *et al.*, 2020; Singh *et al*., 2019). The administration of biofertilisers is considered to be an important component in maintaining fertile soil and crop productivity on an adequately high enough level to allow for sustainable farming (Singh *et al.*, 2019; Singh *et al*., 2018). As such, they will aid in mitigating the negative impacts that have arisen as a result of the growing demand for food from the ever expanding global population (Mącik *et al*., 2020). Biofertilisers will also aid in mitigating environmental pitfalls arising from the widespread chemicalisation in the agriculture industry (Umesha *et al.*, 2018). The changing practices within the agriculture industry in order to assist in combating global warming and climate change means that biofertilisers are now considered to be a critical component to modern day crop production (Mącik *et al.*, 2020). Algae are used as promising and effective biofertilisers (Singh and Sharma, 2012). For example; the alga *Sargassum johnstonii* has been used as a biofertiliser for the production of tomato plants where it has been found to increase the organic composition and essential minerals levels in the soil by more than 100 fold when compared to traditional fertilisers (Thompson *et al*., 2020). In addition to the direct use of algae there are also many indirect applications of algae as biofertilisers. Biochar, the charcoal residue produced during biofuel production, has been regularly applied to the agriculture industry as a biofertiliser (Brennan and Owende, 2010) and the digestate or effluent remaining after the development of algal based feed may also be used. However, this research is ongoing and still in its infancy (Ginni *et al*., 2020; Stiles *et al.*, 2018). Nitrogen fixing cyanobacteria *e.g.*, *Anabaena* sp., are also commonly used as biofertilisers (Umesha *et al.,* 2018).

2.3.3.3. NUTRACEUTICALS & PHARMACEUTICALS

Pharmaceuticals are traditionally used as a medicinal drug to treat disease whilst nutraceuticals are traditionally used as nutritional supplements that are intended to prevent disease (Udayan *et al.*, 2017). Nutraceuticals are used in dietary supplements in most countries and are often considered as

more then food but less than pharmaceuticals. Unlike pharmaceuticals, there is no internationally accepted definition for nutraceuticals and their judgement varies from country to country (Télessy, 2018). However, nutraceuticals are most commonly defined as substances that are foods or part of a food that provides medicinal health benefits that include treatment and prevention of disease (Daliu *et al*., 2019; Dudeja and Gupta, 2017). Pharmaceuticals are defined as medicinal drugs that are prescribed in rational dosages that have been proven to provide high quality, safe and effective treatment against disease (Dmytryk *et al*., 2017).

Despite having been studied for decades, algae are still considered as one of the most poorly understood group of organisms on the planet. However, new research conducted in recent years as part of a search for sustainable and renewable nutritional supplement and pharmaceutical sources, has been promising (Udayan *et al*., 2017). Algae can provide a potential alternative to pharmaceuticals, nutrients and supplements derived from terrestrial plants (Dmytryk *et al.*, 2017; Udayan *et al*., 2017) due to their composition and variety of nutritionally important and biologically active compounds that demonstrate a wide variety of health benefits (Godlewska *et al.*, 2017; Udayan *et al*., 2017). These wide range of compounds present in algae include; nutrients and vitamins, proteins, carbohydrates, lipids and polyunsaturated fatty acids (PUFA's), pigments and bioactive compounds (Daliu *et al.*, 2019; Dmytryk *et al*., 2017; Dudeja and Gupta, 2017; Godlewska *et al.*, 2017; Sasi, 2017; Télessy, 2018; Udayan *et al.*, 2017). In addition to providing a means of supplying vitamins, major and minor trace elements and nutrient supplementation, algae are also high producers of the PUFA's omega-3 and omega-6 (Sasi, 2017; Udayan *et al*., 2017). In fact, it is thought that fish (primary source of omega-3 and -6 for human consumption) are so high in PUFA's due to the fact that they themselves consume algae that already contain such high levels of these PUFA's (Godlewska *et al*., 2017; Udayan *et al.*, 2017; Wells *et al*., 2017). A range of algae have also demonstrated a range of biological activities and benefits including; antioxidant, antimicrobial, anti-inflammatory, antibacterial, antiviral, anticancer, anticoagulant, antidiabetic, anti-obesity, antiallergy, anti-hypersensitivity and anti-hypercholesterolemic activities (Daliu *et al.*, 2019; Dmytryk *et al.*, 2017; Dudeja and Gupta, 2017; Gautam and Mannan, 2020; Godlewska *et al*., 2017; Sasi, 2017; Télessy, 2018; Udayan *et al.*, 2017). Some of the algal species that contain nutraceutical compounds demonstrating biological activities when applied to the pharmaceutical industry include; a range of *Chlorella, Haematococcus, Nostoc, Botryococcus, Anabaena, Chlamydomonas, Scenedesmus, Synechococcus, Parietochloris, Undaria, Scytosiphon, Dunaliella, Crypthecodinium,* and *Porphyridium* (Daliu *et al.*, 2019; Dmytryk *et al*., 2017; Dudeja and Gupta, 2017; Gautam and Mannan, 2020; Godlewska *et al*., 2017; Udayan *et al*., 2017).

2.3.3.4. COSMETICS

The cosmetics industry is a fast-growing industry. In 2015 it had an estimated annual value of $170B United States Dollars or USD (H. M. D. Wang *et al*., 2015) which almost tripled to $507.4 USD by 2018 and is estimated to rise to $758.4 USD by 2025 (Ridder, 2020). Cosmetics have returned to basic or fundamental products due to consumer suspicions about the use of chemicals and as a result there is a rising demand for natural, environmentally friendly and sustainable products (Ariede *et al*., 2017; Joshi *et al*., 2018; H. M. D. Wang *et al*., 2015). As such, the valuable products that algae and cyanobacteria produce have drawn the attention of the cosmetic industry (Yarkent *et al*., 2020). According to Ariede *et al*. (2017) algal based cosmetics are now used by millions on a daily basis. Algae compounds and their secondary metabolites have been found to demonstrate anti-aging activity (Ariede *et al*., 2017; Aslam *et al*., 2021; de Oliveira *et al*., 2021; H. M. D. Wang *et al*., 2015; Yarkent *et al*., 2020), moisturising activity (Ariede *et al*., 2017; de Oliveira *et al*., 2021), de-pigmentation properties (Ariede *et al*., 2017; Joshi *et al*., 2018; H. M. D. Wang *et al*., 2015), as a natural colourant / dye (Aslam *et al*., 2021; de Oliveira *et al*., 2021; Udayan *et al*., 2017), as a skin whitening agent (de Oliveira *et al*., 2021; Yarkent *et al*., 2020), anticellulite properties (de Oliveira *et al*., 2021) and sun / UV protection activity (Ariede *et al*., 2017; Aslam *et al*., 2021; de Oliveira *et al*., 2021; Morone *et al*., 2019; Yarkent *et al*., 2020). Algal based cosmetic compounds have also been successfully applied to hair care products (de Oliveira *et al*., 2021; Udayan *et al*., 2017) such as shampoos (Ariede *et al*., 2017) and have even been found to aid with hair loss issues such as alopecia (de Oliveira *et al*., 2021). *Fucus vesiculosus* extract is used to reduce and eliminate dark circles under eyes stimulating heme oxygenase-1 expression which eliminates the heme catabolites that cause the issue (Ariede *et al*., 2017). Some of the other algal and cyanobacterial species commonly used in cosmetics include; *Isochrysis galbana, Odontella aurita* (B. R. Kumar *et al.*, 2021)*, Chlorella* sp., *Spirulina* sp. (Ariede *et al*., 2017; Morone *et al*., 2019), *Phaeodactylum tricornutum,* and *Chlamydocapsa* sp. (Ariede *et al*., 2017), to name a few.

2.3.3.5. MUNICIPAL WASTEWATER

Municipal wastewater is wastewater from households and / or from industrial origins. Untreated municipal wastewater can contain a range of compounds that are hazardous to human life as well as the environment (Graczyk *et al*., 2009). In fact, municipal wastewater is one of the largest sources of pollution (Paniagua-Michel, 2015). Municipal wastewater does contain relatively small concentrations of dissolved and suspended solids (Pereira *et al.*, 2014). However, they are most notably known for being heavily loaded with nutrients, particularly N and P (Paniagua-Michel, 2015). Municipal wastewater also often has a range of inorganic substances, such as heavy metals, that can display

phytotoxic effects as well as have significant impacts of humans (Pereira *et al.*, 2014). Municipal wastewater treatment plants are usually found in larger urban areas (Speight, 2020). However, the quality and quantity of municipal wastewater does vary greatly depending on the composition of the community using the water *i.e.*, household / domestic wastewater tends to be much less hazardous than industrial waste (Graczyk *et al.*, 2009). Municipal wastewater treatment using algae is considered to be well established (Murry *et al.*, 2019). Wang *et al.* (2010) successfully demonstrated that the application of *Chlorella* sp. can reduce nutrient loads in municipal wastewater and the resulting algae was then used to produce biofuel and feedstock. Similarly, Li *et al.* (2011) confirmed that fourteen algal species from the genus *Chlorella, Chlamydomonas, Scenedesmus, Chloroccum* and *Haematococcus* could all effectively remove wastewater nutrients allowing for the production of biomass that was subsequently used for biodiesel production. In addition to removing nutrients such as N and P, algae have also demonstrated their ability to remove hazardous inorganic substances from municipal wastewater. For example, Zhao *et al*. (2018) demonstrated the use of algal bioreactor films to not only remove significant amounts of N and P waste products but also to effectively remove most metals present in municipal wastewater.

2.3.4. ALGAE IN AQUACULTURE

Algae are utilised diversely in aquaculture, as shown in Figure 2.14 (Priyadarshani *et al*., 2012) due to their range of technical and economic benefits (Han *et al*., 2019; Yang *et al.*, 2020). They are used for oxygen production (Chen and Wang, 2020; Drapcho, 2000; Han *et al*., 2019; Priyadarshani *et al.*, 2012), for water quality and wastewater treatment (Andreotti *et al*., 2020; Aquilino *et al.*, 2020; Cardoso *et al*., 2020; Chen and Wang, 2020; Han *et al*., 2019; Jasmin *et al*., 2020; V. Kumar *et al*., 2021; Peng *et al*., 2020; Zhang *et al*., 2019), for feed / partial feed replacement or supplementation (Han *et al*., 2019; Priyadarshani *et al*., 2012), for pigmentation (Priyadarshani *et al.*, 2012), for immunological properties (Barman *et al*., 2013; Bricknell and Dalmo, 2005; Han *et al*., 2019; Reverter *et al*., 2016; Yaakob *et al*., 2014) and for nutritional purposes (Priyadarshani *et al*., 2012). However, despite the broad and diverse use of algae in aquaculture and its apparent benefits both technically and economically, research into its use in aquaculture is still very limited (Han *et al.*, 2019) and it is only in the last few years that there has been an increased focus in that research. Up to 2012, the main application of algae in aquaculture was related to nutrition (Priyadarshani *et al.*, 2012).

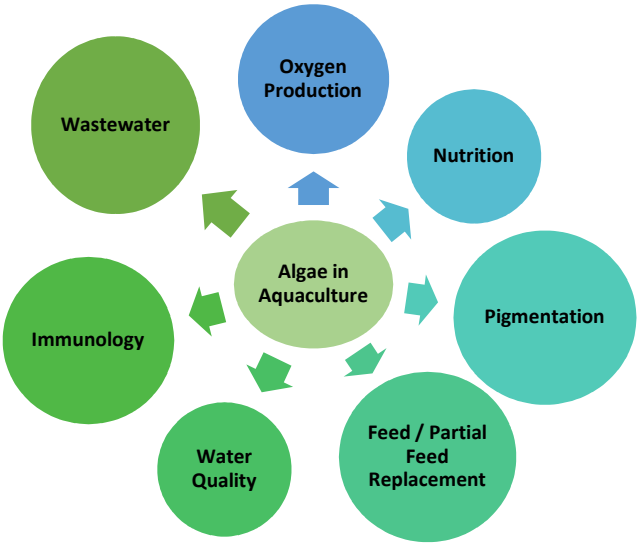


Figure 2.14: Overview of **current algae application to aquaculture**.

Fish produce CO_2 during respiration. High levels of CO_2 can quickly deteriorate the water quality. Algae's ability to produce O_2 via photosynthesis can improve the water quality by eliminating the CO_2 (Priyadarshani *et al.*, 2012). Algae's capability to produce O_2 can also aid in alleviating oxygen depletion (Drapcho, 2000; Han *et al*., 2019; Priyadarshani *et al.*, 2012). Algae's capacity to supply O_2 can help reduce energy consumption overheads from supplementary aeration devices required in aquaculture (Han *et al.*, 2019). In addition to improving water quality via CO_2 removal, algae have also shown to be highly effective in the reduction and removal of nutrient loads. There has been a significant increase in recent years on the successful use of algae for treatment of aquaculture wastewater by bioremediation. *Chaetomorpha linum* (Aquilino *et al.*, 2020)*, Chlorella vulgaris* (Peng *et al*., 2020)*, Tetraselmis suecica* (Andreotti *et al*., 2020) and *Spirulina* sp. (Cardoso *et al.*, 2020) have all been shown to be excellent candidates for aquaculture wastewater bioremediation by efficiently removing N. Algae such as *Chaetomorpha linum* has also been shown to remove P levels (Aquilino *et al*., 2020). However, there is little research done on this but that which has is considered promising (Jasmin *et al*., 2020). Algae nourished by aquaculture wastewater could therefore assist in self-reliance and the sustainability of aquaculture (Zhang *et al.*, 2019).

Algae are a natural food base (Priyadarshani *et al.*, 2012). Integrating the culturing of algae into the aquaculture process provides this natural resource (Yang *et al.*, 2020). Fish can be fed directly or indirectly with algae. The algae can be directly applied to the fish culturing system to partly replace or supplement the traditional synthetic aquaculture feeds (Han *et al.*, 2019) that are necessary and vital in ensuring demands for fish products are met (O'Neill *et al.*, 2019, 2020). For indirect application, algae can also be fed to artemia, daphnids or rotifers (primary consumers) which in turn can be fed to

larger fish (Priyadarshani *et al*., 2012). As a result, the cost of fish rearing can be better controlled *i.e.*, from an economic point of view, partially replacing feed with algae, be it directly or indirectly, can greatly reduce overhead rearing costs (Han *et al.*, 2019). In addition to this, algae are often applied to aquaculture as part of feed in order to advantageously utilise their natural pigments. Algae are often used as a source of natural pigments *e.g.*, carotenoids, astaxanthin, zeaxanthin, lutein (Rout *et al*., 2013) for salmonids, prawns and ornamental fish (Priyadarshani *et al*., 2012; Yusoff *et al*., 2020). For example, *Haematococcus,* which contains astaxanthin, is used to develop the pink colour of salmon flesh and *Spirulina*, which contains carotenoids that are converted to astaxanthin, is used for the bright and vivid pigmentation of ornamental fish such as koi or goldfish (Kalidas and Edward, 2005; Towers, 2013). The application of artificial feeds, especially those which contain these compounds necessary for pigmentation enhancement, is one of the most expensive inputs in aquaculture (Rout *et al*., 2013) therefore, the addition of algae to standard feeds will aid in reducing some of these overhead costs (Han *et al*., 2019).

Algal pigments as well as other biomolecules present, also provide nutritional and immunological benefits (Barman *et al*., 2013; Bricknell and Dalmo, 2005; Han *et al.*, 2019; Priyadarshani *et al.*, 2012; Reverter *et al.*, 2016; Yaakob *et al.,* 2014; Yusoff *et al*., 2020). Pigments such as chlorophylls, phycobiliproteins and carotenoids contain biomolecules associated with increased immunity, high productivity and fast growth (Yusoff *et al.*, 2020). PUFA's, functional amino acids, vitamins and other biomolecules present have also been found to provide health benefits to fish (Yusoff *et al.*, 2020). Algae provide precursors for omega-3 fatty acids in fish (Priyadarshani *et al.*, 2012) thus ensuring fish remains as one of its best sources. Algae also contain β-glucans. β-glucans are polysaccharides found in the cell wall of algae and are becoming more commonly used in aquaculture as immunostimulants (Meena *et al.*, 2013). The required intensification of aquaculture in order to meet demands has led to major issues with disease. Historically, antibiotics have been used to control outbreaks (Owens, 2019). However, due to the broad and frequent over use of antibiotics in the past, the development and spread of antibiotic resistance has since become a major issue (Defoirdt *et al*., 2011; Owens, 2019). Limited studies have been conducted on β-glucans from algae (*e.g., Euglena gracillus*) but have been found to be very promising (Barman *et al.*, 2013; Bricknell and Dalmo, 2005; Reverter *et al.*, 2016; Yaakob *et al.*, 2014). By enhancing fish immunity by algae, the overuse of antibiotics and medications can be reduced thus increasing the safety of the product. These 'pollution-free' fish products are more desirable and have a larger market demand therefore selling for much greater prices (Han *et al.*, 2019).

2.4 GAP IN KNOWLEDGE & RESEARCH CONTRIBUTION

The overall goal of this research was to aid in developing sustainable freshwater aquaculture in Ireland by focusing on some of the main weaknesses and threats (complex environmental issues and requirements leading to slowness and uncertainty in the aquaculture licensing process, failure to protect and measure impacts on the natural environment, insufficient investment in research, spatial restrictions and limitations as a result of Natura 2000 and, issues with climate change variances, eutrophication and algal blooms) highlighted in the Irish National Strategic Plan for Sustainable Aquaculture Development and the needs of the industry.

2.4.1. IRISH FRESHWATER AQUACULTURE

There is a major lack of investment and research being conducted in the Irish aquaculture industry, especially in the freshwater sector. Although several papers that focus on Irish mariculture and aquaculture in general from a European point of view, only five original research papers focusing on Irish freshwater aquaculture could be found in the available literature in the last ten years, as shown in Table 2.1, two of which were as a direct result of this work. As such, this research has already and will further contribute to this gap in knowledge.

Table 2.1: **Breakdown of original research papers focusing on Irish freshwater aquaculture.** Papers have been listed in chronological order.

Reference	Research Description	Similarities to this Research
(Tahar *et al.*, 2018a)	• First study to look at the impact traditional freshwater rainbow trout aquaculture using FTS configuration has on water quality. • Investigated impacts on the whole farm / system (internal, intake and output)	• Used some physicochemical parameters during investigation. • Study conducted on a traditional freshwater FTS aquaculture system.
(O'Neill *et al.*, 2019)	• First study to focus on the ecotoxicological impact of freshwater aquaculture in Ireland. • First study to consider the use of algae as an early warning indicator for climate change.	Not applicable as paper is as a result of this research.
(O'Neill *et al.*, 2020)	• First study on the use of aquaculture in a novel peatland IMTA system.	Not applicable as paper is as a result of this research.
(Naughton *et al.*, 2020)	• Investigated the concept of exploiting microalgae for wastewater treatment. • First study to report on the relationship between wet-lab and *in-situ* technologies.	• Used some physicochemical parameters during investigation. • Used FCM to enumeration algal numbers. • Study conducted on a traditional freshwater FTS aquaculture system.
(Cooney *et al.*, 2021)	• First study on the Life Cycle Assessment (LCA) of perch aquaculture in Ireland. • First study on LCA in RAS in Ireland.	• Study conducted on a traditional freshwater FTS aquaculture system.

Freshwater aquaculture wastewater discharge is currently regulated by the EPA and monitoring is conducted by local authorities, and in some cases Irish Water. However, current regulations may not be specifically applicable to aquaculture, which the EPA are now actively investigating. Hence, there was an urgent need to develop an ecotoxicological toolbox consisting of tests representative of the receiving freshwater aquatic ecosystems downstream of fish farms. This in turn will assist with evaluating environmental requirements that have affected the licensing process, aid in combating the failure to protect and measure impacts on the natural environment, and address spatial restrictions as a result of Natura 2000.

2.4.2. PEATLANDS & AQUACULTURE

There is a commensurate interest in exploiting low-cost environmental-friendly 'natural' processes in aquaculture. Irish aquaculture issues have led to an increased research focus on developing IMTA along with eco-innovation and monitoring of processes. Advances in aquaculture must be balanced by the need to meet commitments as set out by the WFD which aims to achieve good water status in all waters across all EU member countries. Approximately five percent of Ireland is covered in peatlands with Bord Na Móna owning or controlling approximately 80,000 ha. The urgent threat of climate change variances, in addition to some of these peatlands now being listed as important habitats under the EU's Birds and Habitats Directives due to their scarcity, have resulted in dramatic changes in the peat industry including conversion of peatland-usage to wind energy, forestry, biodiversity, amenity and waste management. The use of peatlands for organic aquaculture processes will not only aid in developing a means to increase the sustainable intensification of Irish aquaculture, it will also aid in dealing with the limitations in space and resources facing the industry without compromising any of the environmental EU directives adopted by Ireland.

2.4.3. USE OF ALGAE IN IRISH AQUACULTURE

As mentioned in subsection 2.3.4, the application of algae to aquaculture has been highly beneficial. Three of the five original research papers focusing on Irish freshwater aquaculture are utilising algae. With the increased interest in exploiting low-cost environmentally friendly natural processes in aquaculture and in particular IMTA systems, the use of algae in this process holds great potential. The use of algae (which is most often already present in Irish aquaculture systems) will aid in providing an environmentally friendly approach to developing the Irish freshwater aquaculture industry without compromising the afore mentioned environmental EU directives, but also aid in bridging the gap in the lack of research currently available in algae and its application to aquaculture.

CHAPTER 3

ASSISTING ENVIRONMENTAL CONCERNS & LICENSING DELAYS IN IRISH FRESHWATER AQUACULTURE

3.1. INTRODUCTION

Issues with the adoption of EU environmental protection directives and the subsequent problems and delays in the Irish aquaculture licensing process have hampered the growth of the Irish aquaculture industry, as well as meeting the goals and predictions set out by the Food Wise 2025 Irish agri-food industry development strategy, as outlined in chapter two. A mandatory environmental impact assessment (EIA) now needs to be conducted as part of the Irish aquaculture licensing process (DAFM, 2018b; Office of the Attorney General, 1997).

There is a critical need for non-toxic, reliable and sustainable waste management techniques in order to eliminate potential hazards to health and adverse environmental effects (Earth Link and Advanced Resources Development, 2004). Concerns about the effects human advances have on the environment has radically increased in recent years, resulting in the adoption of EIA protocols that are designed to predict the potential environmental impacts a proposed project may cause (Gilpin, 2013). An EIA highlights and assesses potential environmental issues that are likely to arise from a proposed project (Earth Link and Advanced Resources Development, 2004; Gilpin, 2013; Glasson *et al*., 2005; Glasson and Therivel, 2013; Wood, 2003), as well as propose measures to mitigate the potential negative effects (Earth Link and Advanced Resources Development, 2004). It is a systematic and integrative process, first developed in the United States as a result of the National Environment Policy Act (NEPA), of 1969, for considering potential environmental implications prior to making decisions on whether or not a proposed project is environmentally safe and subsequently given approval to proceed. The NEPA requires an environmental impact assessment report (EIAR) or environmental impact statement (EIS) to be published, describing full details for the EIA and the impacts likely to arise from the proposed project (Wood, 2003). Commonly, permission is granted but is subject to a range of terms and conditions (Gilpin, 2013).

According to the EPAs EIA and EIAR guidelines, one feature of assessing wastewater is evaluating effluent characteristics *i.e.,* physicochemical parameters. As previously mentioned, these physicochemical parameters will be monitored in conjunction with the selected ecotoxicological bioassays for the toolbox. No information to date has been found in relation to an already developed

ecotoxicological toolbox that focuses on both ecotoxicology bioassays and physicochemical analysis to test for and evaluate the water quality of Irish freshwater aquaculture output being released into Irish waters. Freshwater aquaculture wastewater discharge is currently regulated by the EPA and monitoring is conducted by local authorities, and in some cases Irish Water (EPA, 2018a). However, current regulations may not be specifically applicable to aquaculture. Hence, there is an urgent need to develop an ecotoxicological toolbox consisting of tests representative of the receiving freshwater aquatic ecosystem downstream of fish farms. This aims to assist in improving both the aquaculture licensing process and monitoring of concomitant waste-water discharge. The first stage of development of this toolbox focused on a standardised primary producer (*P. subcapitata* - ISO 8692:2012) and primary consumer (*D. magna* - ISO 6341:2012) bioassay. This multi-trophic test battery was run in parallel with the physicochemical parameters currently used to monitor freshwater aquaculture output. This is similar to that utilised in a traditional EIA. The second stage of development of this toolbox focused on primary producers (*M. contortum* and *A. formosa*), and a primary consumer (*D. pulex*), commonly found in Irish waters. These species were then compared to the standardised bioassays. Once developed, the toolbox was then applied to the chosen traditional FTS facility. Development of the ecotoxicological toolbox will assist in monitoring potential environmental issues that have delayed the Irish aquaculture licensing process.

3.2. METHODOLOGY

Environments / ecosystems are vastly complicated networks whose progression and evolution are determined by complex systems of positive and negative feedback loops (Franzle, 2012). Hampering interferences by humans have thus emulated an ever-expanding number of environmental issues that cause major concerns (Twardowska, 2004). Ecotoxicology plays an important role in providing key knowledge that allows for the development and preservation of environmental sustainability and productivity, as well as sustainable economic development (Van Straalen, 2003). It includes transfer pathways of these physical, chemical and biological agents and their interactions with the environment (Boudou and Ribeyre, 1997; Franzle, 2012) and should provide tools for early warning detection and interception systems (Twardowska, 2004).

Toxicity has been found to be trophic level specific and as a result, the protection of environmental resources may not be provided by conducting bioassays entirely at the macro-organism level of a biological organisation / food chain (Wells *et al.*, 2018). Microscale single species test batteries are one of the main tools Ecotoxicologists utilise, covering a wide variety of mechanisms of action of pollutants (Schmitt-Jansen *et al.*, 2008; Wells *et al.*, 2018). Testing at different trophic levels of a food

chain and with different phyla are a critical area of ecotoxicology. This is a rapidly expanding area involving various bio-analytical techniques that have been established and applied at sub-cellular to multi-cellular levels of a food chain (Wells *et al.*, 2018). As indicated in Figure 2.3 in Chapter Two, trophic levels are the feeding positions or food chain levels of all organisms in a specific ecosystem and is commonly presented as a trophic level pyramid. The base / first / lowest trophic level has the highest energy concentration that is dispersed among organisms in the subsequent levels. Trophic levels ultimately describe what organisms eat and are divided into five key levels (*i.e.,* primary producers, primary, secondary and tertiary consumers, and apex predators), in an ecosystem ranging from simple plants at the bottom, to predator animals at the top of a food chain (King, 2018). Many classic or traditional ecotoxicological bioassays used in effect assessment have been established and cover several trophic levels (Schmitt-Jansen *et al.*, 2008) ***e.g.,*** the ISO [8692:2012] *Pseudokirchneriella subcapitata* Algal Bioassay for primary producers, the ISO [6341:2012] *Daphnia magna* Crustacean Bioassay for primary consumers, and the ISO [11348-3:2007] *Vibrio fischeri* Bioluminescent Bacteria Bioassay for decomposers. Including a range of bioassays in toxicity and impact assessment studies should contribute to and provide effective protection to all trophic levels within an ecosystem. Combining bioassay analysis with ambient and community responses provides additional capacity to predict potential adverse effects towards receiving ecosystems (Wells *et al*., 2018).

3.2.1 SAMPLING

Water samples were collected from Keywater Fisheries in five L octagonal carboy HDPE bottles (Lennox) and transported directly to the lab, 70km away, via car. Samples were taken directly from the output source of the farm every two weeks from April 2018 to October 2018 for the pilot study, and once a month from March 2019 to August 2019 for the ecotoxicological toolbox monitoring program. Collection occurred on the same day and at approximately the same time *i.e.,* Thursdays at 10:30 a.m., during the pilot study and Wednesdays at 9:00 a.m. during the ecotoxicological toolbox monitoring program. Intake samples were also collected and analysed so that any potential issues caused by works upstream of the fish farm and not as a result of works within the facility itself would be taken into consideration. Intake samples were taken directly from the intake pipe. A sample from the settlement pond was included in the ecotoxicological toolbox monitoring program to assess its efficacy. See Figure 3.1 for schematic of Keywater Fisheries and the location of the intake, output and settlement pond sampling sites.

Figure 3.1: **Schematic of Keywater Fish Farm**. The location of the hatchery / nursery, mesocosms, culture ponds, settlement pond, which is included in the constructed wetland, and the holding tank at the discharge point are included. Blue lines indicate the direction of the flow of water. The **red circle** indicates the **location of the intake sampling point**. The **green circle** indicates the **location of the output sampling point**. The **yellow circle** indicates the **location of the settlement sampling point.** (Source: Morefish & EcoAqua Project)

3.2.2 PRIMARY PRODUCER ANALYSIS

Planktonic microalgae are primary producers and are a key component in the food chains of aquatic ecosystems (Aruoja, 2011). Sphaeropleales is one of the most dominant groups of green microalgae in the world and contain species that are considered to be very important to freshwater ecosystems (Suzuki *et al.*, 2018). According to Rodgher *et al.* (2012), these microalgae constitute an important group of highly sensitive photosynthetic organisms that are frequently used to assess aquatic ecosystems. One such species in this group is *Pseudokirchneriella subcapitata* (*P. subcapitata*), also commonly known as *Raphidocelis subcapitata* or *Selenastrum capricornutum* (Aruoja, 2011; Rodgher *et al.*, 2012; Suzuki *et al.*, 2018; Yamagishi *et al.*, 2017). This microalgae has a helical or sickle shaped cell ranging from 8 – 14µm in length and 2 – 3µm in width (Aruoja, 2011; Yamagishi *et al.*, 2017) and is found in unicellular form (Suzuki *et al.*, 2018). See Figure 3.2A. The *Pseudokirchneriella subcapitata* algal bioassay was opted for because it is consider the most widely known and used bioindicator in ecotoxicological assessments of freshwater ecosystems due to its high growth rate, high sensitivity and high reproducibility (ISO, 2012a). However, one such issue with this alga is its absence from Irish waters (NBDC, 2021a). In order to compensate for this, two species commonly found in Irish waters were ran alongside the standardised *P. subcapitata*. These were the green alga *Monoraphidium*

contortum (*M. contortum*) and the alga diatom *Asterionella formosa* (*A. formosa*). These two species were opted for based on those such species found in and around the aquaculture facilities utilised for this research. The chosen species were also based on extensive analysis conducted by the National Biodiversity Data Centre (NBDC), as well as through conversations conducted with algal experts in the EPA and the Marine Institute. *M. contortum* (Figure 3.2B) is very similar to *P. subcapitata.* It is also a unicellular green alga in the Sphaeropleale order. They can grow up to 40µm long, have a curved or contorted shape and are commonly found in the temperate freshwater ecosystems of Europe (Durante *et al*., 2013; NBDC, 2021b). Diatoms are a single celled, major group of algae (Diatoms of North America, 2019). *A. formosa* (Figure 3.2C) is a freshwater planktonic diatom (Maberly *et al*., 1994; Sivarajah *et al*., 2016; Spaulding, 2012; Van Den Wyngaert *et al*., 2015). It is a widely distributed species (Sivarajah *et al.*, 2016; Van Den Wyngaert *et al*., 2015), and is a major component of algal production in many temperate water bodies (Maberly *et al.*, 1994). It is most commonly found in mesotrophic and eutrophic waters during spring and autumn but has been reported in these waters in the northern hemisphere during the summer months (Maberly *et al*., 1994; Sivarajah *et al.*, 2016; Spaulding, 2012). *A. formosa* are a long and narrow pennate diatom (ranging from 40 – 68µm in length and 1.1 – 4.5µm in width), which are most commonly linked together forming star shaped colonies (Sivarajah *et al*., 2016; Spaulding, 2012).

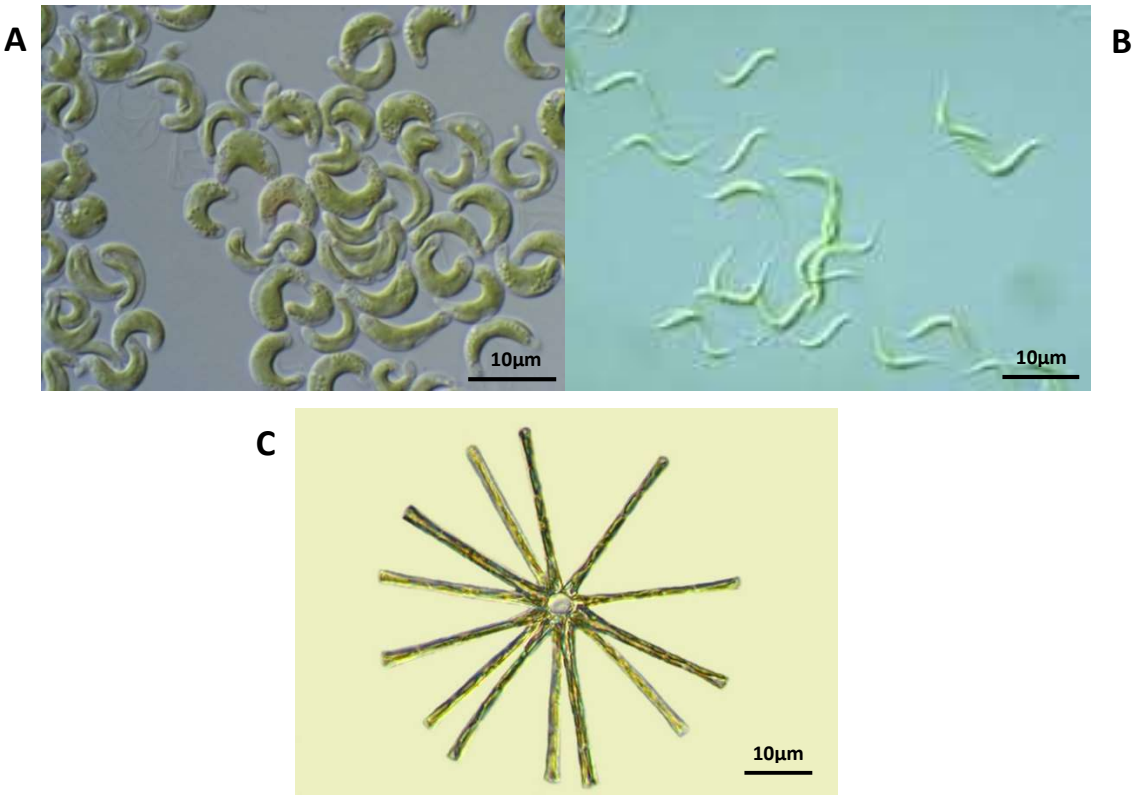


Figure 3.2: Microscopic images of A) ***Pseudokirchneriella subcapitata***, B) ***Monoraphidium contortum*** and C) ***Asterionella formosa***.

ALGAL BIOASSAY METHOD

A starter culture of the *P. subcapitata* (CCAP 278/4), *M. contortum* (CCAP 245/2) and *A. formosa* (CCAP 1005/9) were obtained from The Culture Collection of Algae and Protozoa (SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland). All algal bioassays were carried out in accordance with the ISO [8692:2012] guidelines with some alterations (ISO, 2012a). Due to the limitations in availability of a shaking phytoincubator, an LMS static phytoincubator was used. Due to this limitation, a 96h incubation period was also included to ensure all validation criteria were met. Specific culture medium for the individual algal species were used in addition to the medium set out by the guidelines. See appendix 3 for a more detailed breakdown of all methods and protocols applied. All algal concentrations were manually established using a Superior Marienfeld Neubauer Improved Haemocytometer (0.1mm, 0.0025mm^2, Tiefe Depth Profondeur No: 717810) and a Nikon YS100 light microscopy. Working stock solutions of algae at a concentration of 2x10^5 algal cells mL^{-1} were firstly prepared. Nineteen mL of each chemical / water sample was placed into 25mL Erlenmeyer flasks followed by one mL of the working stock solution resulting in a beginning concentration of 1x10^4 algal cells mL^{-1}. Negative controls were included where only culture medium was used to ensure the bioassays met the validity criterion *i.e.,* a minimum 67-fold growth increase (>6.7x10^5 algal cells mL^{-1}). Water samples from the respective aquaculture facilities were filtered using a Whatman 0.2µm pore membrane filter to remove any contamination that may affect the assay and cause interference. Each flask was set up in triplicate and plugged with cotton wool to prevent evaporation. Flasks were incubated at 23°C ±2°C for 72h and 96h respectively, under continuous illumination (lux 6,000 – 10,000). Results were compared to the negative control and the percent of growth rate inhibition / stimulation were then calculated as follows;

Equation 1:

Algal Cells mL^{-1} = $\frac{n}{0.02}\ x\ 10^3$

where n = number of cells counted using a haemocytometer

Equation 2:

Average Specific Growth Rate (µ) = $\frac{ln\ X_n - ln\ X_0}{T_n - T_0}$

where ln = natural log

X_n = algal cells mL^{-1} at the duration of the test

X_0 = algal cells mL^{-1} at time zero

T_n = duration of the test

T_0 = time zero

Equation 3:

% Inhibition in Growth Rate = $\frac{C\mu - T\mu}{C\mu}$

where $C_\mu = \bar{x}$ specific growth rate for control

$T_\mu = \bar{x}$ specific growth rate for treatment

NOTE: The traditional bioassays were chosen over more modern techniques (polymerase chain reaction or PCR and gene expression) so this work may be reproducible in labs with limited resources.

3.2.3 PRIMARY CONSUMER ANALYSIS

Primary consumerss include invertebrates such as crustaceans *e.g.,* Daphnids (European Commission, 2017b). *Daphnia magna* or *D. magna* (Figure 3.3A) play a key role in aquatic ecosystems and their food chains as they consume algae and provide prey to fish and larger invertebrates (Elenbaas, 2013). They are freshwater planktonic microcrustaceans known as water fleas (Bekker *et al.*, 2018), ranging from 2 - 5mm in length with the females being slightly larger *i.e.,* females are 5mm and males are 2mm (Elenbaas, 2013). They are kidney bean shaped with a transparent shell enclosing their body known as a carapace (Elenbaas, 2013). The *D. magna* crustacean bioassay is one of the most commonly used toxicity bioassays internationally for assessing effluents and contaminated waters as it is highly sensitive and reproducible (Persoone *et al.*, 2009). However, similar to *P. subcapitata*, *D. magna* has not been found in Irish waters (NBDC, 2021c). *Daphnia pulex* or *D. pulex* (Figure 3.3B) is another species of Daphnid similar to *D. magna*. Like *D. magna*, they are freshwater planktonic micro crustaceans, also known as water fleas (Bekker *et al.*, 2018). *D. pulex* is the most common species of water flea found in freshwater bodies and are smaller than *D. magna*, ranging from 0.2 – 3mm in length (Miller, 2000). Like the *D. magna*, the females are larger than the males. They are also kidney bean shaped with a transparent carapace shell (Elenbaas, 2013; Miller, 2000).

Figure 3.3: Microscopic images of A) ***Daphnia magna*** and B) ***Daphnia pulex***.

CRUSTACEAN BIOASSAY METHOD

In-house stock cultures of *D. magna* and *D. pulex* were both used for the development of the ecotoxicological toolbox. All crustacean bioassays were carried out alongside the ISO [6341:2012] guidelines with some alterations (ISO, 2012b). See Appendix 3 for a more detailed breakdown of all methods and protocols applied. Aerated spring water used for culturing the daphnids was used as the diluent as high levels of stress were observed in the daphnids when the suggested guidelines diluent was used resulting in failure to meet the validity criterion *i.e.,* <10% immobilisation observed in the negative control. Daphnids were considered immobile when movement could not be observed for >15s under gentle agitation. Ten mL of each chemical / water sample was placed into a 25mL beaker. Aerated spring water was used as the negative control. Each was set up in quadruplicate. Using a Motic dissecting microscope, five neonates were placed into each beaker using a glass Pasteur pipette, ensuring the neonates were released just below the surface of the liquid to minimise stress conditions. The beakers were then incubated at 20°C ± 2°C and exposed to a light cycle of 16h light and 8h darkness for 24h and 48h respectively, using an LMS phytoincubator. The percent of immobilisation was then calculated as follows;

Equation 4:

$$\% \text{ Immobilisation} = \left(1 - \frac{T_n}{C_n}\right) x\ 100$$

where T_n = number of mobile neonates in the treatment

C_n = number of mobile neonates in the control

3.2.4 PHYSICOCHEMICAL ANALYSIS

Good water quality is critical for the cultivation of fish as well as for the receiving ecosystems attached to aquaculture facilities. Analysing the physicochemical parameters is the most common method of determining the current water quality (Shukla *et al.*, 2013). Each parameter has a standard method therefore continuity can be achieved across the board (Baird *et al*., 2017). No definite physicochemical parameters specific for Irish aquaculture water and wastewater could be found. Therefore, a range of previous studies conducted on aquaculture facilities across the world were researched, as shown in Table A2.7 of appendix 2. The range of parameters investigated in these studies were applied to this research. The standard water and wastewater analysis methods (Table 3.1) were then applied to each parameter. As no values for the individual physicochemical parameter levels could be found in relation to Irish aquaculture, the standard Irish EPA water quality parameters based on the Freshwater Fish Directive [78/659/EEC] , Surface Water Regulations [1989] and surface water regulations [SI 272 of 2009] and amendments [SI 77 of 2019] were used for guidance (EPA, 2001; Irish Statutory Office, 2019, 2009). See Table A2.8 and Table A2.9 of appendix 2 for a breakdown.

PHYSICOCHEMICAL METHODS

Water parameters; temperature, pH, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), orthophosphate (PO_4^{3-}), dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), dissolved solids (DS), hardness, alkalinity, total acidity, and conductivity – were investigated in the laboratory within 24h of collection to prevent the need for preservation. Table 3.1 summarises the physicochemical methods employed in this research. Spectroquant® photometric kits were used to assess the NH_4^+, NO_2^-, $NO3^-$, PO_4^{3-} and COD. Analysis was conducted as per the manufacturer's instructions. Absorbance was analysed using a Shimadzu UV-2250 spectrophotometer. Ammonia (NH_3), nitrogen ammonium (NH_4-N) and orthophosphate as phosphorus (PO_4-P) were manually calculated from the NH_4^+ and PO_4^{3-} results, respectively. Temperature, pH, dissolved solids and conductivity were analysed using a VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe and VWR CO11 conductivity probe. DO and BOD_{5day} were analysed using a Jenway 9500 DO_2 meter and probe. Suspended solids were analysed via filtration using a Buchner flask, Buchner funnel and Whatman 0.45µm pore membrane filter. Hardness was assessed via titration using pH 10 buffer, Erichrome black and EDTA. Alkalinity and / or total acidity were analysed by titration using phenolphthalein indicator, methyl orange indicator, hydrochloric acid and / or sodium hydroxide. See Appendix 3 for a full breakdown of all physicochemical methods and protocols applied.

Table 3.1: Summary of all **physicochemical methods applied to this research**. All parameters / variables, their respective methods, standard analysis of water and wastewater method numbers and the detection limits for parameters where photometric test kits were employed have been included.

Parameter / Variable	Method (standard method number)	Detection Limit (mg L^{-1})
Alkalinity	Titrimetric (2320-B)	-
Ammonium (NH_4^+)	Photometric (4500-NH_3-F)	0.013 – 3.86 2.6 – 193.0
Biochemical Oxygen Demand (BOD)	Membrane Electrode (5210-B)	-
Chemical Oxygen Demand (COD)	Photometric (5220-D)	0 – 150 15 - 300
Conductivity	Electrode (2510-A)	-
Dissolved Oxygen (DO)	Membrane Electrode (4500-O G)	-
Dissolved Solids (DS)	Electrode (2540-C)	-
Hardness	Titrimetric (2340-C)	-
Nitrate (NO_3^-)	Photometric (4500-NO_3)	0.4 – 110.7
Nitrite (NO_2^-)	Photometric (345-1)	0.007 – 3.28
Orthophosphate (PO_4^{3-})	Photometric (4500-P-C)	0.007 – 15.3 1.5 – 92.0
pH	Membrane Electrode (2310-B)	-
Suspended Solids (SS)	Gravimetric (2540-D)	-
Temperature	Thermometer (2550-B)	-
Total Acidity	Titrimetric (2310-B)	-

3.2.5 STATISTICAL ANALYSIS

All statistical analysis and construction of dose response curves, standard curves, *etc*. were performed on GRAPHPAD PRISM 7, 8 and 9, and MINITAB 18 and 19. Linear regression was used to construct all standard curves and calculate R^2 values. Concentration versus response (variable slope) was used to construct all dose response curves. It was also used to calculate E_rC_{50}, IC_{50} and 95% confidence intervals (CI). The data generated were grouped and subject to normality tests (Anderson-Darling), to determine if samples were from a normal distribution ($p > 0.05$ = normal distribution). This in turn would establish whether parametric or non-parametric testing was to be conducted on results. As there was normal distribution, parametric testing was applied. T-tests and ANOVA were used to determine if any significant differences were observed in the variables ($p < 0.05$ = significant difference). Unpaired tests were used as different sets of samples were analysed to assess the quality of the aquaculture water samples. For the correlation studies, the Pearson's correlation coefficient (r) was used to determine whether any relationships existed between any of the parameters investigated. A breakdown of the statistical analysis determined can be found in Appendix 4.

3.3. FINDINGS

3.3.1. VALIDATION - RESULTS & DISCUSSION

Validation is an important step in the research process as it ensures that all bioassays and physicochemical tests to be employed are fit for purpose and performing appropriately. Two independent tests with triplicates per test were conducted for all bioassays and physicochemical tests. An example of a dose response curve (Figure 3.4) for bioassay validation and a standard curve (Figure 3.5) for physicochemical kit validation have been included. All additional graphical results can be found in Appendix 4.

The *P. subcapitata* algal bioassay was validated using two reference chemicals (Table 3.2); 3,5-DCP and $K_2Cr_2O_7$. Continuous shaking conditions are suggested for the algal bioassay, as indicated by the ISO [8692:2012] guidelines. However, due to logistical reasons, the algal bioassay had to be manually shaken. Therefore, to ensure the bioassay met the validation criterion (a 67-fold increase in the growth of the control), an additional 96h time period was conducted. However, as growth conditions were met in the control after 72h exposure, and for ease of reporting only the 72h results have been discussed. This was applied to all algal results in the project. After 72h, an E_rC_{50} value of 3.38 mg L^{-1} (± 1.30) for the 3,5-DCP and 1.19 mg L^{-1} (± 0.27) for the $K_2Cr_2O_7$ respectively, were required for validation. An E_rC_{50} value of 3.09 mg L^{-1} ($p = 0.7728$) and 1.13 mg L^{-1} ($p = 0.8707$) for 3,5-DCP and $K_2Cr_2O_7$

respectively, were established (Table 4.1) and no significant differences were observed. The *D. magna* crustacean bioassay was validated using two reference chemicals; $K_2Cr_2O_7$ and $ZnSO_4$. An EC_{50} value of between 0.60 and 2.10 mg L^{-1} for the $K_2Cr_2O_7$ and between 0.93 and 3.48 mg L^{-1} for the $ZnSO_4$ after 24h were required for validation. An EC_{50} value of 1.12 mg L^{-1} ($p = 0.9727$) and 1.96 mg L^{-1} ($p = 0.9999$) for $K_2Cr_2O_7$ and $ZnSO_4$ respectively, were established and no significant differences were indicated here either (Table 3.2). As the validity criterion for both reference chemicals were established in both bioassays, they were deemed ready for use.

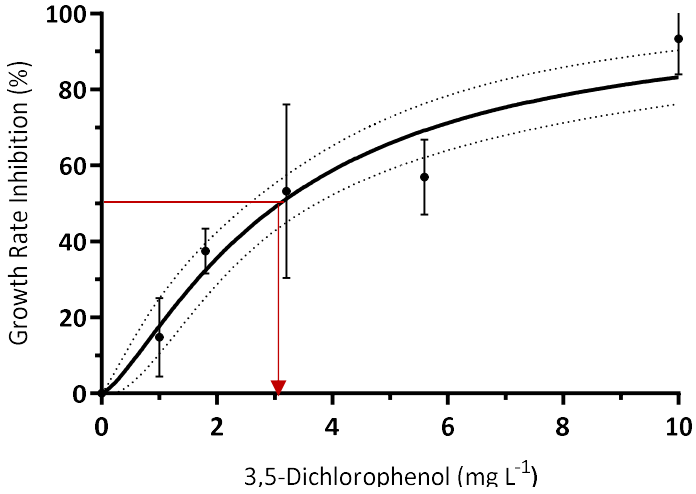


Figure 3.4*: Dose response curve** for the **validation** assay performed on ***P. subcapitata exposed to **3,5-DCP**. The concentration of 3,5-DCP in mg L^{-1} has been plotted against the percent growth rate inhibition. Results display two independent tests with triplicates per test. N = 6, E_rC_{50}, SD & 95% CI indicated. (p= 0.9531). Red line indicates the 72h E_rC_{50} value.

***Table 3.2*: Summary of results for all validation bioassays performed on *P. subcapitata* and *D. magna*.** 3,5-DCP and $K_2Cr_2O_7$ were performed on *P. subcapitata*. $K_2Cr_2O_7$ and $ZnSO_4$ was performed on *D. magna*. Data displayed includes E_rC_{50} values at 72h and 96h for the *P. subcapitata* and EC_{50} values at 24h and 48h for the *D. magna*. The 95% CI, validation criteria and the p value are also displayed.

	***Pseudokirchneriella subcapitata* Validation**		
3,5-DCP	**72h E_rC_{50} = 3.09 mg L^{-1}** (CI = 2.57 – 3.71) p = 0.7728	96h E_rC_{50} = 1.62 mg L^{-1} (CI = 1.52 – 1.72) p = 0.8282	**Validation Criterion** **72h E_rC_{50} = 3.38 mg L^{-1}** **(± 1.30)**
$K_2Cr_2O_7$	**72h E_rC_{50} = 1.13 mg L^{-1}** (CI = 1.02 – 1.25) p = 0.8707	96h E_rC_{50} = 0.82 mg L^{-1} (CI = 0.73 – 0.92) p = 0.8201	**Validation Criterion** **72h E_rC_{50} = 1.19 mg L^{-1}** **(± 0.27)**
	***Daphnia magna* Validation**		
$K_2Cr_2O_7$	**24h EC_{50} = 1.12 mg L^{-1}** (CI = 1.07 – 1.17) p = 0.9727	48h EC_{50} = 0.48 mg L^{-1} (CI = 0.45 – 0.50) p = 0.9999	**Validation Criterion** **24h EC_{50} =** **0.60 – 2.10 mg L^{-1}**
$ZnSO_4$	**24h EC_{50} = 1.96 mg L^{-1}** (CI = 1.74 – 2.22) p = 0.8079	48h EC_{50} = 1.23 mg L^{-1} (CI = 1.06 – 1.42) p = 0.7697	**Validation Criterion** **24h EC_{50} =** **0.93 – 3.48 mg L^{-1}**

Validation of all physicochemical test kits used for this research (NH_4^+, NO_2^-, NO_3^- PO_4^{3-} and COD Spectroquant® kits) was conducted via the wet chemistry standard methods that were initially used to develop the individual kits, as shown in Table 3.3. Statistical analysis indicated no significant differences for either method conducted (kit method and wet chemistry method), nor were significant differences were observed between the two methods when compared (Table 3.3). As a result, all test kits were deemed validated and ready for use.

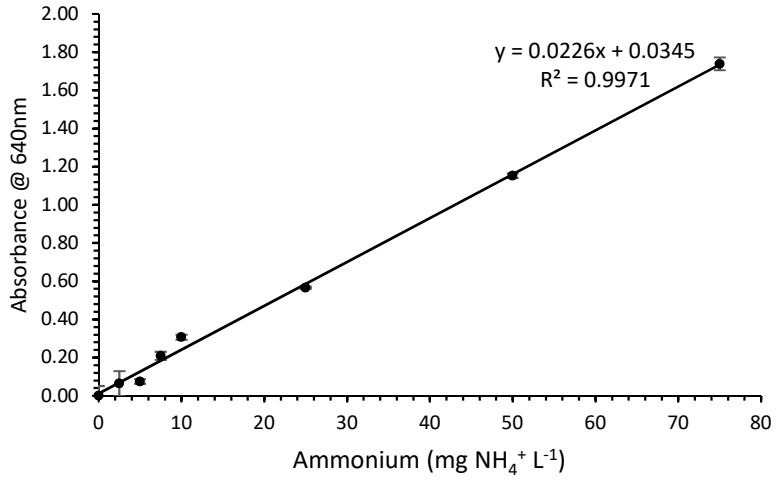


Figure 3.5: **Ammonium standard curve prepared via the Spectroquant Colourimetric Ammonium Test Kit.** The concentration of ammonium in mg NH_4^+ L^{-1} has been plotted against the absorbance. Absorbance has been measured at 640nm. Results display two independent tests with triplicates per test. N = 6, SD, R^2 and equation of the line indicated (p = 0.9958).

Table 3.3: **Overview and comparison of results determined for the validation of physicochemical parameters investigated by use of colourimetric kits.** Results for the standard curves produced from the kit and wet chemistry methods for NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, and COD are displayed. The R^2 value, equation of the line and p value have been for each method are listed. All standard guideline numbers and significant difference between the two methods have been included.

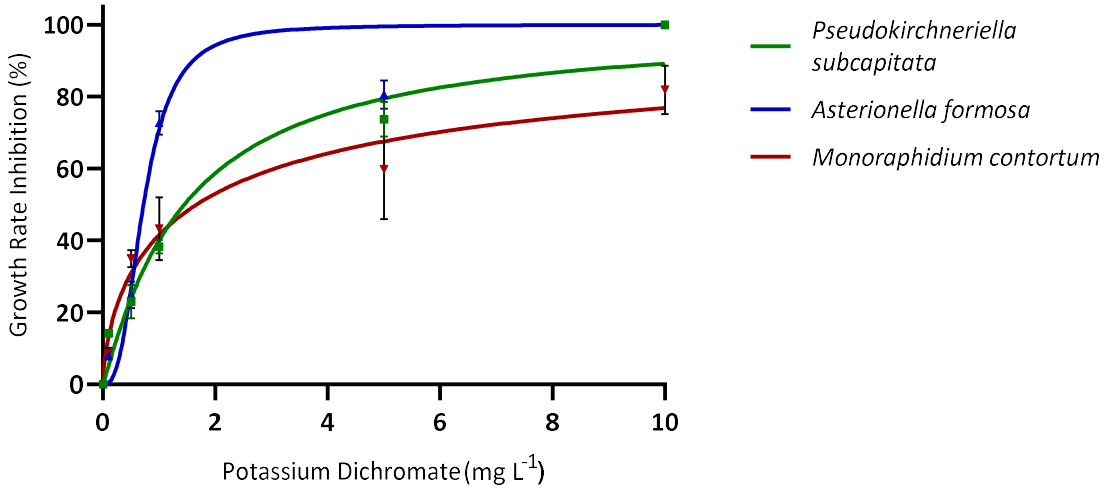
Parameter	Kit Standard Curve	Wet Chemistry Standard Curve	Significant Difference (<0.05)
Nitrate (NO_3^-)	R^2 = 0.9999 Y = 0.0122x – 0.0009 p = 0.9999	APHA 4500-NO_3 R^2 = 0.9990 Y = 0.012x – 0.001 p = 0.9977	p = 0.7567 No significant difference
Nitrite (NO_2^-)	R^2 = 0.9994 Y = 0.8458x + 0.0027 p = 0.9994	EPA 345-1 R^2 = 0.9992 Y = 0.0122x – 0.0009 p = 0.9996	p = 0.9883 No significant difference
Ammonium (NH_4^+)	R^2 = 0.9971 Y = 0.0226x + 0.0345 p = 0.9958	APHA 4500-NH_3-F R^2 = 0.9949 Y = 0.0122x – 0.0009 p = 0.9962	p = 0.9644 No significant difference
Orthophosphate (PO_4^{3-})	R^2 = 0.9991 Y = 0.235x + 0.0014 p = 0.9982	APHA 4500-P-C R^2 = 0.9969 Y = 0.0122x – 0.0009 p = 0.9977	p = 0.9802 No significant difference
Chemical Oxygen Demand (COD)	R^2 = 0.9999 Y = 0.0004x + 0.0039 p = 0.9300	APHA 5220-D R^2 = 0.9998 Y = 0.0122x – 0.0009 p = 0.8678	p = 0.8471 No significant difference

3.3.2. *Representative Species & Comparative Analysis – Results & Discussion*

In order to ensure the standardised bioassays were capable of providing an accurate prediction of the effects aquaculture may have on the receiving Irish freshwater aquatic ecosystem, species found in Irish systems were analysed alongside the standard species. The representative algal species chosen were *A. formosa* and *M. contortum*. The representative daphnia species chosen was *D. pulex*. To establish whether the standard species were fit for purpose *i.e.,* they represent Irish freshwater aquatic ecosystems despite their absence in Irish waters, the above-mentioned species were run alongside the standard species to investigate whether any significant differences were observed. Again, two independent tests with triplicates per test were conducted. *A. formosa* and *M. contortum* were exposed to the two reference chemicals *P. subcapitata* had been validated with. An E_rC_{50} value of 0.72 mg L^{-1} and 1.67 mg L^{-1} for *A. formosa* and *M. contortum*, respectively when exposed to $K_2Cr_2O_7$ (Figure 3.6), and 4.04 mg L^{-1} and 1.74 mg L^{-1} respectively for 3,5-DCP were established. After statistical analysis no significant differences were observed between the three species (Table 3.4). It should be noted that although no statistically significant differences were observed, *A. formosa* had a slower growth rate than *P. subcapitata* and *M. contortum i.e.*, *P. subcapitata* and *M. contortum* demonstrated a similar growth rate, whereas *A. formosa* was much less consistent. No research was found in the available literature demonstrating comparatives between the three different algal species. As a result, the three algal species were exposed to a range of different compounds in order to establish a better comparative. The compounds chosen (NH_4^+, NO_2^-, NO_3^-, and PO_4^{3-}) were done so as these were some of the compounds the algae were likely to encounter during the pilot study and monitoring program. Table 3.4 summarises all E_rC_{50} values obtained. As with the reference chemicals, statistical analysis indicated no significant differences between the three algal species when exposed to NH_4^+ ($p = 0.4943$), NO_2^- ($p = 0.8840$), NO_3^- ($p = 0.8877$) and PO_4^{3-} ($p = 0.9910$).

P. subcapitata is the species of choice for standard ecotoxicological tests, as per ISO [8692:2012]. According to the NBDC (2021a). *P. subcapitata* have not been reported in Irish waters. Both *A. formosa* and *M. contortum* have been reported in Irish waters in great abundance (NBDC, 2021b, 2019a). Additionally, studies conducted by Durante *et al*. (2013), Murnaghan *et al*. (2015), Sparber *et al*. (2015) and, Talling and Heaney (2015) on the algal composition of Irish fresh waters found that *A. formosa* was once of the most common freshwater diatoms detected in Irish waters. Hence, *A. formosa* was further investigated and subsequently chosen as a representative species. As far as the author is aware, previous studies on *A. formosa* or *M. contortum* using a modified version of the ISO [8692:2012] algal bioassay has not yet been conducted. Although some differences were observed in the toxicological responses none were statistically significant, therefore *P. subcapitata* was deemed

suitable to provide an accurate prediction as to the effects freshwater aquaculture may have on the receiving Irish freshwater aquatic ecosystem. However, due to the inconsistencies in growth rates, *A. formosa* was also included in the monitoring process to confirm no significant differences.



***Pseudokirchneriella subcapitata* 72h E_rC_{50} = 1.13 mg L^{-1}** (1.02 – 1.25)
***Asterionella formosa* 72h E_rC_{50} = 0.72 mg L^{-1}** (0.66 – 0.81)
***Monoraphidium contortum* 72h E_rC_{50} = 1.67 mg L^{-1}** (1.32 – 2.12)

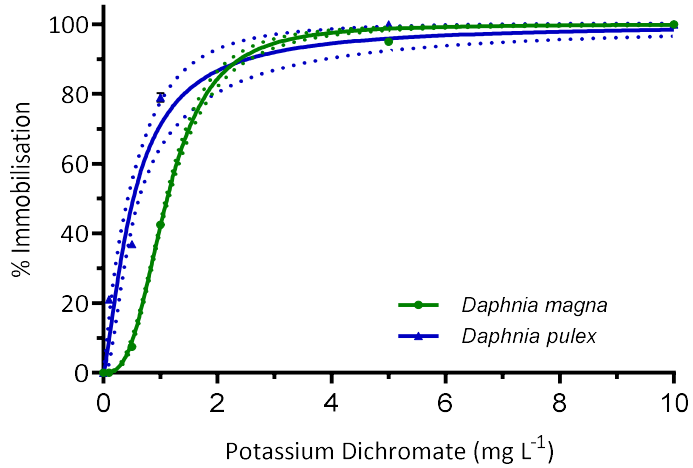
Figure 3.6:* Comparative dose response curve** for ***P. subcapitata (green), ***A. formosa*** (blue) and ***M. contortum*** (red) exposed to **$K_2Cr_2O_7$ for 72h**. The concentration of $K_2Cr_2O_7$ in mg L^{-1} has been plotted against the percent growth rate inhibition. Results display two independent tests with triplicates per test. N = 6, E_rC_{50}, 95% CI & SD indicated. (p= 0.7041). Results display two independent tests with triplicates per test.

Table 3.4:* Summary** of results for **comparative study** between the algae ***P. subcapitata, A. formosa, and ***M. contortum*** after 72h exposure to 3,5-DCP, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and $K_2Cr_2O_7$. P values have been indicated (significant differences = $p < 0.05$).

Chemical	*Pseudokirchneriella subcapitata* E_rC_{50} (mg L^{-1})	*Asterionella formosa* E_rC_{50} (mg L^{-1})	*Monoraphidium contortum* E_rC_{50} (mg L^{-1})	Significant Difference (p value)
3,5-DCP	3.09	4.04	1.74	0.7399
NH_4^+	148.10	6.67	21.69	0.4943
NO_2^-	80.07	11.16	31.39	0.8840
NO_3^-	94.77	238.00	114.20	0.8877
PO_4^{3-}	58.84	39.79	42.93	0.9910
$K_2Cr_2O_7$	1.13	0.72	1.67	0.7041

D. pulex was exposed firstly to the two reference chemicals used for the *D. magna* validation and an EC_{50} value of 0.53 mg L^{-1} (Figure 3.7) and 3.10 mg L^{-1} were observed after 24h exposure to $K_2Cr_2O_7$ and $ZnSO_4$ respectively. For comparative purposes, both daphnid species were then compared using 3,5-DCP due to its availability. After 24h an EC_{50} value of 3.60 mg L^{-1} and 4.06 mgL^{-1} were indicated for the *D. magna* and *D. pulex* respectively. After statistical analysis, no significant differences were observed between either species ($K_2Cr_2O_7$ p = 0.5665, $ZnSO_4$ p = 0.6533, 3,5-DCP p = 0.6388). A more comprehensive study could not be directly conducted due to low numbers of daphnids in the stock cultures. As a result, a 48h incubation period was also conducted with no significant differences observed here either (Table 3.5). A literature search of comparative studies between the two daphnid species was also conducted, a summary of which can be found in Table 3.5.

D. magna is the species of choice for standard ecotoxicological tests, as per ISO [6341:2012]. According to the NBDC (2021c), *D. magna* have not been reported in Irish waters. However, *D. pulex* have been found in Irish waters, with the latest reports in September 2016 (NBDC, 2019b). Additionally, *D. pulex* was also chosen as a representative species after a strong inverse relationship was observed between the crustacean and *P. subcapitata* during the pilot study. See next sub-section. As a result of this, *D. pulex* was further investigated and subsequently chosen as a representative species for a primary consumer in Irish freshwater ecosystems. Comparatives between *D. magna* and *D. pulex* have been routinely conducted. A review of existing literature was conducted to determine whether any significant differences existed between the two Daphnids. In recent times more modern techniques, such as PCR and gene expression, have been used to assess the toxicological status of compounds using Daphnids (Chain *et al.*, 2019; Litoff *et al.*, 2014; Y. Liu *et al.*, 2017; Shaw *et al.*, 2008). In light of this, historical research which use the traditional toxicity assay used in this study has been investigated along with modern studies. The results obtained in this research and the previous comparative studies investigated have suggested that as no differences exist in toxicological responses between the *D. magna* and *D. pulex* (Canton and Adema, 1978; Maki and Bishop, 1979; Winner and Farrell, 2011). Therefore, *D. pulex* was considered a suitable alternative to *D. magna* to represent the potential effects aquaculture output has on primary consumers in Irish aquatic ecosystems.



***Daphnia magna* 24h EC_{50} = 1.12 mg L^{-1}** (1.07 – 1.17)

***Daphnia pulex* 24h EC_{50} = 0.53 mg L^{-1}** (0.48 – 0.59)

***Figure 3.7: Comparative dose response curves** for* the **validation** assays performed on ***D. magna* (Green),** and ***D. pulex* (Blue),** exposed to **$K_2Cr_2O_7$ for 24h**. The concentration of $K_2Cr_2O_7$ in mg L^{-1} has been plotted against the percent immobilisation. Results display two independent tests with triplicates per test. N = 8, EC_{50}, S.D. & 95% CI indicated (p= 0.5665).

Table 3.5*: Summary** of **LC_{50}** results for ***D. magna and ***D. pulex*** at **48h** exposure time after a short literature review was conducted on previous **comparative studies** as well as **IC_{50}** results established in this research with $K_2Cr_2O_7$, $ZnSO_4$ and 3,5-DCP. Results are in mg L^{-1}. LAS = alkyl benzene sulfonates, AE = alkyl ethoxylates and C_n = the length of carbon chain within the compound. The 95% CI and p values for this research have been included.

Test Compound	*Daphnia magna*	*Daphnia pulex*	Reference
Benzene	426	305	
o-Cresol	15.70	9.60	(Maki and Bishop, 1979)
Aniline	0.55	0.11	
Trichloroethylene	65	45	
C_{12} LAS	6.84	8.62	
C_{14} LAS	0.80	0.59	(Winner and Farrell, 2011)
C_{15} LAS	0.20	0.15	
C_{14} AE	0.14	0.10	
Copper	0.47	0.47	(Walsh, 2012)
$K_2Cr_2O_7$	0.48 (0.45 – 0.50)	0.12 (0.02 – 0.22) p = 0.8810	
$ZnSO_4$	1.23 (1.06 – 1.42)	2.53 (1.30 – 3.76) p = 0.4900	This research
3,5-DCP	1.20 (1.07 – 1.35)	2.05 (1.02 – 3.08) p = 0.6264	

3.3.3. Pilot Study – Results & Discussion

The pilot study was conducted in order to establish a baseline for the FTS fish farm at Keywater Fisheries in Co. Sligo, before further development and employment of the ecotoxicological toolbox. Samples were collected every two weeks from April 2018 to October 2018. Due to unavoidable circumstances, samples could not be collected and analysed during the month of June 2018. See Appendix 4 for a summary of all results.

BIOASSAY ANALYSIS

The *P. subcapitata* algal bioassay [ISO 8692:2012] was conducted on intake and output water samples to determine whether growth rate inhibition or stimulation were observed as a result of exposure. Growth rate inhibition was observed in both sample sets (Figure 3.8A). No growth rate stimulation was observed in the intake water however, up to 50.47% was observed in the output samples (Figure 3.8B). Statistical analysis indicated a significant difference between the intake and output water samples ($p = <0.001$). A higher level of growth inhibition was observed in the intake water compared to the output water. This suggested that the intake water would seem unlikely to cause issues such as algal blooms. However, the high level of growth inhibition in the intake water also indicated toxicity and may result in losses to the biodiversity within the receiving water body (Guéguen *et al.*, 2004; Ivanova and Groudeva, 2006; Ma *et al.*, 2006). This toxicity may result in the loss of primary producers (*e.g.,* algae) in the aquatic ecosystem. This may subsequently cause indirect adverse effects on the aquatic food chain (*e.g.*, micro-crustaceans feed on algae and fish in turn, feed on the micro-crustaceans). Loss of the algae removes the food source for the micro-crustaceans, resulting in their potential loss. This in turn, could result in the removal of a valuable food source for the fish. It should be noted that this toxic effect does not occur within the fish farm itself and suggests potential issues upstream of the farm.

The growth stimulation observed in the output water occurred in mid-April and then again from July to September. This coincided with the elevated temperatures and drought conditions experienced in Ireland in the summer of 2018. The ability of the output water to cause growth stimulation suggested that the possibility of algal blooms (resulting in eutrophication) downstream of the fish farm are more likely to occur. This may result in loss of biodiversity, habitat and submerged aquatic vegetation, disruption of the ecosystems functionality, deficiencies in oxygen and modifications in food webs (Rabalais, 2002)**.** The correlation studies (see Appendix 4) demonstrated a moderate to moderately strong negative / inverse relationship between algal growth inhibition and increases in temperature ($r = -0.619$) *i.e.,* algal growth stimulation increased as temperatures increased. No research readily available indicated the potential for using *P. subcapitata* growth stimulation as an indicator of eutrophication suggesting that the alga is being underutilised. Most of the available research involving *P. subcapitata* focused on inhibition of growth (Zhang *et al.*, 2011). However, one study involving the algae and aquaculture wastewater published by Miashiro *et al.* (2012) demonstrated similar results to this study *i.e.,* growth stimulation instead of inhibition was observed. They suggested that the stimulated algal growth may have been due to the high concentration of nutrients that were observed. High levels of nutrients, were also observed in this study. The lack of available research further suggested an under use of *P. subcapitata* as an early indicator of potential issues in aquaculture.

Correlation studies found a strong negative relationship between the algae and concentration of suspended solids (r = -0.727) suggesting that as the concentration of suspended solids increased, the stimulation of algal growth also increased. This was most likely due to the decreases in flow rates as a result of the drought conditions.

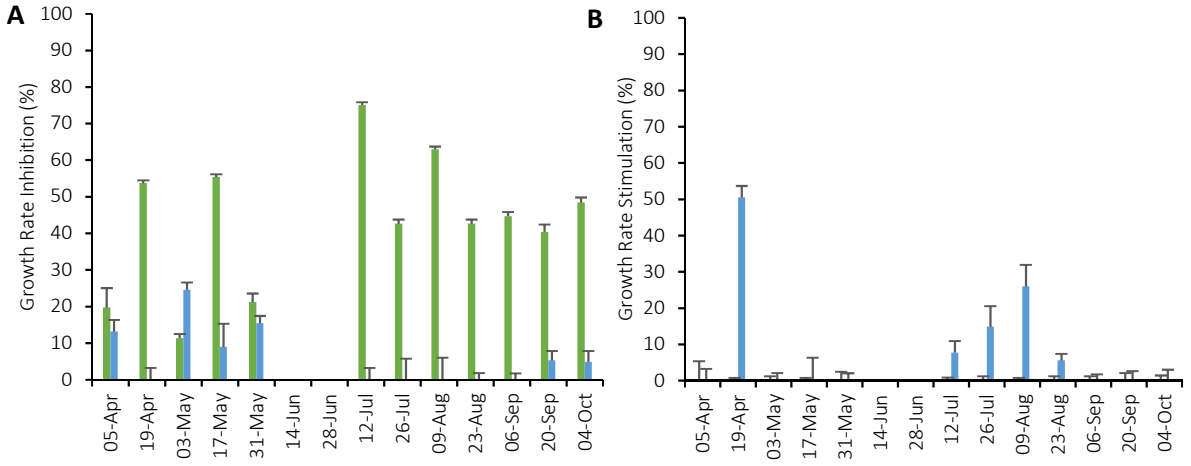


Figure 3.8: (A) **growth inhibition** and (B) **growth stimulation** observed in ***P. subcapitata*** **when exposed to freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries for 72h. Samples were tested every two weeks from April 2018 to October 2018. N = 3 & SD indicated (p = < 0.001 for inhibition & stimulation).

The *D. magna* crustacean bioassay [ISO 6341:2012] was conducted on the samples to determine whether immobilisation was observed after exposure. Due to its availability, *D. pulex* was also exposed to the samples. Low levels of immobilisation were observed in *D. magna* (Figure 3.9A) and *D. pulex* (Figure 3.9B) when exposed to the intake and output water. Statistical analysis demonstrates no significant differences (*D. magna* p = 0.8149, *D. pulex* p = 0.5082). Due to unforeseen technical issues associated with the cultures, the use of *D. pulex* had to cease mid-July and the use of *D. magna* had to cease at the end of August. The low levels of immobilisation observed in the *D. magna* suggested that the crustacean may be more robust than anticipated and might not be the best choice for the toolbox, especially given the fact that no difference in immobilisation was observed during the heat wave and drought conditions experienced when compared to the differences observed in the algal bioassay *i.e.,* the inhibitory / stimulatory effects demonstrated. No studies on the use of *D. magna* as a means of analysing freshwater aquaculture output water could be found in the available literature. Given the apparent robustness of the *D. magna* crustacean, an additional time period of 48h was also conducted. This time period was chosen as the ISO [6341:2012] guidelines followed suggested a 48h time period may also be conducted. No issues were observed during the 48h time period also. Due to issues with the stock cultures, testing with the Daphnid ceased mid-way though the pilot study. As no significant differences were observed between the intake and output water, and very low levels of

immobilisation being observed, the decision to end testing with the *D. magna* was taken in order to save on resources. *S*imilar to the *D. magna*, issues with the *D. pulex* stock culture were experienced and testing ceased mid-way through the pilot study also. As no significant differences were observed between the intake and output water, or between the *D. magna* and *D. pulex* (p = 0.2249), and the fact that very low levels of inhibition were being observed here too, the decision to end testing with the *D. pulex* was also taken in order to save on resources.

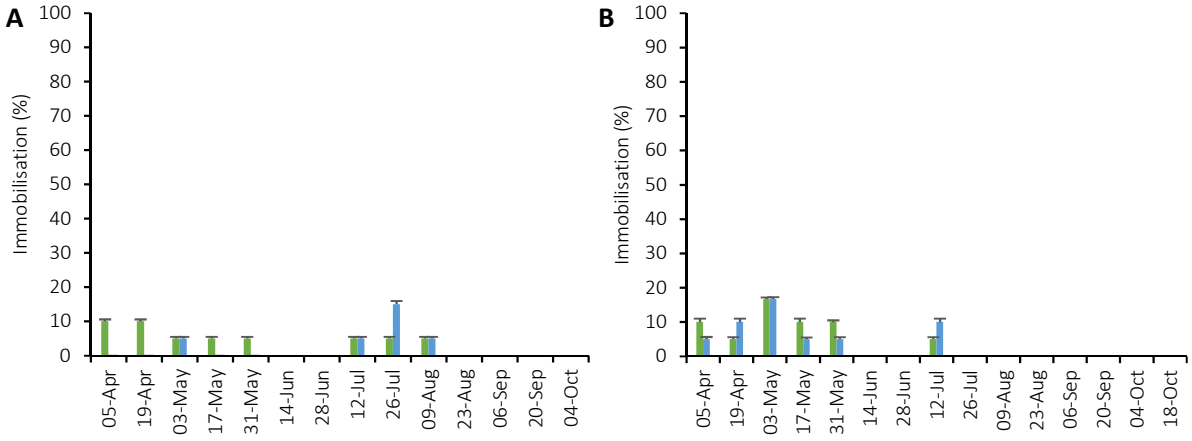


Figure 3.9: **Immobilisation** observed in (A) ***D. magna*** and (B) ***D. pulex*** **when exposed to freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries for 24h. Samples were tested every two weeks from April 2018 to October 2018. N = 4 & SD indicated (*D. magna* p = 0.8149, *D. pulex* p = 0.5082).

PHYSICOCHEMICAL ANALYSIS

Fluctuations in the NH_4^+ (Figure 3.10A), NO_2^- (Figure 3.10B) and NO_3^- (Figure 3.10C) levels were observed in both the intake and output water. Statistical analysis indicated a significant difference between the intake and output water for the NH_4^+ (p = 0.0010) and the NO_2^- (p = <0.001) but not for the NO_3^- (p = 0.0057). The first time point for the NH_4^+ analysis is not present due to delays in receiving the ammonium kit from the supplier. NH_4^+ can be highly toxic to aquatic life (Zhang *et al.*, 2011), and requires treatment before its release into the receiving water body (Celik *et al.*, 2001). When comparing both samples, the concentration of NH_4^+ present in the intake is lower than that of the output. This suggested that the levels of NH_4^+ detected are being generated within the farm itself. However, the small amount detected in the intake water also suggests some form of pollution is being generated upstream of the farm. The concentrations observed in the output water were more than five times greater than the one mg L^{-1} suggested by the EPA (EPA, 2001). However, the concentrations observed in this study were similar to those determined by Boaventura *et al*. (1997) in their study on trout effluent, and Costanzo *et al.* (2004) in their study on shrimp pond effluent. It should also be noted that the dilution factor of the receiving river had not been included and needs to be taken into

consideration. Presence of NH_4^+ is an indicator of recent pollution. It should be noted that no output was released during this period.

As expected, NO_2^- levels detected were very low. These low levels are mainly due to the fact that, although highly toxic to aquatic life (Pollice *et al.*, 2002), NO_2^- is highly unstable and only remains in this form for a short period of time during the transformation of NH_4^+ to NO_3^- (Durborow *et al.*, 1997). When comparing the NO_2^- levels in both samples, levels in the output water were much higher than that of the intake water. Similar with the NH_4^+, the small amount detected in the intake water suggests some form of pollution is being generated upstream of the farm. The higher levels detected in the output water are generated within the farm itself. The concentrations detected were one log dose greater than the 0.03 mg L^{-1} for cyprinid waters, as per the EPAs suggested water quality parameters (EPA, 2001). Despite this, NO_2^- levels determined were in agreement with levels observed by other research conducted on shrimp, trout and prawn effluents (Caramel *et al.*, 2014; Herbeck *et al.*, 2013; Mcintosh and Fitzsimmons, 2003; Moreira *et al.*, 2010; Pulatsü *et al.*, 2004). Similar with NH_4^+, the presence of NO_2^- is an indication of recent pollution.

NO_3^- levels observed in the intake water indicated that NO_3^- may have been entering the river upstream of the fish farm. A spike in the output water at the end of September 2018 was observed and may have been due to the fact that the aquatic plant *L. minor* (duckweed), which uses NO_3^- as a nutrient source, within the farm had been removed. NO_3^- levels were well below the guidance value of 50 mg L^{-1} suggested by the EPA (EPA, 2001). The levels of NO_3^- that were observed in this study were replicated by Boaventura *et al*. (1997), Camargo (1994), Guilpart *et al*. (2012), Lalonde *et al*. (2014) and Pulatsü *et al*. (2004), who all investigated trout aquaculture, and by Biao *et al*. (2004), Costanzo *et al*. (2004), Ferreira *et al*. (2011), Herbeck *et al.* (2013) and, Mcintosh and Fitzsimmons (2003) and their studies on shrimp. A correlation was found between NO_3^- and DO levels (r = 0.578) suggesting that as DO levels increased so did NO_3^- levels. This was expected as oxygen is required for the aerobic conversion of NO_2^- to NO_3^-. Additionally, due to *L. minor*'s ability to use NO_3^- as a nutrient source and the spikes observed in the farm after its removal suggests that the duckweed holds great promise as a potential wastewater treatment option for aquaculture and further research into this possibility needs to be conducted.

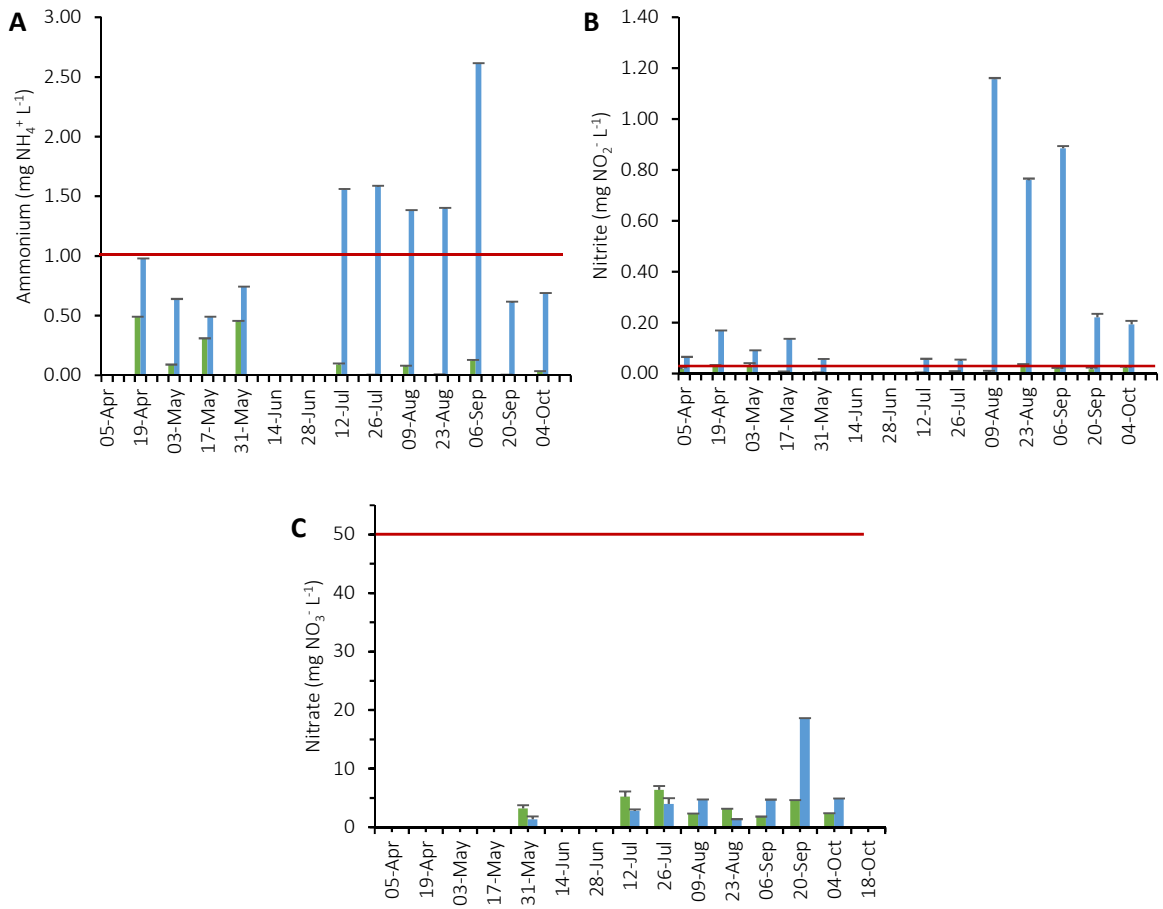


Figure 3.10: Concentrations of (A) **ammonium**, (B) **nitrite** and (C) **nitrate** detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries. Samples were collected and analysed every two weeks between April 2018 and October 2018. N = 6 & SD indicated (NH_4^+ p = 0.0010, NO_2^- p = <0.001, NO_3^- p = 0.0057).

PO_4^{3-} levels were observed in both sample sets (Figure 3.11) where a statistically significant difference was observed (p = 0.0130). PO_4^{3-}, a reactive form of phosphorus (Brogan *et al.*, 2001), was detected in both the intake and output water. The levels observed in the intake water were less than that in the output water. This suggested that phosphorus pollution is entering the river upstream of the fish farm, as well as being generated within the farm itself. Levels in both water sources may be cause for concern as orthophosphates are one of the main causes of algal blooms and the hypoxic conditions which may occur in water bodies (Barcellos *et al.*, 2019; Brogan *et al.*, 2001). Concentrations detected were just over two log doses greater than the recommended value of 0.035 mg L^{-1} set out by the SI 272/2009 and SI 77/2019 for good water status. However, they were in accordance with Stephens and Farris (2004a, 2004b) and Ziemann *et al.* (1992) and their studies on finfish farming effluent, which included studies on catfish. The farm uses a constructed wetland pond for treatment of NO_3^-, PO_4^{3-} and so forth, before being released. However, it has been suggested that the wetland may need to be 0.7 to 2.7 times the size of the culture area to be effective and can be less efficient in the removal of phosphorus wastes (Jegatheesan *et al.*, 2011; Sharrer *et al.*, 2016). Sipaúba-Tavares *et al*. (2017) has

also suggested that constructed wetlands vary with climate change issues. The owner recognised that the constructed wetland needed to be investigated and further research was then conducted in this area. See section 3.3.4. It should again be noted that no output was released during this time.

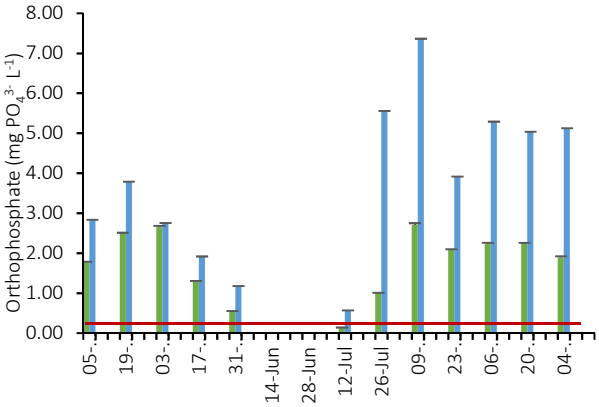


Figure 3.11: **Orthophosphate** levels detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries between April 2018 and October 2018. Samples were collected and analysed every two weeks. N = 6 & SD indicated (p = 0.0130).

Variations in DO (Figure 3.12A), BOD (Figure 3.12B) and COD (Figure 3.12C) levels were observed across the entire pilot study. A statistically significant difference was indicated between the intake and output samples for the DO (p = 0.0010). However, no significant differences were observed with the BOD (p = 0.2127) and the COD (p = 0.2301) results. The recommended dissolved oxygen concentration present in salmonid waters should be ≥9 mg L^{-1} and cyprinid waters (*e.g.,* perch) should be ≥7 mg O_2 L^{-1} (EPA, 2001). There are no issues with the DO levels present in the intake water however there may be cause for concern with levels observed in the output water as they were well below the recommended concentration of ≥7 mg O_2 L^{-1}. Levels below this concentration were only observed during the heat wave and drought conditions and the unusual weather conditions may have played a role. Conditions began to improve once weather conditions had returned to normal. Alam *et al*. (2007) and da Silva *et al.* (2017) have suggested that oxygen concentrations of ≥4 mg L^{-1} are sufficient for the maintenance of aquatic life. DO levels in other studies, which included shrimp, catfish, prawn and trout farming, were similar to the concentrations observed in this study (Biao *et al.*, 2004; Camargo, 1994; da Silva *et al.*, 2017; Mcintosh and Fitzsimmons, 2003; Moreira *et al.*, 2010; Namin *et al.*, 2013; Stephens and Farris, 2004a, 2004b). BOD is the amount of oxygen used by bacteria in breaking down organic matter in the water (EPA, 2001). SI 272/2009 and SI 77/2019 recommend a mean BOD concentration of 1.3 mg L^{-1} for high water status and 1.5 mg L^{-1} for good water status (Irish Statutory Office, 2019, 2009). However, the EPA has suggested ≤3 mg L^{-1} and ≤6 mg L^{-1} for salmonid and cyprinid waters, respectively (EPA, 2001). The current BOD levels detected in the intake water suggested no

issues. The concentration of BOD detected in the output water may be cause for concern. Although the level was below that suggested by the EPA for cyprinid water, it was greater than that suggested in the SI 272/2009 and SI 77/2019. This research was then compared to results determined by other researchers. Although not many studies included BOD, those that were revised demonstrated higher levels than the concentrations detected in this study (Ansah *et al*., 2012; Boaventura *et al*., 1997; Mcintosh and Fitzsimmons, 2003; Miashiro *et al*., 2012). With regards to the COD Levels, a spike of 151.66 mg O_2 L^{-1} in May, were observed in the intake water and spikes of 169.19 and 197.49 mg O_2 L^{-1} in May and July respectively, were observed in the output water and were most likely due to drought conditions. COD measures the stress a quantity of organic matter puts on a receiving water body (Lee and Nikraz, 2015). COD was detected in both the intake and output water. The levels observed in both sets of samples may be cause for concern, especially the output water. The mean concentration was almost double the suggested 40 mg L^{-1} set out by the Irish EPA (EPA, 2001). Very few studies included COD as a parameter in their investigations. However, research conducted by da Silva *et al*. (2017) reported some COD levels similar to those determined in this research.

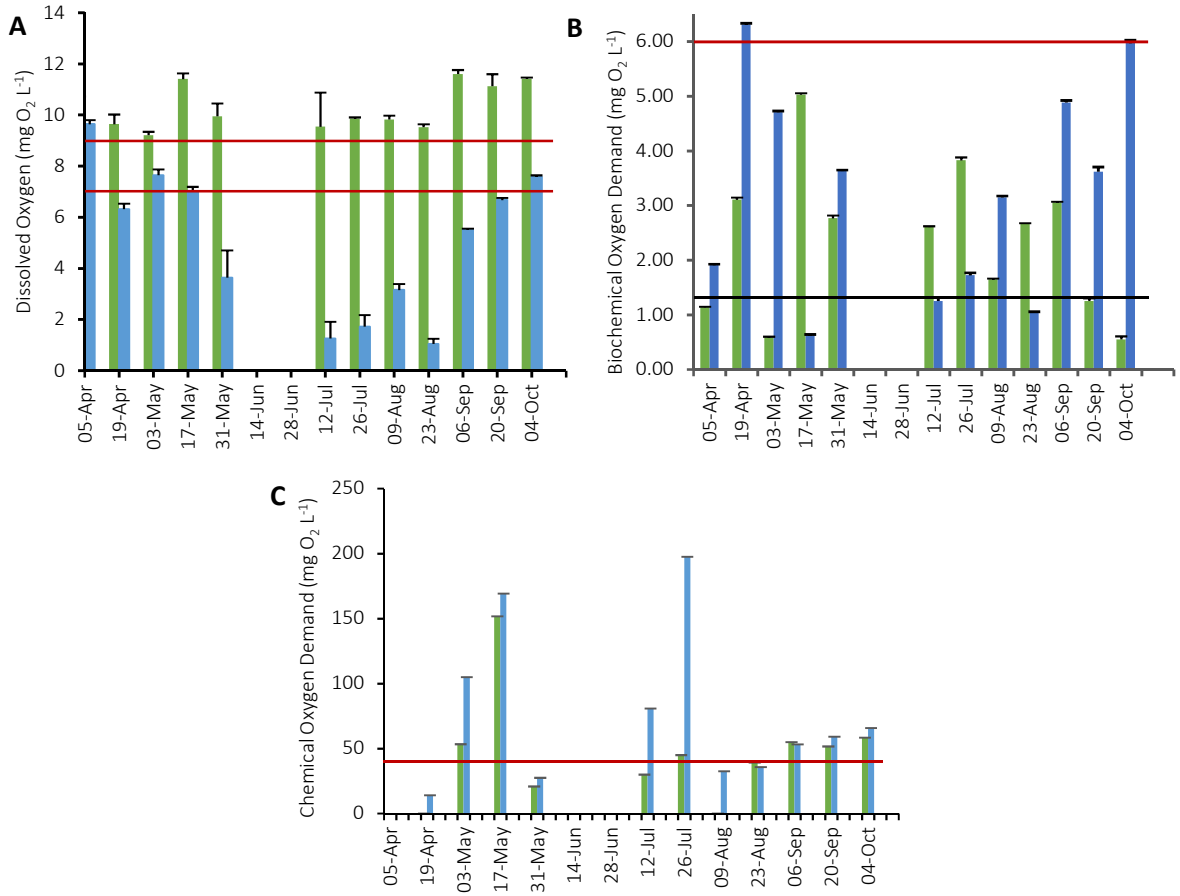


Figure 3.12: (A) **Dissolved oxygen**, (B) **biochemical oxygen demand** and (C) **chemical oxygen demand** levels detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries. Samples were collected and analysed every two weeks from April 2018 and October 2018. N = 3 & SD indicated (DO p = 0.0010, BOD p = 0.2127, COD p = 0.2301).

Calcium carbonate ($CaCO_3$) levels were analysed for the hardness and alkalinity, as shown in Figure 3.13. Some variations were observed however, no statistically significant differences were indicated between both set of samples for the hardness results (p = 0.0922) or the alkalinity results (p = 0.9153). $CaCO_3$ improves conditions for benthic animals and microbial activity, increases CO_2, phosphorus and other nutrient availability, improves survival and production, and enhances phytoplankton growth. The alkalinity is the buffering capacity of the water body and is related to important factors in aquaculture (Ferreira *et al.*, 2011). The $CaCO_3$ alkalinity results from shrimp and catfish studies demonstrated similar results (Ferreira *et al.*, 2011; Mcintosh and Fitzsimmons, 2003). Water hardness is the amount of dissolved calcium and / or magnesium present in the water. The $CaCO_3$ levels were measured for this study. Results suggested that the water is slight to moderately hard. This correlates with water hardness maps of Ireland which demonstrated water around Boyle, Co. Roscommon was slightly too moderately hard. There are no guidance values for alkalinity or hardness as the alkalinity and buffering capacity of water bodies vary throughout the country, as does the water hardness. Similar hardness results were observed in revised studies on catfish and Atlantic salmon (Lalonde *et al.*, 2014; Stephens and Farris, 2004a, 2004b).

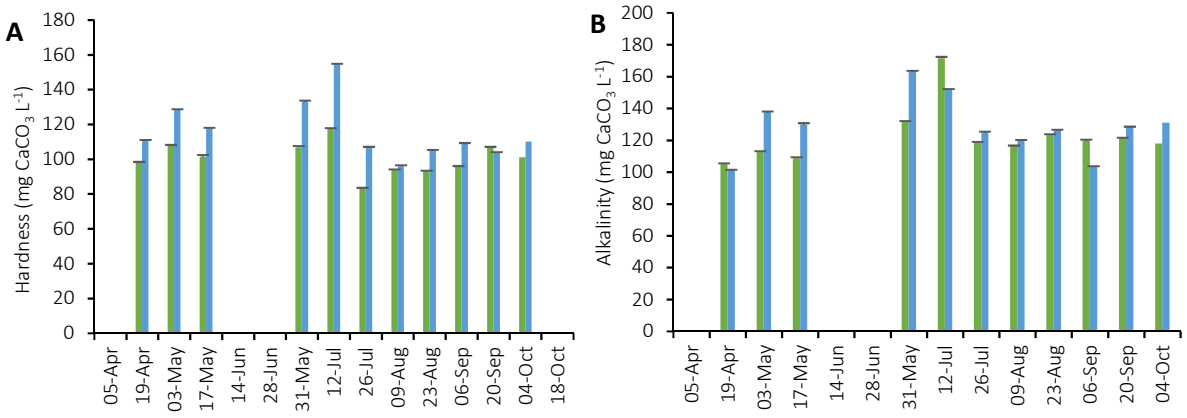


Figure 3.13: Calcium carbonate levels for (A) **hardness** and (B) **alkalinity** detected **in freshwater aquaculture finfish intake** (green) and **output** (blue) **water** from Keywater Fisheries. Samples were collected and analysed every two weeks from April 2018 and October 2018. N = 3 & SD indicated (hardness p = 0.0922, alkalinity p = 0.9153).

Fluctuations were observed in the temperature range (Figure 3.14A) and the pH range (Figure 3.14B) in the intake and output water samples. However, after statistical analysis was conducted between both sets of samples, no significant differences were indicated (temperature p = 0.8539, pH p = 0.7896). With growing concerns associated with climate change and global warming, increases in temperatures may become more frequent. Temperature is a critical environmental factor for

aquaculture due to its effect on growth, metabolism, survival, immune responses and oxygen consumption (Ferreira *et al.*, 2011). Fluctuations in temperature were observed in both sets of samples. These rises in temperatures were only observed during the elevated temperatures experienced in Ireland in 2018. The results for the pH indicated that the intake water was slightly more alkaline than the output water, which held a pH of just above neutral (pH 7). The recommended pH levels should be between pH 6 and pH 9. Levels in both sample sets are well within this level and therefore present no issues. The mean temperature and pH results observed were similar to those recorded in the revised studies that focused on freshwater finfish *i.e.,* catfish, brown trout and rainbow trout (Boaventura *et al.*, 1997; Caramel *et al.*, 2014; Namin *et al.*, 2013; Noroozrajabi *et al.*, 2013; Pulatsü *et al.*, 2004; Stephens and Farris, 2004b; Živić *et al.*, 2009).

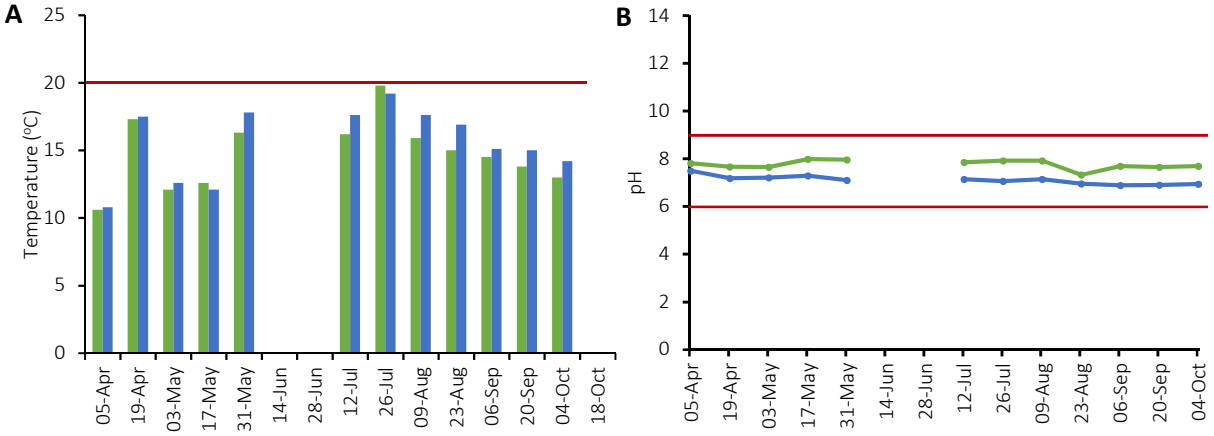


Figure 3.14: (A) **temperature** and (B) **pH** levels detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries. Samples were collected and analysed every two weeks from April 2018 and October 2018. N = 3 & SD indicated (temperature p = 0.8539, pH p = 0.7896).

Suspended solids in the intake water were between 4 and 30 mg L^{-1}, with a spike of 290 mg L^{-1} in mid-April, whilst in the output water levels were between 11 and 70 mg L^{-1}, with spikes of 170 and 520 mg L^{-1} observed in mid-April and mid-May respectively (Figure 3.15A). Dissolved solids were found to be between 106 mg L^{-1} and 206 mg L^{-1} in the intake water and between 129 and 235 mg L^{-1} in the output water (Figure 3.15B). Statistical analysis was conducted between the intake and output water and no significant differences were observed for either the suspended solids (p = 0.0730) or the dissolved solids (p = 0.5130). Suspended solids often consist of organic matter and elevated levels can be an indicator of eutrophic conditions (Bilotta and Brazier, 2008). Two concentrations of suspended solids have been suggested by the Irish EPA (EPA, 2001). Fifty mg L^{-1} as per the Irish Surface Water Regulations [1989], and 25 mg L^{-1} as per the Freshwater Fish Directive [78/659/EEC], and Irish Salmonid Waters Regulations [1988]. Suspended solids can increase gill irritation and blanket the benthos (Bilotta and Brazier, 2008), therefore the lower concentration of 25 mg L^{-1} was used as the

guidance value for the maximum allowable concentration (MAC). The average levels detected in both the intake and output water may be cause for concern as they were above the 25 mg L^{-1}. Increased levels were most likely due to reduced flow rates as a result of the drought conditions. Again, wastewater was not released during this time period. Suspended solid concentrations in a range of studies on shrimp, prawn, salmonid, catfish, brown trout and rainbow trout (Boaventura *et al.*, 1997; Camargo, 1994; Caramel *et al.*, 2014; Costanzo *et al.*, 2004; Guilpart *et al.*, 2012; Lalonde *et al.*, 2014; Mcintosh and Fitzsimmons, 2003; Pulatsü *et al.*, 2004; Ziemann *et al.*, 1992) were similar to those established in this study. Water is an excellent solvent (*i.e.*, it is the universal solvent) and can pick up impurities easily. Dissolved solids generally consist of inorganic salts and small levels of organic matter and, is the sum of cations and anions in the water. It is considered as more of a qualitative measure and does not indicate the nature or ion relationship. Therefore, it is used as a general indicator of water quality (Oram, 2020; WHO, 2003). The EPA's parameters for water quality have indicated no reference or recommendation for levels however, according to WHO (2003) concentrations <300 mg L^{-1} indicate excellent water quality. As all dissolved solid levels were below this value no concerns were indicated with regards to the general quality of the water.

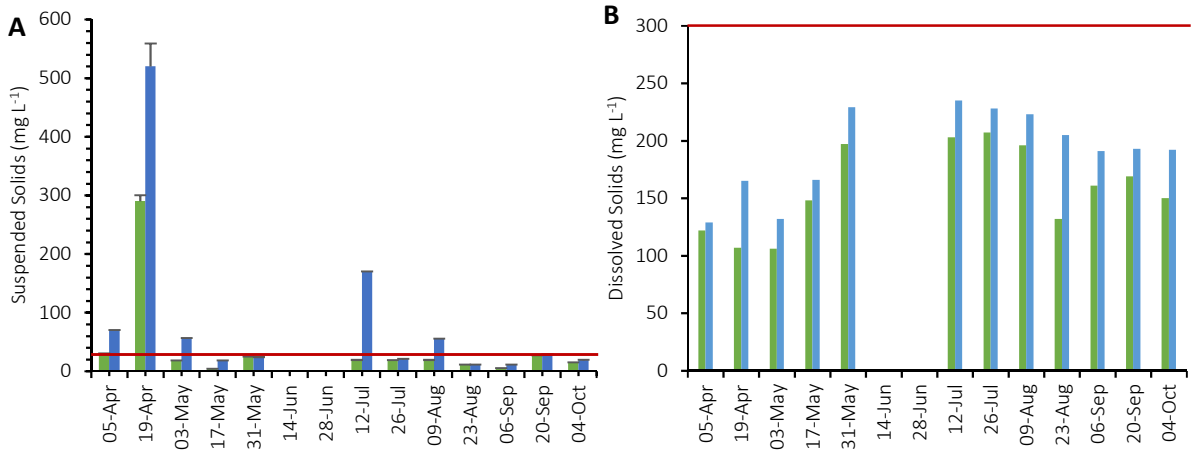


Figure 3.15: Levels of (A) **suspended solids** and (B) **dissolved solids** detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries. Samples were collected every two weeks between April 2018 and October 2018. N = 3 & SD indicated (suspended solids p = 0.0730, dissolved solids p = 0.5130).

Conductivity in the intake water was between 165 and 324 µS cm^{-1}, whilst in the output water, levels were between 202 and 368 µS cm^{-1} (Figure 3.16). Statistical analysis was conducted between the intake and output water and a significant difference was observed (p = 0.0410). Conductivity represents the ability of water to conduct electrical currents and is therefore associated with dissolved solids, among other parameters. According to the EPA's parameters for water quality, <1000 µS cm^{-1} is indicative of good water quality (EPA, 2001) and levels observed were below this.

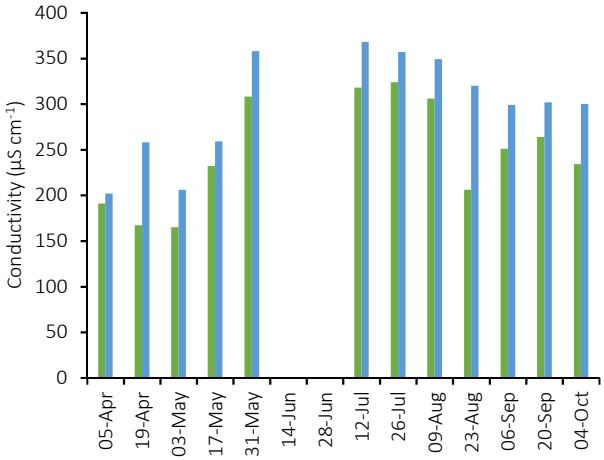


Figure 3.16: Levels of **conductivity** detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Keywater Fisheries. Samples were collected and analysed every two weeks from April 2018 to October 2018. N= 3 & SD indicated (p = 0.0410).

The variances in the nutrients in the output water is most likely to be predominantly from uneaten feed and fish faeces present in the water coupled with the low flow rates due to drought conditions. The intake water variation on the other hand is most likely from forestry and agricultural practices upstream as well as potentially from horticultural processes being conducted in the school grounds immediately next door and upstream of the farm. Results determined during the pilot study were published, in part, in the technical report generated as part of the EcoAqua project for DAFM and BIM, entitled "Supporting the sustainable development of the Irish freshwater aquaculture industry". See Appendix 1.

3.3.4. MONITORING PROGRAM – RESULTS & DISCUSSION

The samples for the monitoring program were collected from Keywater Fisheries from March 2019 to August 2019. Due to limitations in travel availability samples could only be collected and analysed once a month unlike the bimonthly (twice a month) analysis conducted during the pilot study. In addition to the intake and output water, samples from the then newly renovated settlement pond were also included to establish the efficacy of the constructed wetland being used to assist in wastewater treatment. Due to unforeseen circumstances within the farm, a sample from the settlement pond could not be collected and analysed during the month of June. See Appendix 4 for a summary of all results.

BIOASSAY ANALYSIS

P. subcapitata was exposed to the freshwater finfish aquaculture intake, output and settlement pond water for 72h. As previously mentioned, *A. formosa* was also included due to inconsistencies in growth

rates during the comparative analysis. *P. subcapitata* growth inhibition was observed in the intake, output and settlement pond water (Figure 3.17A). Growth inhibition was also observed in the *A. formosa* when exposed to the sample sets (Figure 3.17B). Statistical analysis identified no significant differences (*P. subcapitata* p = 0.5129, *A. formosa* p = 0.0549). The growth inhibition observed in the sample sets suggested that the intake and output water would be unlikely to cause issues such as algal blooms. However, growth inhibition would also indicate toxicity and may potentially result in losses to the biodiversity of the receiving water body (Guéguen *et al.*, 2004; Ivanova and Groudeva, 2006; Ma *et al.*, 2006). Although levels were low for the most part, this toxicity may result in the loss of primary producers in the aquatic ecosystem, subsequently causing indirect adverse effects on the aquatic food chain. The higher levels of growth inhibition observed in the intake water also suggested potential issues upstream of the fish farm and not in the farm itself. This is further indicated by the reduction in the rate of growth inhibition between the settlement pond and the output water samples by demonstrating that treatment processes within the farm *i.e.*, the constructed wetland, are effective in reducing the rate of growth inhibition associated with aquaculture processes. No previously published research could be found in the available literature for *A. formosa* and, although a small amount of research found involving the use of *P. subcapitata* demonstrated similar results (O'Neill *et al.*, 2019; Zhang *et al.*, 2011), research into the use of algae as an early bioindicator for potential issues in aquaculture is still lacking.

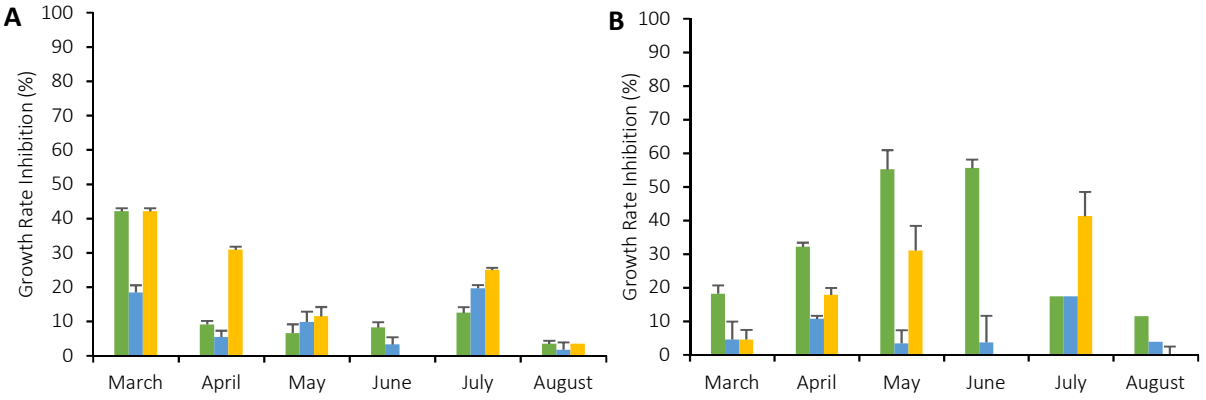


Figure 3.17*: Growth inhibition** observed in (A) ***P. subcapitata and (B) *A. formosa* **when exposed to freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries for 72h. Samples were tested once a month from March 2019 to August 2019. N = 3 & SD indicated (*P. subcapitata* p = 0.5129, *A. formosa* p = 0.0549).

Due to the loss of *D. magna* cultures and the presence of *D. pulex* in AIT house stocks, the latter was used for the crustacean bioassay from this point onwards. As there were no significant differences observed between the species during the comparative study, this was not considered to be an issue. *D. pulex* was utilised to determine the immobilisation effects observed as a result of exposure to the intake, output and settlement pond water for 24h. Immobilisation was observed in the intake, output

and settlement pond water (Figure 3.18). Statistical analysis identified no significant differences (p = 0.4676). As with the algae, the low levels of immobilisation observed in the intake, output and settlement pond wayer have suggested that the samples were unlikely to cause issues downstream of the fish farm. However, as immobilisation has been detected it should be noted that, although levels were low, this is still an indication of toxicity. Similarly, with the primary producers this toxicity may result in the loss of primary consumers in the aquatic ecosystem, subsequently causing indirect adverse effects on the aquatic food chain. Loss of the crustacean could result in the build-up of algae within systems resulting in potential eutrophic conditions as the crustaceans are no longer present to aid in keeping algal levels in check.

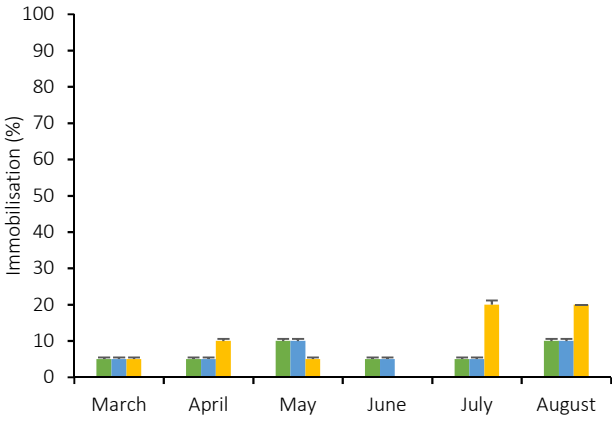


Figure 3.18: **Immobilisation** observed in ***D. pulex*** **when exposed to freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries for 24h. Samples were tested once a month from March 2019 to August 2019. N = 4 & SD indicated (p = 0.4676).

PHYSICOCHEMICAL ANALYSIS

NH_4^+ (Figure 3.19A) NO_2^- (Figure 3.19B) and NO_3^- (Figure 3.19C) levels were observed in the intake, output and settlement pond water. Statistical analysis was conducted between the three sets of samples. A significant difference was observed in the NH_4^+ samples (p = 0.0170). However, no significant differences were observed in the NO_2^- samples (p = 0.3272) or the NO_3^- samples (p = 0.4123). When comparing the samples, the concentration of NH_4^+ present in the intake water is lower than that of the output and settlement pond water suggesting the levels of NH_4^+ detected are being generated within the farm itself. However, the small amount detected in the intake water also suggests some form of pollution is periodically being generated upstream of the farm. Production of fish was at a minimum for the months of March and April. This may explain the increase in NH_4^+ detection observed from May onwards. The settlement pond had also not been filled to capacity during this time and therefore the constructed wetland was not being utilised to its maximum potential. Once the constructed wetland was filled to capacity, the levels of NH_4^+ in the output water

was lower than that of the settlement pond suggesting that the wetland was effectively removing the NH_4^+. The levels of NH_4^+ were gradually decreasing, the longer the constructed wetland was at maximum capacity. Despite the reduced capacity of the wetland, the concentrations observed in the output water were not greater than the one mg L^{-1} suggested by the EPA (EPA, 2001). The concentrations observed in this study were also lower than those determined by Boaventura *et al.* (1997) in their study on trout effluent and Costanzo *et al*. (2004) in their study on shrimp pond effluent.

NO_2^- levels detected were very low, as expected, due to its high instability. It is an indication of recent pollution. When comparing the NO_2^- levels in the samples, levels in the output water were much higher than that of the intake water. Similar with the NH_4^+, the small amount detected in the intake water suggested some form of pollution was being generated upstream of the farm. The higher levels detected in the output water were generated within the farm itself. The concentrations detected were greater than the 0.03 mg L^{-1} for cyprinid waters, as per the EPA's suggested water quality parameters (EPA, 2001). Despite this, NO_2^- levels determined were in agreement with, and on occasion lower than levels observed by other research conducted on shrimp, trout and prawn effluents (Caramel *et al.*, 2014; Herbeck *et al.*, 2013; Mcintosh and Fitzsimmons, 2003; Moreira *et al.*, 2010; Pulatsü *et al.*, 2004). The settlement pond levels were below that of the output water suggesting that the constructed wetland was not efficient in the removal of NO_2^-. However, this was only observed in times when the wetland was not completely filled and therefore not working to its maximum potential. A spike was also observed in the settlement pond during July. However, this occurred when the farm had just increased production. Once production stabilised, the concentration of NO_2^- in the settlement pond returned to levels previously observed. This drop may also be due to the introduction of *L. minor* to the constructed wetland.

Low levels of NO_3^- were observed in all water samples. The levels observed in the intake water indicated that low levels of NO_3^- may have been entering the river upstream of the fish farm. A spike in the settlement pond was observed during July and may have been due to the fact that the production had just increased within the farm. It also may have been due to difficulties observed in obtaining the sample and as a result debris not associated with the actual aquaculture production may have also been present in the sample. The level of NO_3^- dropped to previous reported levels once production stabilised. This drop may also have been due to the introduction of *L. minor,* which has the ability to use NO_3^- as a nutrient source. The concentration of NO_3^- in the output water was below that of the settlement pond suggesting that the constructed wetland was effectively reducing the NO_3^-. The NO_3^- levels were well below the guidance value of 50 mg L^{-1} suggested by the EPA (EPA, 2001), in the output water. Similar output levels of NO_3^- detected in this study were also indicated by

Boaventura *et al*. (1997), Camargo (1994), Guilpart *et al*. (2012), Lalonde *et al*. (2014) and Pulatsü *et al*. (2004), who all investigated trout aquaculture, and by Biao *et al*. (2004), Costanzo *et al*. (2004), Ferreira *et al*. (2011), Herbeck *et al*. (2013) and, Mcintosh and Fitzsimmons (2003) and their studies on shrimp.

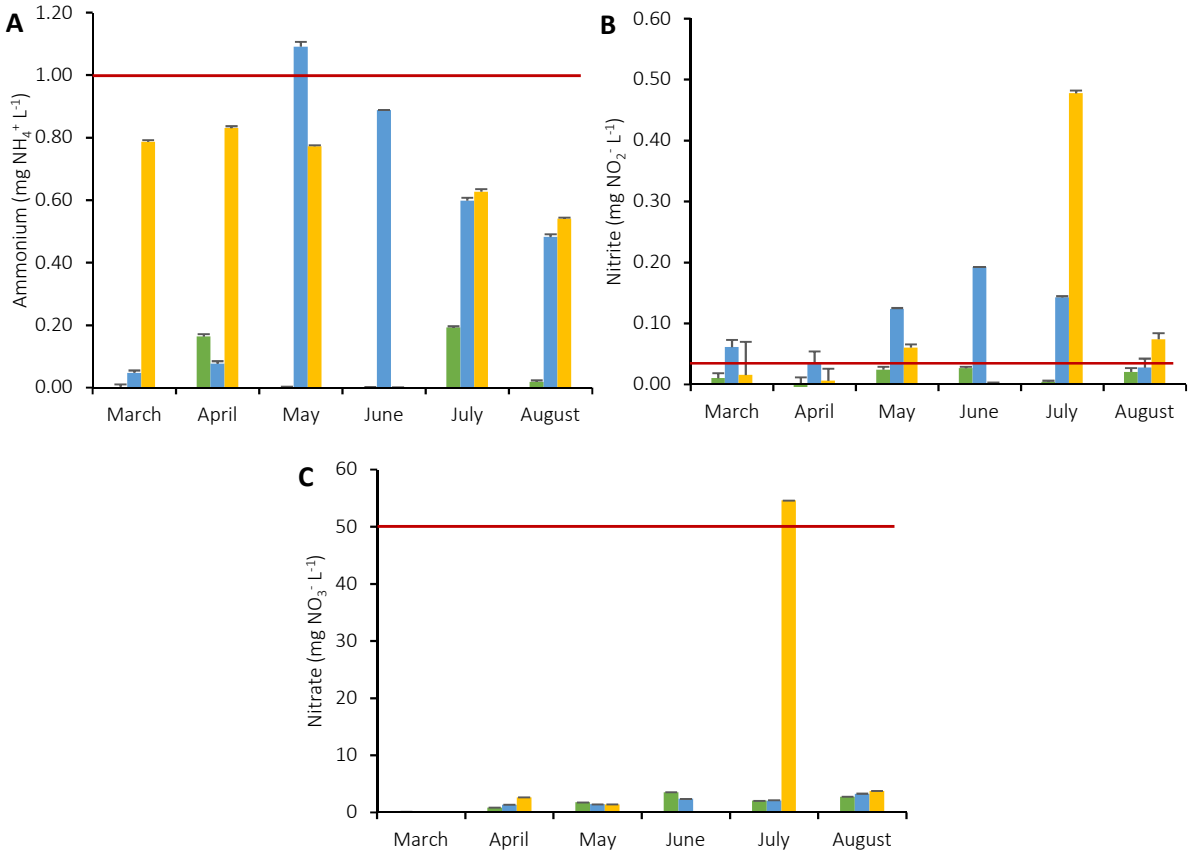


Figure 3.19: Concentrations of (A) **ammonium**, (B) **nitrite and** (C) **nitrate** detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected and analysed once a month between March 2019 and August 2019. N = 6 & SD indicated (NH_4^+ p = 0.0170, NO_2^- p = 0.3272, NO_3^- p = 0.4123).

Alterations in the PO_4^{3-} concentrations were observed in the intake, output and settlement pond water (Figure 3.20). Statistical analysis indicated that there were no significant differences observed between the intake, output and settlement pond water samples (p = 0.5268). The levels observed in the intake water did not occur until May, which coincided with the commencement of agricultural processes occurring upstream. A high spike in June may have been as a result of increased forestry activity also occurring upstream. This work was reported to the author by the fish farm workers. This suggested that phosphorus pollution is entering the river upstream of the fish farm, as well as being generated within the farm itself. Levels in both sample sets may be cause for concern due to its ability to cause algal blooms and hypoxic conditions (Barcellos *et al*., 2019; Brogan *et al*., 2001). Once the agricultural and forestry processes upstream ceased and the constructed wetland was in full

operation, the PO_4^{3-} levels dropped back to previous levels. Concentrations detected were just over two log doses greater than the recommended value of 0.035 mg PO_4^{3-} L^{-1} set out by the SI 272/2009 and SI 77/2019 for good water status (Irish Statutory Office, 2019, 2009). However, once back to previous levels, they were also in accordance with Stephens and Farris (2004a, 2004b) and Ziemann *et al*. (1992) and their studies on finfish farming effluent. It should also be noted that the dilution factor of the receiving river had not been included and needs to be taken into consideration. The settlement pond levels were greater than that of the output suggesting that the constructed wetland was reducing the levels of PO_4^{3-} before being released. Although it did not reduced levels to below recommended levels, increases in reduction were observed after May. This also may have been due to the constructed wetland became fully functional or to the introduction of the *L. minor*.

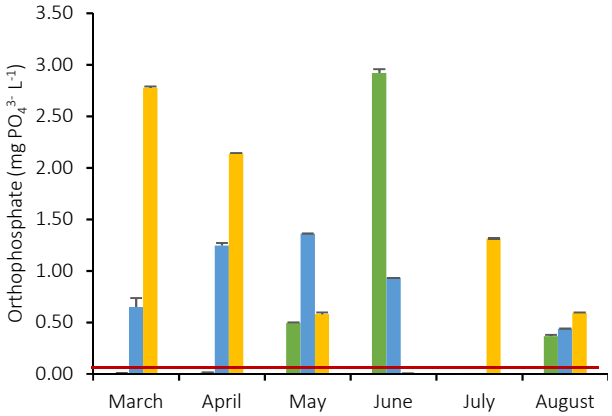


Figure 3.20: **Orthophosphate** levels detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries between March 2019 and August 2019. Samples were collected and analysed once a month. N = 6 & SD indicated (p = 0.5268).

Fluctuations in the DO, BOD and COD levels were observed in the intake, output and settlement pond samples (Figure 3.21). Statistical analysis found no significant differences between the three sample sets for any of the oxygen parameters (DO p = 0.1872, BOD p = 0.6308, COD p = 0.3214). There are no issues with the DO levels present in the intake water. However, there may be cause for concern with levels observed in the output water as they were below the recommended concentration of ≥7 mg O_2 L^{-1} from May onwards (EPA, 2001). Levels below this concentration were observed during warmer conditions. A longer monitoring period would need to be conducted to determine whether lower levels were as a result of the seasonal changes. It has been widely confirmed that dissolved oxygen levels are affect by changes in season and temperature, as well as the daily cycle. However, concentrations were also higher than the ≥4 mg L^{-1} required for the maintenance of aquatic life as suggested by Alam *et al*. (2007) and da Silva *et al*. (2017). DO levels in other studies, which included shrimp, catfish, prawn and trout farming, were similar to or above the concentrations observed in this study (Biao *et al*., 2004; Camargo, 1994; da Silva *et al*., 2017; Mcintosh and Fitzsimmons, 2003; Moreira

et al., 2010; Namin *et al*., 2013; Stephens and Farris, 2004a, 2004b). BOD levels in all samples were above the SI 272/2009 and SI 77/2019's recommended values (1.3 mg L^{-1} for high water status and 1.5 mg L^{-1} for good water status) but below the EPA's values (≤3 mg L^{-1} and ≤6 mg L^{-1} for salmonid and cyprinid waters, respectively) (EPA, 2001; Irish Statutory Office, 2019, 2009). This suggested that levels may be cause for concern. However, the presence of BOD in the intake water indicated that the BOD levels were as a result of works upstream of the farm, in addition to works conducted within the farm itself. This research was then compared to results determined by other researchers. Those that were revised demonstrated slightly higher levels than the concentrations detected in this study (Ansah *et al*., 2012; Boaventura *et al*., 1997; Mcintosh and Fitzsimmons, 2003; Miashiro *et al*., 2012). The COD levels observed in both the intake and output water were below the suggested 40 mg L^{-1} set out by the Irish EPA (EPA, 2001) and were therefore deemed not to be any cause for concern. COD levels were however, periodically above this level in the settlement pond samples. As levels dropped in the output water, treatment processes within the farm appeared to be efficient in the reduction of COD levels. Research conducted by da Silva *et al*. (2017) reported some COD levels greater than those determined in this research. It should also be noted that the dilution factor of the receiving water system has not been included and therefore needs to be taken into consideration.

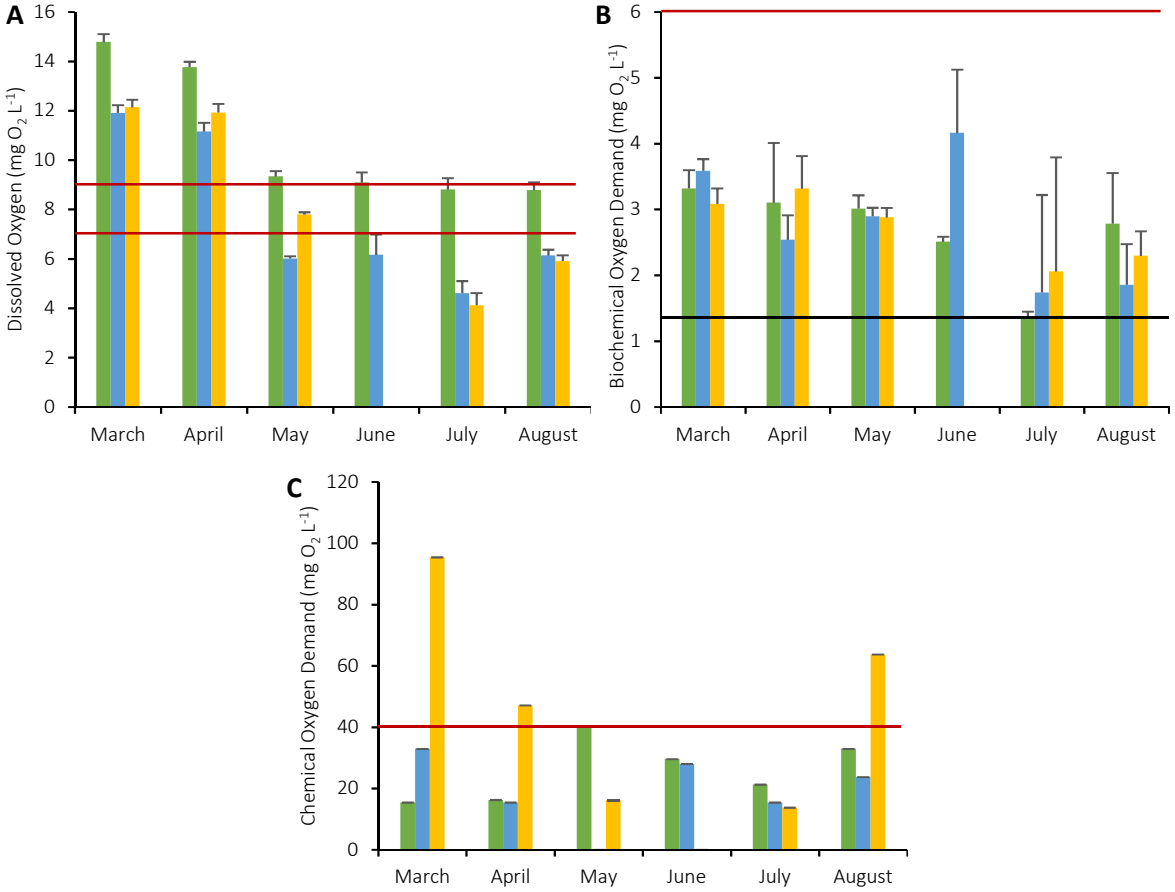


Figure 3.21: (A) **Dissolved oxygen**, (B) **biochemical oxygen demand** and (C) **chemical oxygen demand** levels detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected and analysed once a month from March 2019 to August 2019. N = 3 & SD indicated (DO p = 0.1872, BOD p = 0.6308, COD p = 0.3214).

Temperature and pH levels fluctuated in the samples, as shown in Figure 3.22. However, no significant differences were observed in either parameter (temperature p = 0.6766, pH p = 0.2794). With growing concerns associated with climate change and global warming, increases in temperatures may become more frequent. Small fluctuations in temperature were observed in the samples. This was primarily due to seasonal changes, as would be expected. Observed temperatures were not considered to be any cause for concern. The results for the pH indicated that the intake water was slightly more alkaline than the output water. Levels in the settlement pond fluctuated between both. The recommended pH levels should be between pH 6 and pH 9. Levels in all samples are well within this level and therefore present no issues. The mean temperature and pH results observed were similar to the those recorded in the revised studies that focused on freshwater finfish *i.e.,* catfish, brown trout and rainbow trout (Boaventura *et al*., 1997; Caramel *et al*., 2014; Namin *et al*., 2013; Noroozrajabi *et al*., 2013; Pulatsü *et al*., 2004; Stephens and Farris, 2004b; Živić *et al*., 2009).

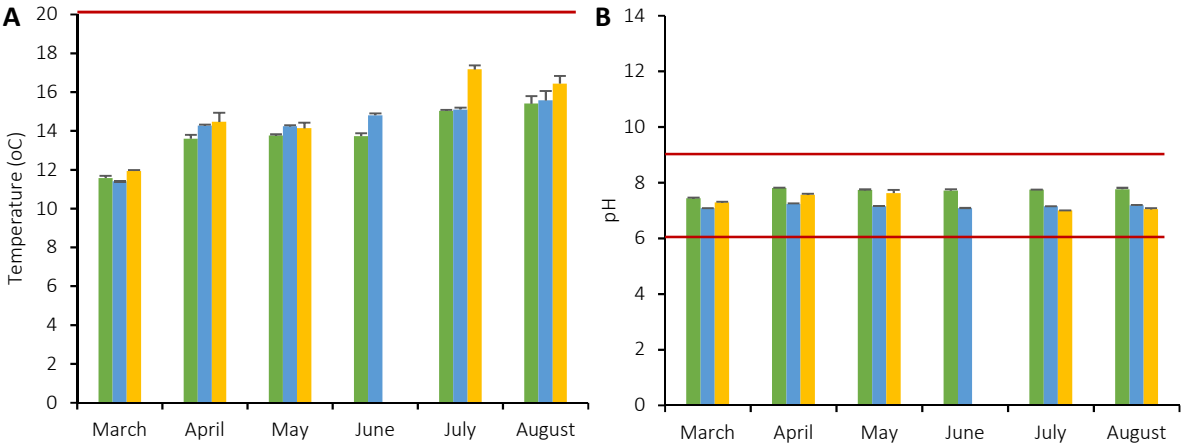


Figure 3.22: A) **temperature** and B) **pH** levels detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected and analysed once a month from March 2019 and August 2019. N = 3 & SD indicated (temperature p = 0.6766, pH p = 0.2794).

Although there were some variations in the $CaCO_3$ levels detected for hardness (Figure 3.23A) and alkalinity (Figure 3.23B), no statistically significant differences were observed (hardness p = 0.4660, alkalinity p = 0.5831). Alkalinity results from shrimp and catfish studies (Ferreira *et al.*, 2011; Mcintosh and Fitzsimmons, 2003) demonstrated similar findings to this study. Hardness results suggested that the water is slight to moderately hard. This correlates with water hardness maps of Ireland which demonstrated water around Boyle, Co. Roscommon was slightly to moderately hard. Similar hardness results were observed in revised studies on catfish and Atlantic salmon (Lalonde *et al.*, 2014; Stephens and Farris, 2004a, 2004b).

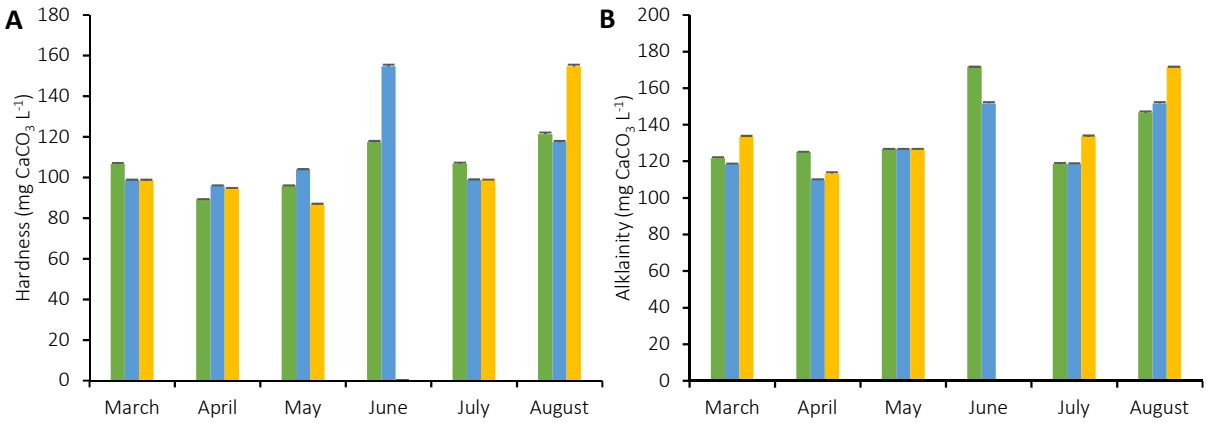


Figure 3.23: Calcium carbonate levels for (A) **hardness** and (B) **alkalinity** detected **in freshwater aquaculture finfish intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected and analysed once a month from March 2019 to August 2019. N = 3 & SD indicated (hardness p = 0.4660, alkalinity p = 0.5831).

Suspended and dissolved solids were detected in the intake, output and settlement pond water, as shown in Figure 3.24. After statistical analysis, a significant difference was indicated in the suspended solids (p = 0.0337). However, no significant difference was observed in the dissolved solids (p = 0.5237).

The average levels detected in both the intake and output water were not considered to be any cause for concern as they were below the 25 mg L^{-1} guidance value. Levels rose above this value in May. This may have been due to the agricultural and forestry processes occurring upstream. It should also be noted that levels were greater in the intake water than the output water indicating that the processes upstream were causing higher levels of suspended solids than the works within the farm itself. It also indicated that the treatment processes within the farm were improving conditions. Results from this study were compared to previous aquaculture effluent studies. Suspended solid concentrations in a range of studies on shrimp, prawn, salmonid, catfish, brown trout and rainbow trout (Boaventura *et al.*, 1997; Camargo, 1994; Caramel *et al.*, 2014; Costanzo *et al.*, 2004; Guilpart *et al.*, 2012; Lalonde *et al.*, 2014; Pulatsü *et al.*, 2004; Ziemann *et al.*, 1992) were similar to those established in this study. Levels in the settlement pond were below that of the output water suggesting that the treatment processes within the farm were not reducing levels. However, this may be due to the fact that the constructed wetland was not fully functional until the latter stages of the study. Dissolved solid levels were well below the guidance value of 300 mg L^{-1} indicating no issues with the general water quality.

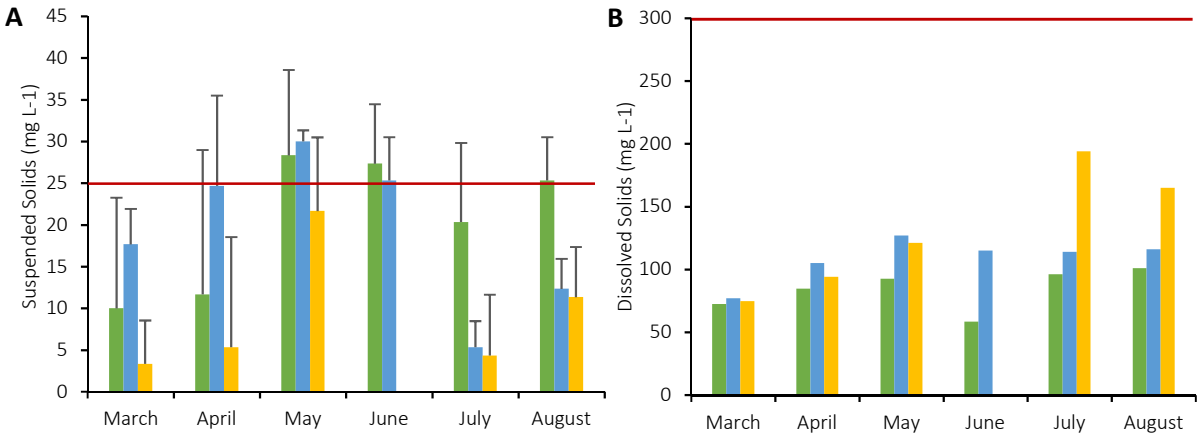


***Figure 3.24*: Levels of (A) **suspended solids** and (B) **dissolved solids** detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected once a week from March 2019 to August 2019. N = 3 & SD indicated (suspended solids p = 0.0337, dissolved solids p = 0.5237).**

Conductivity in the intake water was between 91.50 and 158 µS cm^{-1}, between 120.30 and 198.40 µS cm^{-1} in the output water and between 116.80 and 304 µS cm^{-1} in the settlement pond (Figure 3.25). No statistically significant differences were observed between the samples (p = 0.5280). As conductivity levels observed were <1000 mg L^{-1}, no issues were foreseen.

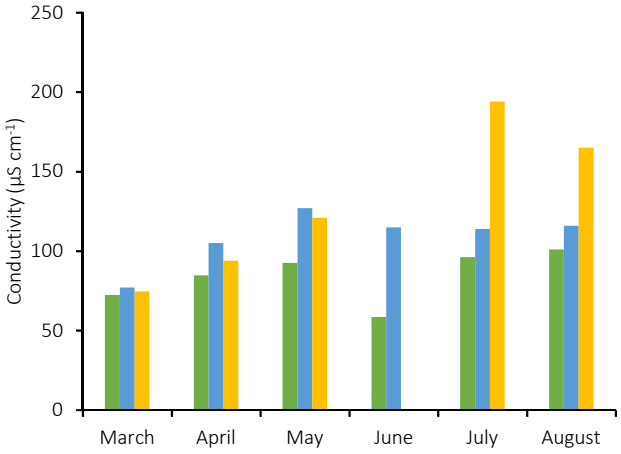


***Figure 3.25*: **Conductivity** levels detected **in freshwater finfish aquaculture intake** (green), **output** (blue) **and settlement pond** (yellow) **water** from Keywater Fisheries. Samples were collected and analysed once a week from March 2019 to August 2019. N= 3 & SD indicated (p = 0.5280).**

The variations in the nutrient composition in the settlement and output samples are most likely due to the presence of uneaten feed and fish faeces. However, this is unlikely the case with the intake water. The variance here is most likely as a result of forestry and agricultural practices upstream of the farm. As issues were observed with grass clippings from the next-door school accidently entering

the stream and subsequently blocking filters within the farm during and after the pilot study, the conscious decision to pump water upstream of the school was conducted. Therefore, it is unlikely that the variations have anything to do with the horticultural processes being conducted within the school grounds.

A comparative investigation was conducted between the pilot study and the monitoring program. See Table A4.26 of Appendix 4. Analysis was conducted on all bioassays and physicochemical parameters investigated on the intake and output water in both studies. The only parameter that indicated a significant difference was the results obtained in the *P. subcapitata* algal bioassay. This was expected given that very high levels of inhibition in the intake water and growth stimulation in the output water that were observed during the pilot study. Whereas low levels of inhibition were observed in both samples during the monitoring program. Reduction in inhibitory effects in the intake water may be due to the fact that water entering the farm is now pumped from upstream of an old national school located next door (upstream) to the farm. Thus, eliminating complications associated with the maintenance of the school grounds resulting in filter blockages. The national school also has an old overflow wastewater treatment system which may have been also affecting the quality of the water. However, further research would need to be conducted in this area.

The pilot study highlighted that on review of the performance of the constructed wetland, it was envisaged that further research was required. Also highlighted was the importance and potential use of *L. minor* as a wastewater treatment system within aquaculture facilities. This was the main reason for including settlement pond samples for testing in the monitoring program. After conducting this monitoring program, it was found that the constructed wetland in Keywater Fisheries was effectively reducing the waste levels once it was fully functional and also had *L. minor* introduced into it. However, the farm was not operating to its full capacity and increases to the size of the wetland may need to be conducted in order for the treatment process to be entirely effective.

3.4. ISSUES ENCOUNTERED

The ISO [8692:2012] required the use of a shaking phytoincubator. However, access to a static phytoincubator was only available. To compensate for this, all algal culture flasks were manually shaken as often as possible throughout the day. Commencement of testing was also conducted on Mondays as often as possible to offset for the fact that test culture flasks could not be manually shaken over the weekends. Additionally, given the fact that manual shaking may not allow for the validity

criterion (67-fold increase in control growth after 72h) to be met, a 96h time period was also included to ensure validation was achieved.

Issues were encountered with successfully achieving growth in the *A. formosa* and *M. contortum* starter cultures from CCAP. Culturing was conducted as per the culture media suggested by CCAP however, lower growth rates were achieved when compared to the expected growth rates CCAP indicated. To aid with these issues, a range of different culture media (see Appendix 3) were investigated in order to achieve successful growth. Successful growth was observed in both algal species when exposed to modified Jarworski's medium (JM). The JM was prepared as per CCAP and ISO (8692:2012) guidelines. However, the pH was altered to 7.4 for the *M. contortum* and 6.8 for the *A. formosa*.

Supply and delivery of all started algae cultures is dependent on the concentration and availability of the individual species *i.e.*, not all algae species are continuously grown therefore some species require additional time and extensive culturing before they are ready to be dispatched. Delays were experienced in delivery of the *M. contortum*. This resulted in the unavailability of the *M. contortum* during the monitoring program conducted in Keywater Fisheries between March 2019 and August 2019. However, as no significant differences were observed between the standardised algae, *P. subcapitata*, and the *M. contortum* during the comparative study, this was not deemed to be an issue.

Delays were observed in the supply and delivery of the nitrate photometric test kit. As a result, an ion selective electrode (ISE) was used until the kit arrived. The standard water and wastewater analysis method (4500-NO_3^-D) was conducted. The method was validated and standard curve constructed prior to commencement (See Appendix 3 for a breakdown of the protocols applied and Appendix 4 for graphical results). Continued use of the ISE could not be conducted due to high demand for its use.

Despite the fact that wastewater discharge regulations may not be applicable to aquaculture, current discharge licences do provide daily maximum limits for some of the physicochemical parameters including; PO_4-P, NO_3^- and suspended solids. However, these limits are based on composite samples collected over a 24h period. Access to a composite sampler was not available and grab sampling could only be conducted therefore, the indicated daily maximum limits were not considered applicable. To compensate for the use of grab samples over composite samples, larger grab samples (5L) were collected over approximately 30 min.

3.5. CONCLUSION

Water quality parameters specific for aquaculture wastewater have not yet been established in Ireland. The EPA has begun the process of regulating aquaculture wastewater. Results observed in this research have demonstrated that water quality parameters suggested by SI 272/2009, SI 77/2019 and the EPA may not be applicable to aquaculture wastewater as these results were similarly displayed in other aquaculture studies. However, the dilution factor of the receiving aquatic ecosystem is important and therefore also needs to be taken into consideration when this research is to be interpreted and these water quality parameters are to be determined. Results have also indicated that intake water quality is also as important when assessing aquaculture wastewater as it may indicate potential environmental issues as a result of works upstream and not that which is occurring within the farm *e.g.*, agricultural or forestry practices being conducted on or near the freshwater source, upstream.

The use of *P. subcapitata* as an early warning indicator of potential environmental issues associated with aquaculture has been revealed as a more responsive model than physicochemical parameters alone. Evaluation of aquaculture output water could include ecotoxicological bioassays in order to determine any potential effects the output water may have on the receiving aquatic ecosystem. Inclusion of the *P. subcapitata* algal bioassay in this research has demonstrated the potential eutrophication implications as a result of releasing untreated output water from fish farms. The additional bioassays that focus on different trophic levels should also be considered in order to develop a broader picture of the potential effect's aquaculture output water poses on its receiving ecosystems.

This research has demonstrated that despite the absence of *P. subcapitata* in Irish waters, it should still provide an accurate prediction of the effects of Irish freshwater aquaculture wastewater on its receiving aquatic ecosystem. Therefore, in order to assist the Irish aquaculture licensing process and monitoring of wastewater for discharge licensing, *P. subcapitata* and *D. pulex* should be considered for use in addition to the traditional physicochemical parameters already used for monitoring water quality. This, it is hoped, will assist in helping Ireland comply with the EU directives adopted for environmental protection. The use of physicochemical parameters on their own is not sufficient to determine the exact potential aquaculture effluent has on its receiving aquatic ecosystem. To save on costs, the traditional physicochemical parameters that were tested in this research should continue to be routinely monitored. During instances of variation in the physicochemical parameters, the bioassays should then be employed in order to determine if there are any potential ecotoxicological issues associated with the change in parameters.

CHAPTER 4

ADDRESSING SPACE AND LOCATION LIMITATION ISSUES IN THE IRISH FRESHWATER AQUACULTURE INDUSTRY

4.1. INTRODUCTION

Peatlands are natural wetland ecosystems formed by the accumulation of organic matter that is produced from dead and decaying plant material under wet conditions. The majority of Ireland's peatlands are raised bogs (NPWS, 2015). These bogs were formed thousands of years ago (Ward *et al*., 2019). However, the bogs of Ireland are now considered endangered places. In the early 1960's, 17.2 % of the Republic of Ireland's land cover were peatlands (Hammond, 1981). Now it is down to just over 5 % of Ireland's landscape (Ward *et al*., 2019). Approximately 103 bogs across Ireland are now considered SACs as part of the Natura 2000 initiative and a further 75 are considered Natural Heritage Areas or NHAs (NPWS, 2015; Ward *et al*., 2019). Bord Na Móna, a state company that was originally developed to establish Irish peat resources for economic benefit, owns or controls approximately 80,000 ha of bog. The urgent threat of climate change, in addition to some of these bogs now being listed as important habitats under the EU's Birds and Habitats Directives due to their scarcity, have resulted in dramatic changes in the peat industry, including conversion of peatland usage to wind energy, forestry, biodiversity, amenity and waste management (Bord na Mona, 2019a; Irish Peatland Conservation Council, 2019; O'Neill *et al.*, 2019, 2020; Ward *et al.*, 2019).

There is a commensurate interest in exploiting low-cost environmentally-friendly 'natural' processes in aquaculture (Han *et al*., 2019). These issues have led to an increased research focus on developing IMTA (Granada *et al*., 2016) along with eco-innovation and monitoring of processes (Rowan, 2019; Tahar *et al*., 2018b, 2018a, 2018c). Advances in aquaculture must also be balanced by the need to meet commitments as set out by the WFD (Voulvoulis *et al*., 2017; WFD Ireland, 2018a). Recently, Bord Na Mona, in conjunction with BIM, has expanded use of these cutaway bogs to develop Ireland's first IMTA adhering to organic principles, known as Oasis. This IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), *L. minor* and *L. gibba*, and exploits use of microalgae for waste removal (Bord na Mona, 2019a). This IMTA process differs from traditional aquaculture practices that use water from rivers and lakes where the latter traditional systems must consider potential pollutants from agricultural runoff, industry and wastewater treatments plants (Barrett *et al*., 2016; Hayes *et al*., 2013; Rowan, 2011; Tahar *et al*., 2018c, 2017; Tiedeken *et al*., 2017).

Bord na Móna are currently reducing the level of harvesting at its bogs and will cease all harvesting by 2025. This will, in turn, result in hundreds, if not thousands of job redundancies (Ganly, 2017; Lee, 2018). The potential use of bogs for aquaculture facilities may provide a means to limit the level of redundancies facing employees. In addition, if aquaculture in peatlands proves viable, this could also assist in aiding the government meet its goals of increasing its aquaculture production by assisting with the space and location limitations facing the industry.

4.2. METHOD APPROACH

In order to determine viability, the previously developed ecotoxicological toolbox, as discussed in chapter three, was firstly applied to the novel process to ensure no unforeseen environmental issues would arise for the application of aquaculture processes to these protected locations. The characterisation and profiling of the rearing and treatment water was then conducted to optimise the novel IMTA process and ensure it was adhering to environmentally friendly, organic principles. Relationships exist between algae, microbes and nutrients present in aquaculture rearing water. The presence of one is connected to or affected by one or all of the others. Developing a comprehensive understanding of the role of algal communities and a baseline for the emergence/predominance of specific species in freshwater aquaculture would specifically inform and guide the development of the innovative peatland cut-away IMTA process at Oasis. This would thereby provide the aquaculture industry with a holistic understanding of the critical role of algal communities in maintaining optimal IMTA conditions and enable augmentation of this trial process at Oasis in an environmentally sustainable manner that positively influences the industry. In order to analyse the biological community present, methods of preservation, enumeration and identification needed to be applied.

4.2.1 SAMPLING

Water samples were collected from Oasis in five L octagonal carboy HDPE bottles (Lennox) and transported directly to the lab, 62km away, via car. Samples were taken directly from the output source of the farm once a month from May 2019 to August 2019 during the pilot study. Collection occurred on the same day (Wednesdays) and at approximately the same time (08:30 a.m.). Intake samples were also collected and analysed in order to determine the quality of the bog water entering the fish farm. Intake samples were taken directly from the intake pipe. See Figure 4.1 for schematic of Oasis Fish Farm and the location of the intake and output channels. Samples were then collected every two weeks from December 2019 to February 2020 and then once a week until October 2020 for the characterisation of the novel IMTA system. Samples were taken from each of the culture ponds, the

entry and exit points of the duckweed lagoon during this study. Samples were also taken from the overflow tank during times when there may have been a potential for discharge. Samples from the reservoir began in June due to the commencement of culturing in it as a result of issues being observed in the ponds. See Figure 4.1 for the locations of all sample points within the farm.

Figure 4.1: **Aerial view of Oasis Fish Farm**, located in Ballycon, Co. Offaly. The culture ponds, water reservoir, algae & duckweed wastewater treatment channels, overflow tank and bog river are all visible. Blue lines indicate the direction of the flow of water. The **red circle** indicates the **location of the intake sampling point**. The **yellow circle** indicates the **location of the output sampling point**. The **green squares** indicate **all sampling points within the farm** to monitor the IMTA process.

4.2.2 Ecotoxicological Bioassays & Physicochemical Analysis

The ecotoxicological analysis was conducted with two algal species (*P. subcapitata* and *A. formosa*) and *D. pulex*. Protocols for all algal bioassays were conducted as per **subsection 3.2.2** and protocols for the crustacean bioassay was conducted as per **subsection 3.2.3** of Chapter Three. Protocols for the respective physicochemical parameters were conducted as per **subsection 3.2.4** of Chapter Three. Statistical analysis was conducted as per **subsection 3.2.5** of Chapter Three.

4.2.3 Sample Preservation

Ideally cells need to be analysed as soon as possible after collection (Marie *et al.*, 2005) and cells should preferably be analysed fresh. However, if that is not possible preservation can be conducted

(Marie *et al.*, 2000). Preserving samples minimises the loss of the biological composition (Nachimuthu *et al*., 2020). As enumeration and identification could not be conducted within 24h of collection, preservation methods were employed in order to maintain as close to *in-situ* conditions as possible. Based on the success Guillard and Sieracki (2005), Naughton *et al*. (2020) and, Noble and Fuhrman (1998) observed with preservation methods, Lugols iodine and formaldehyde were used to preserve the algae and bacteria samples, respectively. A 2% formaldehyde (Sigma-Aldrich) and 1% Lugols iodine (Merck) end concentration was opted for based on the successful application of these concentrations by Naughton and her colleagues work on characterising phytoplankton and microbial communities in a traditional Irish aquaculture facility. The use of Lugols iodine and formaldehyde can alter the shape and fluorescence properties and become unstable over extended storage periods *i.e.*, >3-6 months (Marie *et al*., 2005, 2000; Naughton *et al*., 2020). Viable preservation methods for long term storage and analysis of phytoplankton has yet to be fully developed (Naughton *et al*., 2020). However, as analysis was to be conducted within the week of collection this was not deemed to be an issue. Samples were then stored at 4°C until analysed in order to slow down any physical and chemical reactions that could cause deterioration of cell structures (Marie *et al.*, 2005, 2000).

SAMPLE LABELLING & PRESERVATION METHODS

From each of the five L grab samples taken from the individual locations within the farm, two separate 500mL samples were placed into 500mL carboy HDPE bottles (Lennox). One set was for algae analysis and one set for bacteria analysis. Each bottle was then labelled with the date, sample location and sample type. To preserve the individual samples, one mL of Lugols iodine was added per 500mL water sample to preserve the algal composition. One mL of formaldehyde was added to a 500mL water sample to preserve the bacterial composition. All samples were well mixed and then stored at 4°C until analysis was conducted.

4.2.4 PHYTOPLANKTON ENUMERATION ANALYSIS

Both the traditional method of manual enumeration and the modern method of automated enumeration was investigated and applied to this research. The traditional means of counting (manual counting) was also conducted to validate that the automated machine counts being achieved.

Manual Cell Enumeration

Manual cell counting via light microscopy is the classic method for cell enumeration (ChemoMetec, 2020) and is the standard method employed by many laboratories (especially those with low resources) around the world (Green and Wachsmann-Hogiu, 2015). However, it does have its setbacks

and limitations. This method can be both time consuming and laborious, and there is often a large variation in determining cell concentration and viability (ChemoMetec, 2020) which can be statistically significant (Naughton *et al.*, 2020). There are also a range of drawbacks as a result of human error *e.g.*, what individual people perceive as cell definition varies leading to issues distinguishing between cells and debris. Also, tasks such as cell dilution, dispensing volumes and even general pipetting skills can vary from person to person (ChemoMetec, 2020). To minimise human errors, the same techniques for dilution and dispensing were conducted by the same individual during every cell count. Additionally, a minimum of three replicates were counted per manual count to reduce any significant differences.

MANUAL CELL ENUMERATION METHOD

All manual enumeration was conducted using a Superior Marienfeld Neubauer Improved Haemocytometer (0.1mm, 0.0025mm^2, Tiefe Depth Profondeur No: 717810) and a Nikon YS100 light microscope. Ten µL of the sample was loaded onto each side of the haemocytometer and then manually counted under a magnification of 400X. Counts for both sides were then combined. This was conducted in triplicate. The average cell count was then placed into equation one (see subsection 3.2.2 in Chpater Three) and the concentration of algal cells was calculated.

Automated Algae Enumeration

In order to establish a complete and comprehensive baseline analysis of the phytoplankton community present in the IMTA system, samples from a range of different locations within the farm were required. These samples also required regular monitoring and analysis. Given that manual counting can be very time consuming and a quick turnaround of analysis was requested by the farm, automated cell enumeration methods were also required to meet demands. Automated cell counting has greatly improved the speed and accuracy of cell enumeration (Büscher, 2019; Green and Wachsmann-Hogiu, 2015). It minimises human error and improves the statistical significance of findings (Naughton *et al*., 2020). Where possible, automated cell counting has become the preferred method for cell enumeration (Marie *et al*., 2005; Naughton *et al*., 2020). Flow cytometry (FCM) was opted for as the most appropriate method for automated enumeration because it eliminated the limitations present in the automated cell counters designed for phytoplankton enumeration. For example; the Beckman Coulter cell counter and the high accuracy (HIAC) cell counter were both developed for phytoplankton counting however both are limited in their abilities (Marie *et al.*, 2005). With the Beckman counter, distinction between phytoplankton, bacteria and cell debris can be difficult, the instrument is not suitable for counting picoplankton and similar sized cells overlap making the analysis of mixed samples difficult (Beckman Coulter, 2021; Marie *et al*., 2005). With the HIAC

counter, much like the Beckman counter, similar sized cells overlap making analysis of mixed samples difficult as only one parameter can be measured (Marie *et al.*, 2005).

FCM is a more advanced technique as it allows for the measurement and analysis of several parameters at once *e.g.,* shape, size and fluorescence intensity, which allows for sorting of sub-populations (Bonnevier *et al.*, 2018; Marie *et al.*, 2005; Naughton *et al.*, 2020). Although it was originally developed in the 1980's for the analysis of protein expression and to phenotype live cells for histological and immunological work (Bonnevier *et al*., 2018) it is now used for the analysis of a range of cell types, including phytoplankton (Marie *et al*., 2005). The principle of FCM is based on the Coulter Principle whereby the detection and measurement of changes in electrical resistance produced by a cell suspended in a conductive liquid travelling through an aperture (Beckman Coulter, 2021; Bord na Mona, 2019b; Büscher, 2019; Coulter, 1953; Green and Wachsmann-Hogiu, 2015). When cells individually pass through the aperture, they momentarily alter the electrical resistance or impedance of the electrical path between two electrodes located on each side of the aperture, creating an electrical pulse (Beckman Coulter, 2021; Büscher, 2019). Wolfgang G*ö*hde refined the principle by developing a flow cell that allows cells to be directed into a focal point allowing for individual analysis. He also developed the first fluorescence based FCM whereby fluorescence molecules (fluorochromes) could be added and the light emission could be measured (Bonnevier *et al*., 2018). Leonard Herzenberg then quickly built on this by developing fluorescence activated cell sorting (FACS) which allowed cells to be sorted based on fluorescence rather than just size (Bonnevier *et al*., 2018; Goetz *et al*., 2018). In modern FCM instruments, cells in a liquid suspension are passed through a narrow stream where a light source (single or multiple lasers) is focused. Once cells pass through the laser, the light is scattered differently depending on the cells size, shape and fluorescence properties (Büscher, 2019; Marie *et al.*, 2005; McKinnon, 2018). The light signals generated as cells pass are known as events. These events are then detected by photodiodes or photomultipliers, depending on which instrument is used. The detectors are usually positioned at 180^{o} and 90^{o} (Marie *et al.*, 2005). Visible light is measured in two different directions; forward scatter (FSC) light at 180^{o} and side scatter (SSC) light at 90^{o}. These light signals are converted to electronic signals that are then analysed by special computer software programs with the data written as a standardised format (McKinnon, 2018).

Fluorescent compounds have a range of specific wavelengths at which they absorb light energy. Absorption causes electrons to rise from ground state energy to a higher (excited) energy state. These excited electrons then quickly drop back to their ground state and emit light. This energy transition is

called fluorescence (Adan *et al.*, 2017). Fluorochromes are used to extend the application of FCM considerably. There are many fluorochromes that can be used to analyse phytoplankton including protein stains or cellular activity stains. However, they have received limited application to phytoplankton. Nucleic acid stains on the other hand are considered by many to be one of the most useful fluorochromes for phytoplankton FCM analysis (Marie *et al.*, 2005). SYBR Green dye was opted for in this research due to its availability and also to the success Naughton *et al.* (2020) reported in their study that performed enumeration of algae using FCM. SYBR Green is a highly sensitive nucleic acid specific stain (Marie *et al.*, 2001) that binds to the minor groove of double stranded DNA in a sequence-independent way emitting 1000x fluorescence than when unbound (Kim *et al.*, 2013).

The Miltenyi Biotec MACSQuant® Analyser 10 Flow Cytometer (Figure 4.2) was used for the automated enumeration of the phytoplankton. This FCM instrument has three lasers with eight different channels that are designed to be used with several different dyes as well as FSC and SSC channels as previously outlined. See Table 4.1 for a breakdown of the channels and lasers. The main channels used for enumeration were FSC, SSC, B1 to detect the SYBR Green fluorescence dye, B3 to detect chlorophyll and R1 to detect phycocyanin.

***Figure 4.2**:* Miltenyi Biotec **MACSQuant® Flow Cytometer** Instrumentation (Source: Miltenyi Biotec).

Table 4.1: Summary of **lasers** and **channels** present in the **MACSQuant® 10 Flow Cytometer**. Channels used for this research have been indicated in bold.

Laser	Channel	Filter (nm)	Parameter / Dye
Violet 405nm	V1	450/50	CFP, VioBlue
	V2	525/50	Pacific Orange™, VioGreen
Blue 488nm	**B1**	525/50	GFP, FITC
	B2	585/40	PE
	B3	655-730	PI, PerCP, PE-Cy™5.5, PerCP-Vio700, PE-Vio615, EDC, PE-CF594, PE/Dazzle™ 594, PE-eFluor® 610
	B4	750LP	PE-Cy7, PE-Vio770
Red 635nm	**R1**	655-730	APC
	R2	750LP	APC-Cy7, APC-Vio770
Blue 488nm	**FSC**	488/10	Size
	SSC	488/10	Granularity

FLOW CYTOMETRY METHOD

Preparation of phytoplankton samples for flow cytometry was adapted from (Naughton *et al.*, 2020). All samples were prepared in the following manner. A ten mL aliquot of each sample preserved with Lugols Iodine were centrifuged at 3500X G for 20 min. The supernatant was removed and the algae pellet was re-suspended in flow buffer. The flow buffer was prepared by adding 1mM EDTA, 0.2% Tween and 0.1% NaN_3 to 1L phosphate saline buffer or PBS (Merck). The buffer was filtered using a 0.20µm filter (Sigma-Aldrich) to remove impurities which may interfere with the flow cytometer. The re-suspended sample was divided into two aliquots (one three mL aliquot and one seven mL aliquot). The three mL aliquot was used for the unstained negative control samples. The seven mL aliquot was used for the stained samples. 200µL of 10X SYBR Green was added to the seven mL aliquot and incubated for 15 min in the dark at room temperature. (The SYBR Green was used to distinguish between cells (DNA containing) and debris (sediment and organic matter) present in the samples). Using two mL Eppendorf's (Merck), 1.5mL from the unstained aliquot and three 1.5mL's of the stained aliquot were centrifuged at 3500x g for 15 min. The supernatant was removed and the pellets were re-suspended in 1.5mL of fresh flow buffer. Samples were then loaded onto a round bottomed 96 well plate. 200µL of each aliquot was loaded onto the plate *i.e.,* four wells containing one unstained and three (triplicate) stained aliquots were loaded for each sample. Samples were tested in triplicate to ensure there was an appropriate representation and distribution of each species present in the different locations.

The flow cytometry instrument settings and gating were conducted in the following manner. MACSQuant® calibration beads were used to calibrate the flow cytometer prior to every run to ensure the machine was functioning correctly and ensuring results were reliable. The instrument was set at a medium flow rate with high mixing to ensure adequate mixing. The instrument was set to uptake 100µL of each sample for analysis. The trigger point for the FSC laser was set at 1.0 to eliminate the detection of as much debris as possible in the samples. The FlowJo™ v10.7 software program was used for the analysis of the data generated from the MACSQuant® Analyser 10 flow cytometer.

Gating was used to enumerate the algal and cyanobacterial populations. The gating method was adapted from Haynes *et al.* (2016), Moorhouse *et al.* (2018), Naughton *et al.* (2020) and Read *et al.* (2014). The unstained sample (negative control) was first gated (Figure 4.3A) to eliminate as much autofluorescence interference as possible. Algae contain chlorophyll which naturally fluoresces (autofluorescence). Eliminating this autofluorescence interference as much as possible reduces the risk of false positives. This gate was then applied to the stained samples (Figure 4.3B) in order to identify and enumerate the cells present in each sample. However, this population of cells contains both algae and cyanobacteria. Therefore, the individual populations needed to be gated and separated out in order for enumeration to be conducted. Both algae and cyanobacteria contain chlorophyll however, cyanobacteria can be distinguished by the presence of phycocyanin. There are only two groups of red algae that also contains phycocyanin, both of which belong to the Cryptophyta phylum (Brient *et al.*, 2008; Naughton *et al.*, 2020; van Vuuren *et al.,* 2006). However, as these two groups were not observed / identified during microscopic analysis it was deemed that the majority of the phycocyanin positive cell population represented the cyanobacteria present. As per Moorhouse *et al.* (2018) and Naughton *et al.* (2020), the blue B3 channel which was used to identify chlorophyll positive cells was plotted against the red R1 channel which was used to identify phycocyanin positive cells to distinguish between algae and cyanobacteria. Chlorophyll positive, phycocyanin negative cells represented the algae population whilst, chlorophyll and phycocyanin positive cells represented the cyanobacteria population (Figure 4.3C). Enumeration of the algal and cyanobacterial populations were then established (Figure 4.3D).

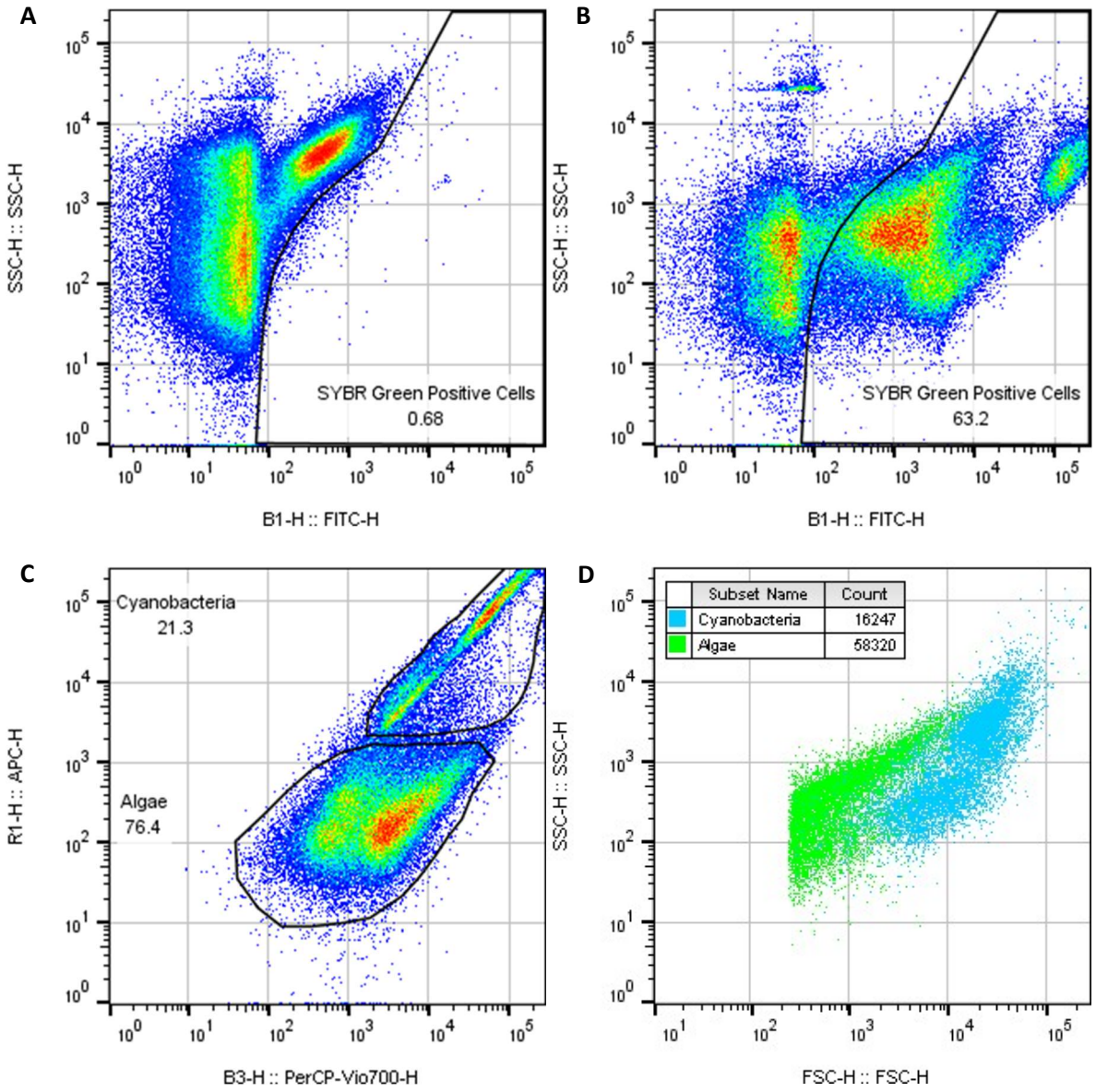


Figure 4.3: **Flow cytometry dot diagrams for the enumeration of algae and cyanobacteria.** A) Unstained samples to eliminate autofluorescence interference, B) cells stained with SYBR Green for the enumerations of algal and cyanobacterial populations, C) chlorophyll and phycocyanin levels used to distinguish between algae and cyanobacteria, D) enumeration of both the algae and the cyanobacteria populations.

4.2.5 PHYTOPLANKTON IDENTIFICATION ANALYSIS

There are two main ways to identify algal species; physical or molecular analysis. Using morphological data and identification keys is the most traditional method of identifying organisms (Hulcr *et al.*, 2015). However, advancements in DNA sequencing has made molecular analysis more popular (Waikagul and Thaenkham, 2014). The level of detail and knowledge required is the main factor in determining which form of analysis is best suited. For physical analysis, specimens are microscopically examined and key physical features are recorded, including; size, shape, colour, morphology (internal and external) and motility, which are grouped together. Key features and photographic imagery and then used for

identification (Bellinger and Sigee, 2015a, 2015b, 2015c). This method is the most common and rapid method used to identify the more frequently occurring freshwater algae (Bellinger and Sigee, 2015b) but it does have its limitations. Morphologies can be difficult to distinguish between due to a high level of similarities between many species of the same genus making species identification difficult (Manoylov, 2014; Waikagul and Thaenkham, 2014). Therefore, microscopic identification using physical features can usually only identify as far as the genus level thus allowing for partial speciation. As such, a more detailed analysis may be required as many species with a given genus can provide beneficial or neutral effects whilst others of the same genus can induce adverse effects.

Identification of algae using molecular methods provides a more in-depth picture as to which species are present by providing full speciation. DNA sequencing is used to determine the order of the four chemical building blocks or bases (adenine, thymine, guanine and cytosine) that make up the DNA molecule. The DNA sequence is unique to every living thing. Thanks to the completion of the Human Genome Project (HGP) DNA sequencing has become much faster and less expensive. Sanger sequencing (method used for the HGP) is a first generation sequencing (FGS) method and is the most well established method of DNA sequencing (Heather and Chain, 2016; Kchouk *et al.*, 2017). Although it is still considered the gold standard (Grada and Weinbrecht, 2013) by providing 99.9% base accuracy (CD Genomics, 2020) it is much more expensive that next generation sequencing (NGS) methods (Grada and Weinbrecht, 2013). Despite Sanger sequencing's high base accuracy, as the DNA is analysed base by base, the Illumina sequencing method is also highly accurate (Mahajan, 2018).

Illumina sequencing is an NGS method used to generate millions of highly accurate reads and is much faster and cheaper than other methods such as the Sanger method. The DNA is broken down into fragments of double stranded DNA (dsDNA) between 200 and 600 base pairs (bp) long. Adaptors, which are short sequences of DNA, are attached to the DNA fragments and converted from dsDNA to single stranded DNA (ssDNA). The ssDNA is then washed across a flow-cell which contains complementary primers which binds the ssDNA. Polymerase extends the primer by adding complementary unlabelled nucleotide bases or deoxynucleotide triphosphates (dNTPs: A, T, G and C) to the template DNA. This lengthens and joins the strands of DNA on the flow cell creating bridges of dsDNA between the primer and the surface of the flow-cell. This dsDNA is then broken down again to ssDNA. Dideoxynucleotide triphosphates (ddNTPs: A, T, G and C), labelled with distinct fluorescent dyes which terminates synthesis, is then used to determine which nucleotide has been incorporated into the chain of nucleotides. These ddNTPs are known as fluorescently labelled terminators. Primers and the terminators are added where the DNA polymerase first attached the primer to the DNA and

then binds the terminator to the first group of bases (A, T, C or G). Each dNTP are added separately. Lasers pass over the flow-cell to activate the label and the fluorescence is recorded. The first terminator is remove and the next is added. This process continues until all bases have been sequences. The generated sequences are then aligned with reference sequences for identification (Mahajan, 2018; Your Genome, 2015). See Figure 4.4 for a breakdown of the method.

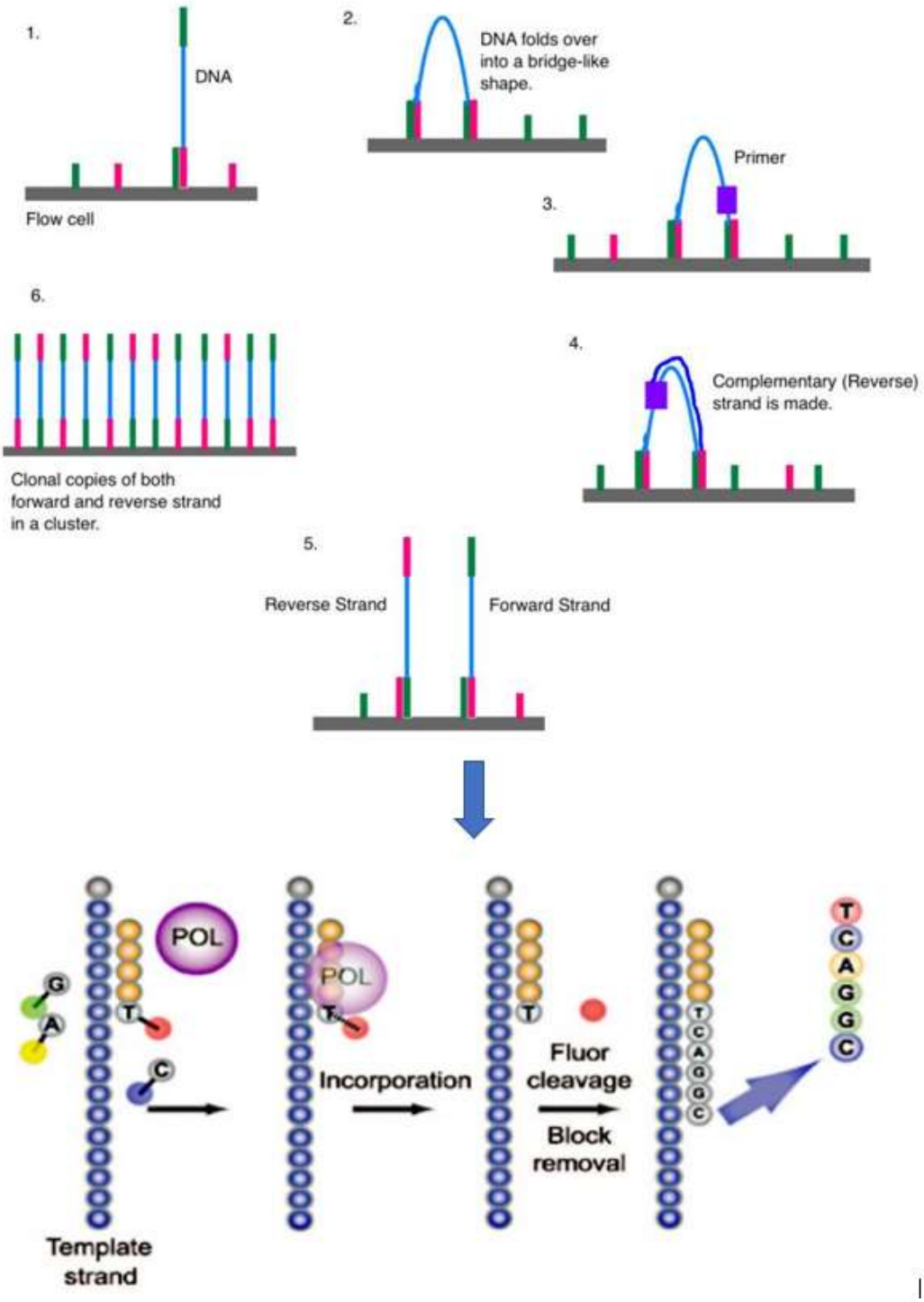


Figure 4.4: Breakdown of the **Illumina Sequencing Method**. (Source: NCBI & Alchetron)

As algae was a key component for wastewater assimilation and a means to determine water quality within the novel peatland IMTA system, it was important to determine which species were present. Identification of the algae would indicate whether beneficial species complementing the system or potentially hazardous species that could compromise the system were present. Given the importance of identification, molecular analysis was to be the main method of identification. However, as issues within the farm occurred quite quickly throughout the course of the study, a rapid means of identification was required. Therefore, the traditional microscopic method was also applied.

MICROSCOPIC IDENTIFICATION PROTOCOL

For the microscopic analysis, six 5mL aliquots were taken from the sample and placed into the wells of a 6 well plate. This was conducted for each sampling point. This was also conducted on fresh and preserved samples. During times of high algal concentration, aliquots were diluted to 1:2, 1:5 and 1:10. The plates were left to sit for 48h which allowed for the algae to settle out. Plates were then examined extensively using an Olympus CKX41 inverted microscope. Twelve images per well were taken using the ISCapture software to ensure as many algae as possible could be observed and identified. These images were then analysed whereby the physical features were recorded and then identified using identification keys and cross comparison images from the Algae Base data bank.

MOLECULAR IDENTIFICATION PROTOCOL

Due to the COVID-19 lockdown and the subsequent limited access to laboratory facilities, samples were sent for DNA sequencing to Macrogen Bioinformatics. Macrogen were chosen as they provided the most comprehensive service for the lowest price. DNA extraction, primer development and DNA sequencing would all be conducted by Macrogen. Fifty mL aliquots of each sample were centrifuged at 3500X G for 20 min. Samples were re-suspended in 1.5mL of filtered sample water and then transferred to a two mL Eppendorf before being sent to Macrogen Bioinformatics in Seoul, South Korea, where Illumina sequencing was conducted. Bioinformatics was then conducted with the assistance of Dr. Robert Pogue. The DNA sequences that were returned from Macrogen were first ran through the DAD2 Pipeline v1.8 software. This is an open-source software package than is used to model and correct Illumina sequenced amplicon errors. For taxonomic classification, denoising was carried out. This separates sequencing errors from biological variants, generating Amplicon Sequence Variants (ASVs), which were saved in a fasta file (Laehnemann *et al*., 2016). The fasta file was analysed using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) which rapidly aligns the DNA sequences with a sequence database to identify the genus / species of algae present.

4.3. FINDINGS

4.3.1. PILOT STUDY & ECOTOXICOLOGICAL EVALUATION – RESULTS & DISCUSSION

In order to assess the potential implications of conducting aquaculture practices in peatlands, which are now protected under the Birds Directive and Habitats Directive, the ecotoxicological toolbox was applied to the novel trial IMTA aquaculture facility developed in the peatlands at Mount Lucas Wind Farm.

BIOASSAY RESULTS

P. subcapitata and *A. formosa* were exposed to intake and output water samples for 72h as per the ISO [8692:2012] guidelines. Growth inhibition was observed in both algal species when exposed to the intake water and the output water samples (Figure 4.5). Statistical analysis indicated no significant differences with either algal species (*P. subcapitata* p = 0.4022, *A. formosa* p = 0.3978). *D. pulex* was exposed to the water samples for 24h as per the ISO [6341:2012] guidelines. Immobilisation was observed in both sets of water samples (Figure 4.6). No statistically significant difference was observed between both sets of water samples (p = 0.3903).

Low levels of inhibition observed suggested that the quality of water in the intake samples and the holding tank output water, in terms of its effect on the algae, remained unchanged and that the water within the bog was unlike to cause growth inhibition, therefore the recovery of the bog would not be affected. No previous research could be found on *A. formosa* and most of the previous research using *P. subcapitata* in assessing water were based on polluted river systems and drainage water (Guéguen *et al*., 2004; Ivanova and Groudeva, 2006). These studies displayed higher growth rate inhibition levels than those observed in this study. Only two previous studies could be found in the available literature that focused on the use of the algae in the context of aquaculture. The research conducted by O'Neill *et al.* (2019) found considerably higher inhibition levels than those reported in this study. Miashiro *et al.* (2012) and O'Neill *et al.* (2019) also reported stimulation of growth in the algae which could results in eutrophic conditions. As no stimulation of the algae was observed in this study, issues with eutrophication would seem highly unlikely.

Low levels of immobilisation in the *D. pulex* observed in both samples suggested that the water quality seemed unlikely to cause any adverse effects on any of the aquatic organisms present in the bog. However, no studies in the available research focused on the use of *D. pulex* to assess aquaculture discharge. Despite the low levels of toxicity observed in this short study, further ecotoxicological tests would need to be conducted in order to fully determine the effects of the aquaculture process on the

receiving natural wetland. Equally important is the potential effects the quality of the bog water may have on fish themselves. Therefore, additional ecotoxicological assessments also needs to be conducted in order to ascertain any possible health risks to the fish.

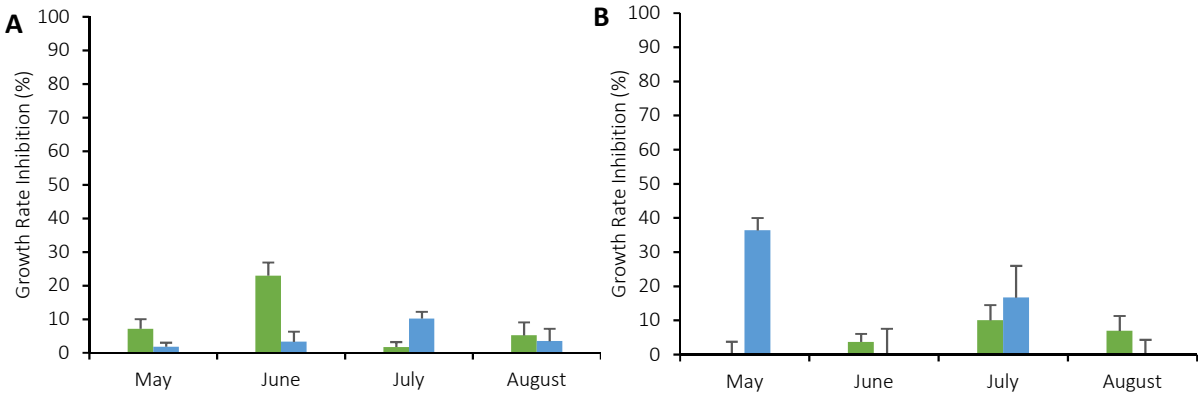


Figure 4.5: **Growth inhibition** observed in A) ***P. subcapitata*** and B) ***A. formosa*** **when exposed to novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis after 72h. Samples were tested once a month from May 2019 to August 2019. N = 3 & SD indicated (*P. subcapitata* p = 0.4022, A. formosa p = 0.3978).

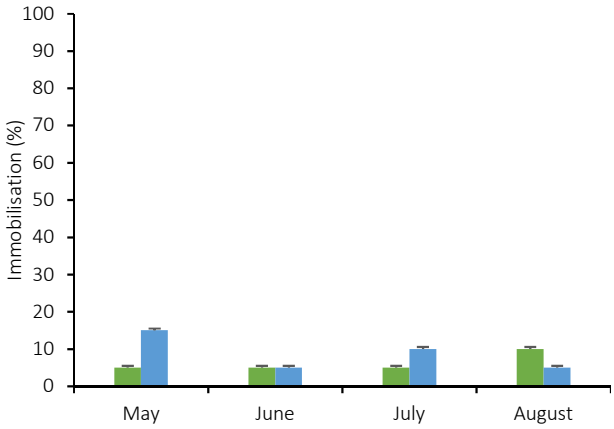


Figure 4.6: **Immobilisation** observed in ***D. pulex*** when **exposed to novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis after 24h. Samples were tested once a month from May 2019 and August 2019. N = 4 & SD indicated (p = 0.3903).

PHYSICOCHEMICAL RESULTS

Fluctuations in the NH_4^+ (Figure 4.7A), NO_2^- (Figure 4.7B) and NO_3^- (Figure 4.7C) levels were observed in both the intake and output water. No statistically significant difference was observed in the NH_4^+ results (p = 0.2644). However, significant differences were indicated in the NO_2^- (p = <0.001) and the NO_3^- results (p = 0.0164).

When comparing both samples, the concentration of NH_4^+ present in the intake water was greater than that of the output water. This suggested that the levels of NH_4^+ present in the farm were being

produced on the bog itself. It also demonstrated that, as a small level of NH_4^+ was only detected during the month of June, the treatment processes within the farm were effective in the removal of NH_4^+ by way of conversion to NO_2^-. Although NH_4^+ was only detected during the months of June and July in the intake water, levels were greater than the suggested freshwater fish directives recommended value of 1 mg NH_4^+ L^{-1} (EPA, 2001). However, there are no causes for concern between the intake and output water given the fact that the water quality in relation to NH_4^+ was improved. The concentrations observed in this study were compared to previous aquaculture effluent studies. Although the studies were compared to traditional aquaculture settings, the results determined by Boaventura *et al.* (1997), in their study on trout effluent, and Costanzo *et al*. (2004), in their study on shrimp pond effluent were above that of this study.

When observing the NO_2^- levels in both samples, levels in the output water were much higher than that of the intake water. The lack of NH_4^+ detected in the output water suggested that it had successfully been converted to NO_2^-. However, the higher levels of NO_2^- present in the output water suggested that it had not yet been completely converted to the less toxic and more stable NO_3^-. The farm uses a large duckweed lagoon for treatment processes. The results suggested that the NO_2^- may not be present in the lagoon for long enough. Further research into the lagoon needed to be considered (see section 4.3.2). However, it should be noted that this was still a trial farm. The higher levels in the output water have also demonstrated that the NO_2^- is being generated within the farm itself. The concentrations detected in the intake and output water were greater than the 0.01 mg L^{-1} for salmonid waters and 0.03 mg L^{-1} for cyprinid waters, as per the EPAs suggested water quality parameters (EPA, 2001). However, as the output is only periodically released from the farm during times of high rainfall this was not considered to be a cause for concern. Additionally, NO_2^- levels determined were in agreement with levels observed by other research conducted on shrimp, trout and prawn effluents (Mcintosh and Fitzsimmons, 2003; Pulatsü *et al.*, 2004; Moreira *et al.*, 2010; Herbeck *et al.*, 2013; Caramel *et al.*, 2014).

NO_3^- levels observed in the intake water indicated that NO_3^- was entering the farm via the bog. Levels detected in the output water were greater than that of the intake water suggesting that, although not all of the NO_2^- had yet been successfully converted to NO_3^- (see previous paragraph) some already had been. Levels were well below the guidance value of 50 mg L^{-1} suggested by the EPA (EPA, 2001), so there were no issues envisaged. Similar levels of NO_3^- were detected when compared to those observed by Camargo (1994), Boaventura *et al.* (1997), Pulatsü *et al.* (2004), Guilpart *et al.* (2012), and Lalonde *et al.* (2014), who all investigated trout aquaculture, and by Mcintosh and Fitzsimmons

(2003), Biao *et al.* (2004), Costanzo *et al.* (2004), Ferreira *et al.* (2011) and Herbeck *et al.* (2013), and their studies on shrimp.

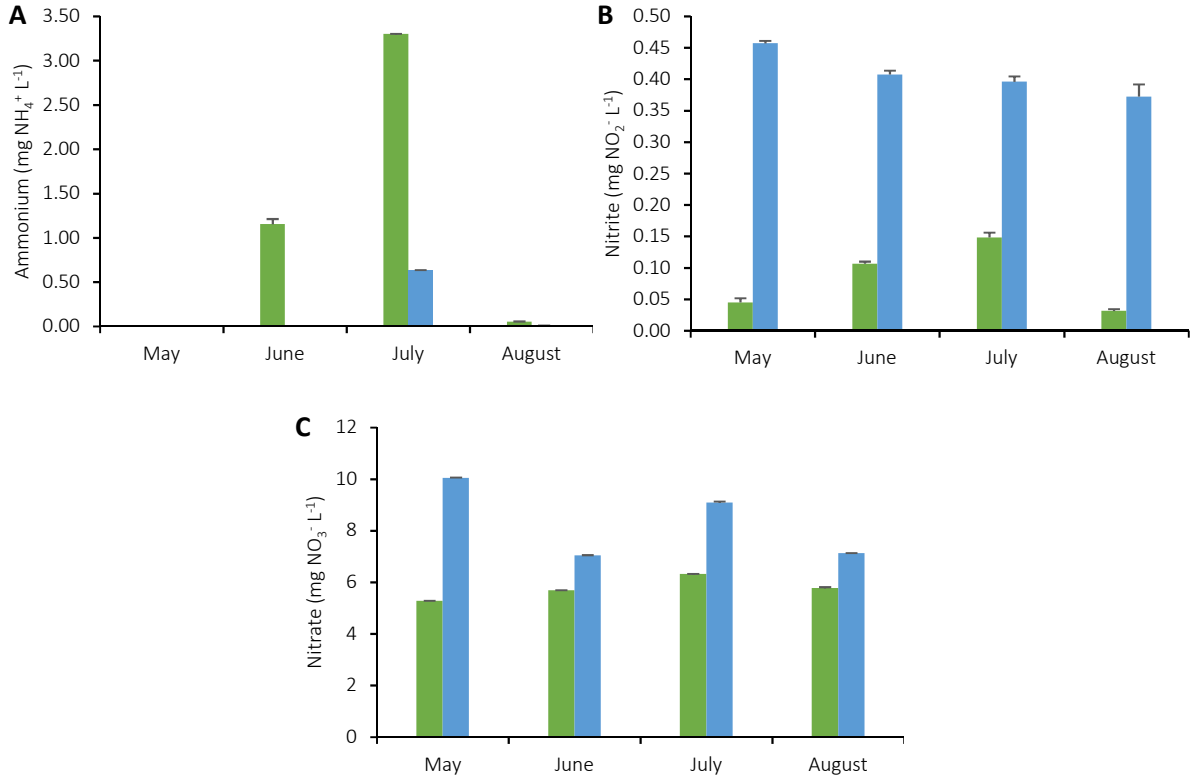


Figure 4.7: Concentrations of A) **ammonium**, B) **nitrite** and C) **nitrate** detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis. Samples were collected and analysed once a month between May 2019 and August 2019. N = 6 & SD indicated (NH_4^+ p = 0.2644, NO_2^- p = <0.001, NO_3^- p = 0.0164).

PO_4^{3-} levels were observed in both sample sets (Figure 4.8) and no statistically significant difference was observed (p = 0.2114). The levels observed in both samples were very similar to one another. This suggested that PO_4^{3-} is entering the fish farm by way of the bog, as well as being generated within the farm itself. Additionally, as there was very little difference observed in the levels, the fish farm did not increase PO_4^{3-} levels, unlike other studies (Stephens and Farris, 2004b; 2004a; and Ziemann *et al.*, 1992). Concentrations detected were greater than the recommended value of 0.035 mg L^{-1} set out by the SI 272/2009 and SI 77/2019 for good water status (Irish Statutory Office, 2019, 2009). However, as the water is returning to the natural wetland of the bog, and no increases in concentrations were observed, no causes for concern had been indicated. It should be noted that the levels were in accordance with Stephens and Farris (2004b, 2004a), and Ziemann *et al.* (1992) and their studies on finfish farming effluent. Similarly, with the NO_2^-, the PO_4^{3-} may not be present in the lagoon for long enough to be removed, or the reed bed may not be capable of removing it. Further testing would need to be conducted to confirm or deny this.

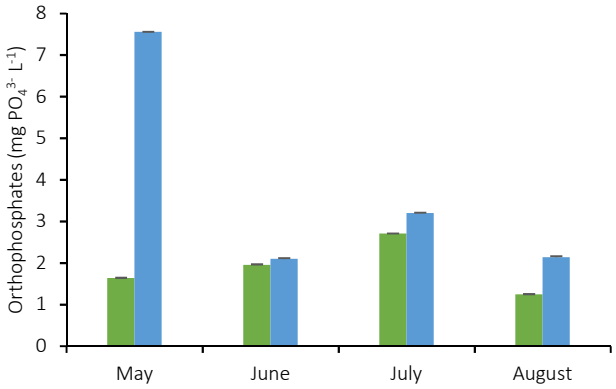


Figure 4.8: **Orthophosphate** levels detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis between May 2019 and August 2019. Samples were collected and analysed once a month. N = 6 & SD indicated (p = 0.2114).

Fluctuations in the DO (Figure 4.9A), BOD (Figure 4.9B) and COD (Figure 4.9C) levels were observed in the intake and output samples. Statistical analysis indicated no statistically significant differences were observed in the DO results (p = 0.3218) or the BOD results (p = 0.0913). However, a significant difference was indicated in the COD results (p = 0.0112). There were no issues with the DO levels present in either sample as water is entering and exiting directly from and to the bog. Additionally, DO levels are closely monitored on the farm itself, and the use of air lifts and paddle wheels to aerate the water are continuously used to ensure levels remain at a high level. DO levels in other studies, which included shrimp, catfish, prawn and trout farming, were slightly below the concentrations observed in this study (Camargo, 1994; Mcintosh and Fitzsimmons, 2003; Biao *et al.*, 2004; Stephens and Farris, 2004b, 2004a; Moreira *et al.*, 2010; Namin *et al.*, 2013; da Silva *et al.*, 2017). The current BOD levels detected in the intake water suggested no issues as they were below both of the suggested values. The concentration of BOD detected in the output water were above the SI value (1.3 mg L^{-1} for high water status and 1.5 mg L^{-1} for good water status) but below the EPA value (≤3 mg L^{-1} and ≤6 mg L^{-1} for salmonid and cyprinid waters, respectively). This research was then compared to results determined by other researchers. Those that were revised demonstrated higher levels than the concentrations detected in this study (Boaventura *et al.*, 1997; Mcintosh and Fitzsimmons, 2003; Ansah *et al.*, 2012; Miashiro *et al.*, 2012). COD was detected in both sets of samples. The levels observed in the intake water were not considered to be cause for alarm as they were below the mean concentration of 40 mg L^{-1} set out by the Irish EPA (EPA, 2001). Levels observed in the output water fluctuated above and below this value but are also deemed not to be any great cause for concern as water was not being released from the system during this time. Research conducted by da Silva *et al.* (2017), reported some COD levels greater than those determined in this research.

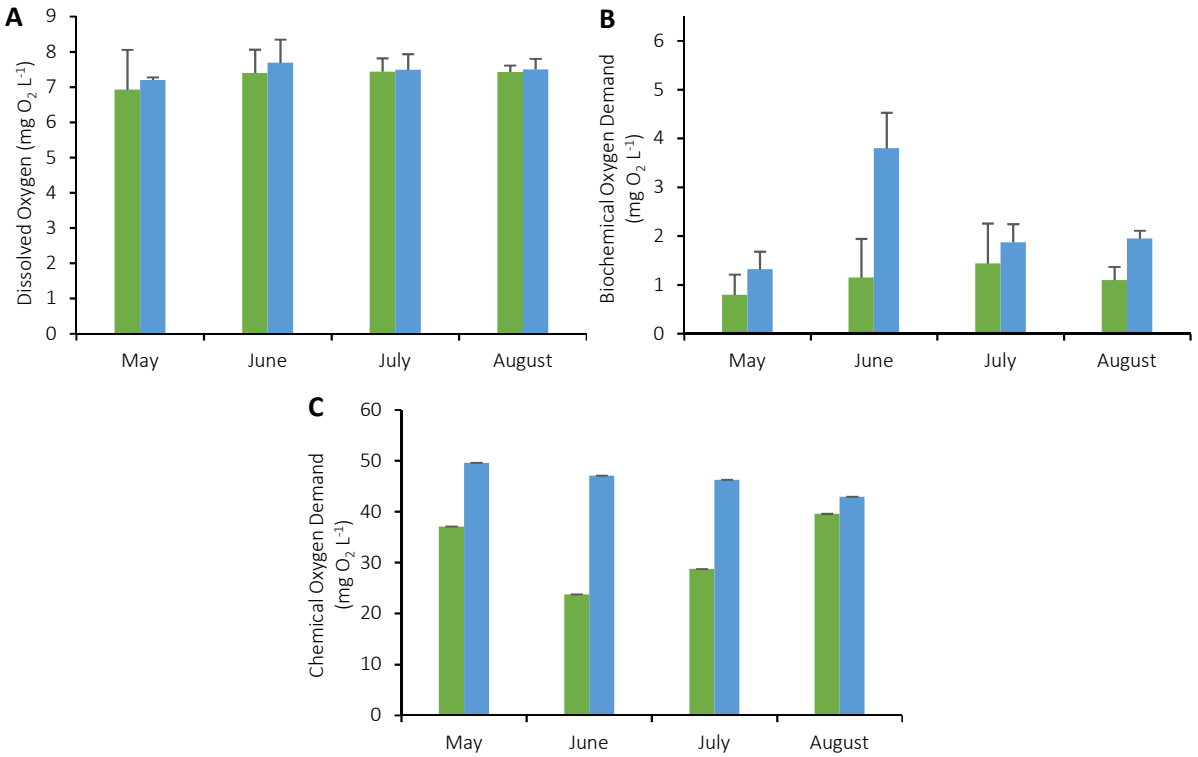


Figure 4.9: A) **Dissolved oxygen**, B) **biochemical oxygen demand and** C) **chemical oxygen demand** levels detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** in Oasis. Samples were collected and analysed once a month from May 2019 to August 2019. N = 3 & SD indicated (DO p = 0.3218, BOD p = 0.0913, COD p = 0.0112).

Temperature and pH levels fluctuated in the samples, as shown in Figure 4.10. However, no significant differences were observed in either parameter (temperature p = 0.1172, pH p = 0.5351). The temperature remained relatively constant in both samples throughout the study and were not at a level to indicate any causes for concern. The results for the pH indicated that the intake and output equally demonstrated consistently similar levels. The recommended pH levels should be between 6 and 9. Levels in both the intake and output were well within this range and therefore presented no issues. The mean temperature and pH results observed were similar to the those recorded in the revised studies that focused on freshwater finfish *i.e.,* catfish, brown trout and rainbow trout (Boaventura *et al.*, 1997; Pulatsü *et al*., 2004; Stephens and Farris, 2004a; Živić *et al.*, 2009; Namin *et al.*, 2013; Noroozrajabi *et al.*, 2013; Caramel *et al.*, 2014).

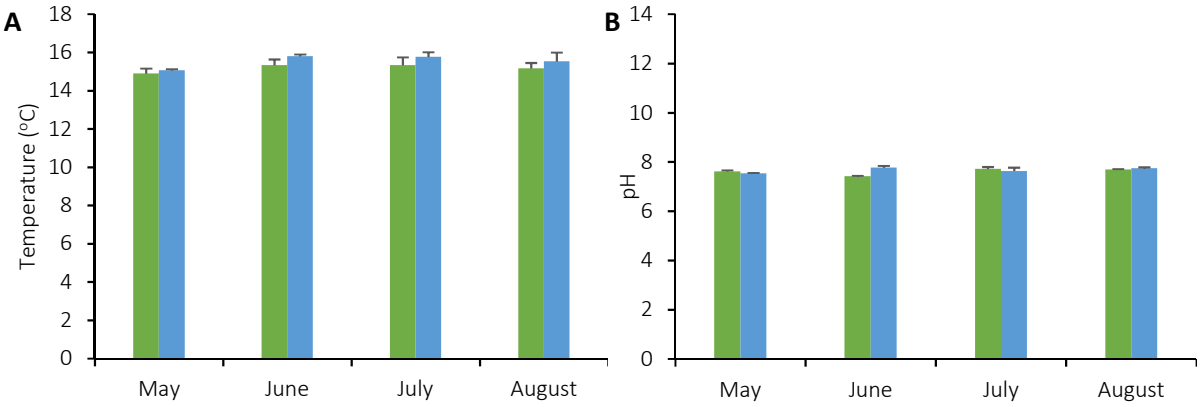


Figure 4.10: A) **temperature** and B) **pH** levels detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis. Samples were collected and analysed once a month from May 2019 and August 2019. N = 3 & SD indicated (temperature p = 0.1172, pH p = 0.5351).

$CaCO_3$ levels were observed in the intake and output water samples in order to determine hardness and alkalinity levels (Figure 4.11). Statistical analysis was conducted between both sample sets and no significant differences were observed (hardness p = 0.5405, alkalinity p = 0.8742). Observations from shrimp and catfish studies demonstrated similar alkalinity results to this study (Mcintosh and Fitzsimmons, 2003; Ferreira *et al.*, 2011). Hardness results suggested that the water is slight to moderately hard. Similar hardness results were observed in revised studies on catfish and Atlantic salmon (Stephens and Farris, 2004a, 2004b; Lalonde *et al.*, 2014).

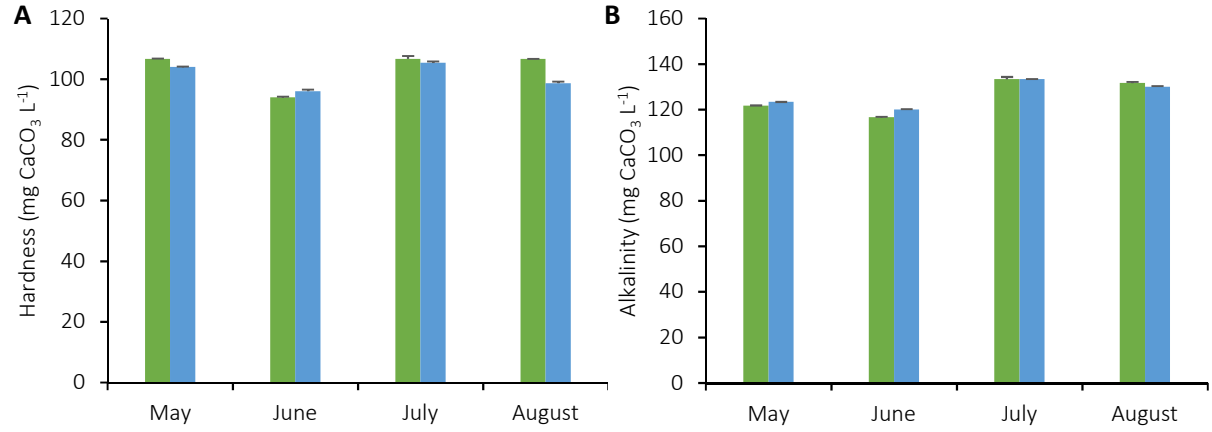


Figure 4.11: A) **hardness** and B) **alkalinity** levels detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis. Samples were collected and analysed once a month from May 2019 and August 2019. N = 3 & SD indicated (hardness p = 0.5405, alkalinity p = 0.8742).

Suspended and dissolved solids observed in the intake and output water samples are displayed in Figure 4.12. After statistical analysis, no significant differences were observed in either parameter (suspended solids p = 0.1580, dissolved solids p = 0.2300). With the exception of the output sample in May, the suspended levels detected in both samples were below the MAC of 25 mg L^{-1}, indicating that

there were no issues. Additionally, suspended solid concentrations in a range of studies (shrimp, prawn, salmonid, catfish, brown trout and rainbow trout), were greater than those established in this study (Boaventura *et al.*, 1997; Camargo, 1994; Caramel *et al.*, 2014; Costanzo *et al.*, 2004; Guilpart *et al.*, 2012; Lalonde *et al.*, 2014; Mcintosh and Fitzsimmons, 2003; Pulatsü *et al.*, 2004; Ziemann *et al.*, 1992). Dissolved solid levels were well below the guidance value of 300 mg L^{-1} indicating no issues with the general water quality.

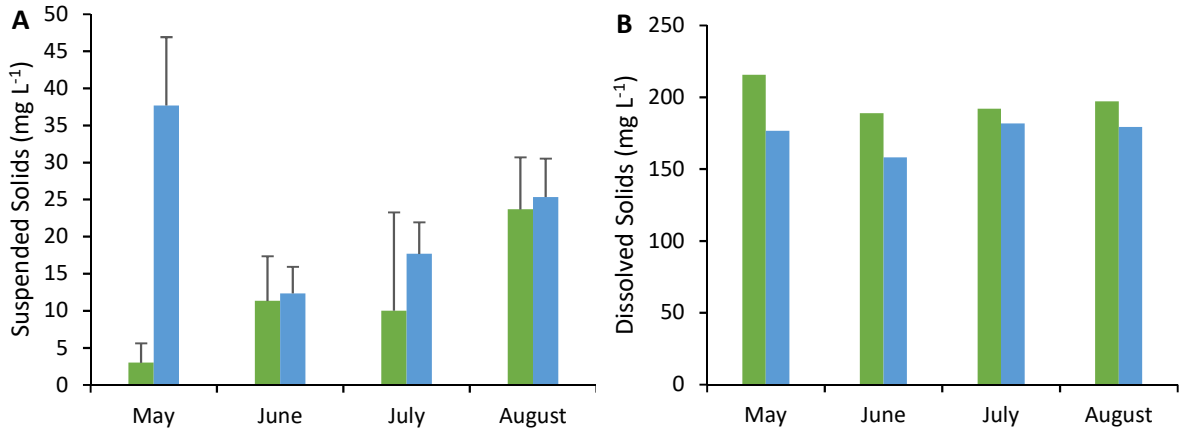


Figure 4.12: A) **suspended solid** and B) **dissolved solid** levels detected **in novel peatland freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis. Samples were collected and analysed once a month from May 2019 and August 2019. N = 3 & SD indicated (suspended solids p = 0.1580, dissolved solids p = 0.2300).

Conductivity in the intake water samples was between 295 and 337 µS cm^{-1}, and between 247 and 284 µS cm^{-1} in the output water (Figure 4.13). No statistically significant differences were observed between the samples (p = 0.2380). As conductivity levels observed were <1000 mg L^{-1} no issues were foreseen.

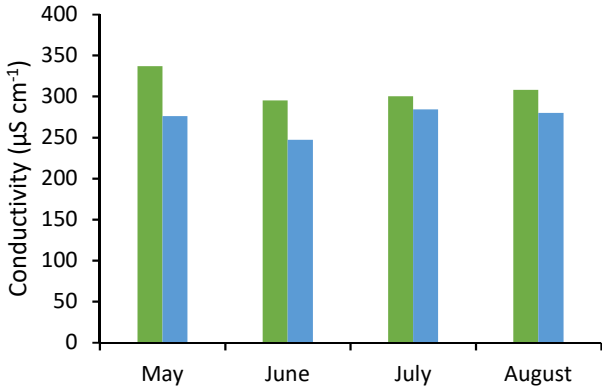


Figure 4.13: **Conductivity** levels detected **in freshwater finfish aquaculture intake** (green) **and output** (blue) **water** from Oasis. Samples were collected and analysed once a month from May 2019 to August 2019. N= 3 & SD indicated (p = 0.2380).

Variances in the nutrients present in the output water is most likely as a result of the presence of fish feed and faeces. However, given that variances were observed in the input water also, it is likely that the bog itself is also playing a role. Additional research will need to be conducted.

Some of the results observed (NO_2^-, NO_3^-, PO_4^{3-} and COD), suggested potential issues. No research conducted on the use of peatlands for aquaculture could be found in the available literature. Additionally, this research only focused on one fish farm in a peatland setting. As a result, all physicochemical results were then compared to a range of studies conducted on traditional fish farms. All results obtained were similar to or below the concentrations determined in the previous aquaculture studies investigated, including NO_2^-, NO_3^-, PO_4^{3-} and COD. This indicated that there appeared to be no observable differences between the water quality after traditional aquaculture settings, and that of peatland settings.

As no research has been previously conducted on the use of peatlands for aquaculture practices (as far as the author is aware), a comparative investigation was conducted between the study conducted in Keywater Fisheries during the same time period as the pilot study conducted in Oasis. See Table A4.27 in Appendix 4. Statistical analyses were conducted between the intake and output samples of both farms. Due to logistical reasons sampling could not be conducted for the full six months on Oasis Fish Farm. Therefore, results from May to August 2019 from both fish farms were analysed. Several significant differences were observed in both the intake (*Asterionella formosa*, NO_3^-, DO, BOD, suspended solids and conductivity), and output (pH, NH_4^+, NO_2^-, NO_3^-, DO, COD and conductivity) water. These differences may have been due to the fact that many differences existed with the farms themselves. Keywater Fisheries is a small facility located in the west of Ireland, culturing cyprinids only, uses freshwater from an adjacent stream / river as its water source, uses an FTS as its primary method of culture, and has a settlement pond and constructed wetland for wastewater treatment processes. It also has a small RAS facility that is only used for hatching and nursery purposes. Oasis Fish Farm on the other hand, is a much larger scale farm located in the middle of a bog in the midlands, cultures both cyprinids and salmonids, uses water from the bog itself as its source, uses a recirculating IMTA as its primary method of culture, and has a large algae and duckweed bed to treat wastewater. This facility also reuses up to 100% of its water and discharge is only release during times of heavy rainfall. Water is only taken up into the farm once a month during cooler, winter conditions and up to once a week during warmer, summer conditions due to loss via evaporation.

Results for the novel pilot study led to the publication of a research paper entitled "Use of peatlands as future locations for the sustainable intensification of freshwater aquaculture production – A case study from the Republic of Ireland" in the Science of the Total Environment Journal. See appendix 1 for a copy of the published paper.

4.3.2. Characterisation of Novel IMTA Water – Results & Discussion

Once it was ascertained that the application of aquaculture production within a peatland setting was deemed unlikely to induce any adverse environmental impact, a better understanding of the novel IMTA process within the aquaculture facility was necessary in order to specifically inform and guide the development of the process. This was done so by characterising the process by way of physicochemical monitoring in conjunction with the quantification and identification of algae over a year-long case study.

All eight sampling points have been displayed in all physicochemical, algal and cyanobacterial analysis. For ease of reporting the same colour designation has been used for all results. See figure 4.14 for a breakdown of the colour legend applicable to all results (Figure 4.15 to Figure 4.23) with the exception of the total acidity results (Figure 4.20C).

Figure 4.14: Legend with **colour designation** for all **physicochemical**, **algal** and **cyanobacterial** analysis conducted on the **eight samples** collected from **Oasis** between December 2019 and October 2020. Legend is applicable from Figure 4.14 to Figure 4.21.

Physicochemical Analysis

This research is novel as there were no previous studies conducted on aquaculture facilities located within peatland settings found in the available literature. As a result of this no direct comparatives to previous studies could be conducted. Therefore, levels detected in this research were compared to previous studies conducted on traditional aquaculture facilities across the globe and to guidance/recommended values for the physicochemical parameters set out by the EPA and the Irish Statutory Office in order to establish a baseline for the novel system.

Fluctuations across the eight sampling points were observed in the NH_4^+ (Figure 4.15A), NO_2^- (Figure 4.15B) and NO_3^- (Figure 4.15C) levels detected. The reservoir (light blue) displayed the greatest variation across all sampling points. However no statistically significant differences were observed

across the eight sampling points in the NH_4^+ ($p = 0.5520$) and NO_2^- ($p = 0.4867$) results. A significant difference was observed in the NO_3^- ($p = <0.0001$). Statistical analysis was also conducted between the four culturing ponds and between the two locations within the duckweed lagoon only and no significant differences were observed, as shown in Table A4.48 of Appendix 4.

Prior to the first COVID-19 national lockdown in March 2020, very little to no NH_4^+ levels were detected within the farm *i.e.,* levels were below the limit of detection of the kit used (0.01 mg NH_4^+ L^{-1}). This was believed to be due to a combination of issues associated with cyanobacteria levels which will be discussed in the algae and cyanobacteria analysis section (next section) of this chapter, and increased levels of rainfall experienced in February 2020 which will be further discussed in the climate change section of Chapter 5. NH_4^+ levels increased across all sampling points from the end of July 2020 with spikes of up to 0.90 mg NH_4^+ L^{-1} observed at the beginning of October 2020. As levels did not rise to greater than the recommended value of 1 mg NH_4^+ L^{-1} set out by the Freshwater Fish Directive (EPA, 2001) no concerns or issues associated with the levels of NH_4^+ detected in the farm were indicated or foreseen. Additionally, no NH_4^+ was detected at the discharge point during times of possible overflow and release indicating no potential issues associated with NH_4^+ for the receiving peatlands. Levels of NH_4^+ also decreased between the culture ponds and the duckweed lagoon (treatment lagoon) suggesting that the treatment process was effective at reducing NH_4^+ levels in wastewater. Levels detected were then compared to previous aquaculture studies. With the exception of the spike observed in October, NH_4^+ levels observed in this study were similar to research conducted by Boaventura *et al.* (1997) and their research on three rainbow trout farms in Portugal where up to 0.70 mg L^{-1} was detected, Cao *et al.* (2007) and their study on a range of different aquaculture farms (including rainbow trout) in China where an average of up to 0.48 mg L^{-1} was observed, Guilpart *et al.* (2012) and their research on eight different rainbow trout farms across France where an average level of up to 0.70 mg L^{-1} was detected, Noroozrajabi *et al.* (2013) and their study of a rainbow trout farm in Iran where levels of up to 0.45 mg L^{-1} were observed and Živić *et al.* (2009) and their research on three different rainbow trout farms on the same river in Serbia where an average concentration of 0.55 mg L^{-1} was indicated. Levels detected in this study were also less than studies conducted by O'Neill *et al.* (2019) and their research on perch aquaculture in Ireland where levels up to 1.09 mg L^{-1} were detected, Stephens and Farris (2004a) and their study on catfish in the US where levels of up to 4.87 mg L^{-1} were observed and Costanzo *et al.* (2004) and their research on shrimp in Australia were levels reached up 18.50 mg L^{-1}. However, these last three studies where NH_4^+ levels were greater focused on different species of fish.

Before the lockdown period, NO_2^- levels fluctuated between 0 mg NO_2^- L^{-1} and 0.03 mg NO_2^- L^{-1}. However, levels increased greatly after this period, with concentrations spiking to between 0.25 mg NO_2^- L^{-1} and 0.30 mg NO_2^- L^{-1} in mid-July and mid-September, respectively. This was a tenfold increase on previous levels as well as being tenfold greater than the Freshwater Fish Directive recommended value of 0.03 mg L^{-1} (EPA, 2001). NO_2^- is highly toxic to aquatic life (O'Neill *et al.*, 2019; Pollice *et al.*, 2002) but is extremely unstable and would not remain in this form for long as it would be quickly transformed to NO_3^- (Durborow *et al*., 1997; O'Neill *et al.*, 2019, 2020). As no overflow and release occurred during the times of high levels, the NO_2^- would not cause issues within the bog. With regards to effects on the farm itself, this study was compared to previous aquaculture research. The majority of studies investigated demonstrated NO_2^- levels tenfold less than levels indicated in this research *i.e.,* levels in these previous studies displayed similar results to those observed prior to lockdown in this study. For example; Caramel *et al.* (2014), Noroozrajabi *et al.* (2013) and Živić *et al*. (2009) and their work with rainbow trout, da Silva *et al.* (2017) and Moreira *et al.* (2010) and their research on shrimp/prawns and Stephens and Farris (2004a, 2004b) and their studies with catfish, all demonstrated levels of 0.03 mg NO_2^- L^{-1} or below. However, two studies working with rainbow trout did display similar results to the higher levels observed in this study. Boaventura *et al.* (1997) and Pulatsü *et al.* (2004) both reported levels of just above 0.22 mg NO_2^- L^{-1}.

NO_3^- levels dropped considerably the month prior to lockdown (February 2020) going from >8 mg NO_3^- L^{-1} to 0 mg NO_3^- L^{-1}. This coincided with changes in weather conditions and excessive rainfall experienced throughout the month. This will be further discussed in the climate change section of Chapter 5. Once analysis recommenced, NO_3^- levels slowly increased reaching levels >8 mg NO_3^- L^{-1} in September before dropping back to between 2 mg NO_3^- L^{-1} and 4 mg NO_3^- L^{-1} in October. Levels were well below the guidance value of 50 mg L^{-1} suggested by the EPA (EPA, 2001) as a result no issues or concerns were foreseen. Guilpart *et al.* (2012)and their research on eight rainbow trout farms across France was the only study that demonstrated levels greater than those observed in this study whereby concentrations of up to 44.60 mg NO_3^- L^{-1} were detected. All other research on aquaculture facilities that included analysis of NO_3^- levels in and around a range of different farms displayed lower levels of NO_3^- (Boaventura *et al.*, 1997; Camargo, 1994; Costanzo *et al.*, 2004; da Silva *et al.*, 2017; Noroozrajabi *et al.*, 2013; Pulatsü *et al.,* 2004; Stephens and Farris, 2004a, 2004b; Živić *et al.*, 2009). Concentrations ranged from 0.01 mg NO_3^- L^{-1} observed by Moreira *et al.* (2010) and their research on freshwater prawns in Brazil, to 4.20 mg NO_3^- L^{-1} detected by Lalonde *et al.* (2014) and their research surrounding Atlantic salmon in Canada. The increased levels in NO_3^- observed in this study may be due to the increased levels of NO_2^- also observed.

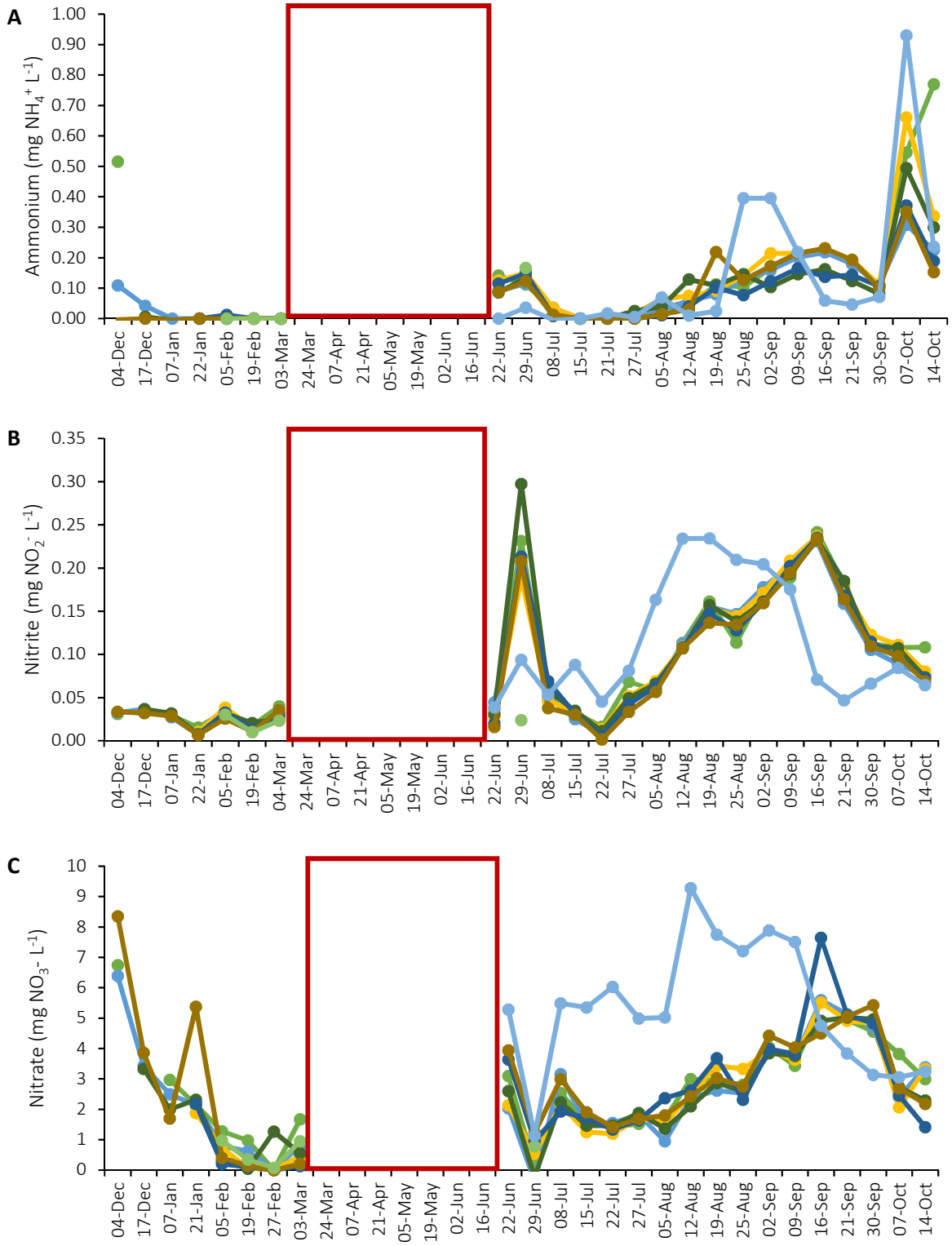


Figure 4.15: Levels of A) **ammonium**, B) **nitrite** and C) **nitrate** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 6 and SD indicated (NH_4^+ p = 0.5520, NO_2^- p = 0.4867, NO_3^- p = <0.0001).

PO_4^{3-} levels were observed across all sampling points within the farm as indicated in Figure 4.16. Statistical analysis demonstrated no significant difference between the different sampling points (p = 0.2160). No significant differences were indicated between the different locations either *i.e.,* culturing ponds or the duckweed lagoon. See Table A4.48 in Appendix 4. PO_4^{3-} levels detected were above the recommended value of <0.035 mg L^{-1} set out by the SI 272/2009 and SI 77/2019 for good water status (Irish Statutory Office, 2019, 2009) indicating there may be potential issues within the farm and additional treatment processes to reduce PO_4^{3-} levels may need to be considered as the duckweed lagoon is not effectively removing it. Levels in this research were greater than those observed in many of the studies investigated in the comparative (Caramel *et al.*, 2014; Guilpart *et al.*, 2012; Lalonde *et al.*, 2014; Noroozrajabi *et al.*, 2013; Ziemann *et al.*, 1992; Živić *et al.*, 2009). However, they were in accordance with Stephens and Farris (2004a, 2004b) and their studies on catfish where levels of up to 6.80 mg PO_4^{3-} L^{-1} and 2.76 mg PO_4^{3-} L^{-1} were observed, respectively, and were well below concentrations of up to 194.10 mg PO_4^{3-} L^{-1} observed by da Silva *et al.* (2017) and their research on shrimp aquaculture in Brazil.

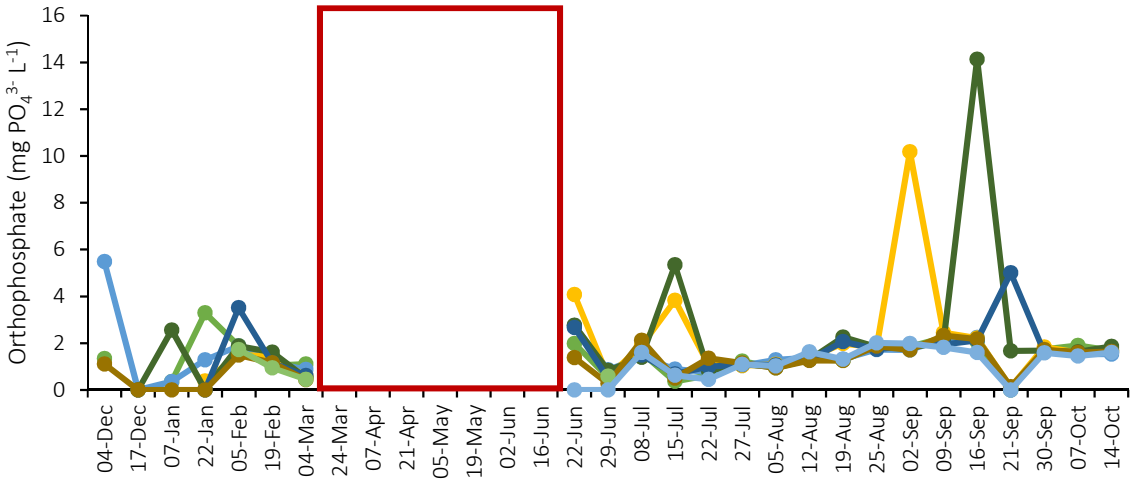


Figure 4.16: Levels of **orthophosphate** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 6 and SD indicated (PO_4^{3-} p = 0.2160).

Variations in DO levels (Figure 4.17A) and BOD levels (Figure 4.17B) were observed across the eight sampling points. No statistically significant differences were observed across both parameters (DO p = 0.1421, BOD p = 0.5464). DO levels fluctuated between 4 mg O_2 L^{-1} and 10 mg O_2 L^{-1}. The recommended DO concentration present in salmonid waters is ≥9 mg O_2 L^{-1} and in cyprinid waters is ≥7 mg O_2 L^{-1} (EPA, 2001). Levels continually increased and decreased above and below these

recommended levels. However, they did not drop below the threshold of 4 mg O_2 L^{-1} required for sufficient maintenance of aquatic life (Alam *et al.*, 2007; da Silva *et al.*, 2017; O'Neill *et al*., 2019, 2020). Despite these fluctuations, no issues were foreseen with DO levels as O_2 is supplemented within the farm. Air lifts and paddlewheels are located throughout the farm and levels are closely monitored to maintain the ideal and optimum levels of O_2 within the farm. DO levels observed in other studies demonstrated higher levels of O_2 (Boaventura *et al.*, 1997; Camargo, 1994; da Silva *et al.*, 2017; Moreira *et al*., 2010; Namin *et al.*, 2013; Noroozrajabi *et al.*, 2013; Pulatsü *et al.*, 2004; Stephens and Farris, 2004b, 2004a) ***e.g.,*** Živić *et al.* (2009) reported DO levels of 10.20 mg O_2 L^{-1} in their study on rainbow trout in Serbia. The differences in O_2 levels between this and previous studies may be due to that fact that the other studies were situated in rivers where O_2 levels will be naturally higher than peatland systems.

The SI 272/2009 and SI 77/2019 recommended a mean BOD concentration of 1.30 mg O_2 L^{-1} for high water status and 1.50 mg O_2 L^{-1} for good water status (Irish Statutory Office, 2019, 2009). However, the EPA suggested ≤3 mg O_2 L^{-1} and ≤6 mg O_2 L^{-1} for salmonid and cyprinid waters, respectively (EPA, 2001). Issues were indicated with the BOD levels observed across all sampling points. In addition to levels reporting greater (up to 34.60 mg O_2 L^{-1}) than the recommended values mentioned above, levels were also greater than those reported in previous studies. For example; Boaventura *et al.* (1997) observed BOD levels up to 1.60 mg O_2 L^{-1} in their study on rainbow trout, Lalonde *et al.* (2014) reported levels of up to 5.00 mg O_2 L^{-1} in their research with Atlantic salmon, Moreira *et al.* (2010) indicated that concentrations of up to 2.93 mg O_2 L^{-1} were displayed in their work with freshwater prawns, Pulatsü *et al.* (2004) observed levels of just below 5.00 mg O_2 L^{-1} in their study with rainbow trout and O'Neill *et al.* (2019) reported levels of up to 6.31 mg O_2 L^{-1} in their research with Perch. The BOD is caused by microorganisms using O_2 when consuming organic matter (dead algae, fish waste, uneaten feed, *etc.*) therefore organic matter needs to be reduced in order to decrease BOD levels (EPA, 2001; Gupta *et al*., 2017; Kasuya *et al.*, 1998; Lee and Nikraz, 2015; Mcintosh and Fitzsimmons, 2003; Sultana *et al.*, 2017). Increasing O_2 levels and the addition of filtration to remove some of the organic matter have been found to decrease BOD levels (Gupta *et al.,* 2017; Lee and Nikraz, 2015). Work is ongoing to reduce BOD levels. However, as no water was released from the farm during times of increased BOD levels, no concerns associated with this issues affecting the surrounding peatland habitat were foreseen.

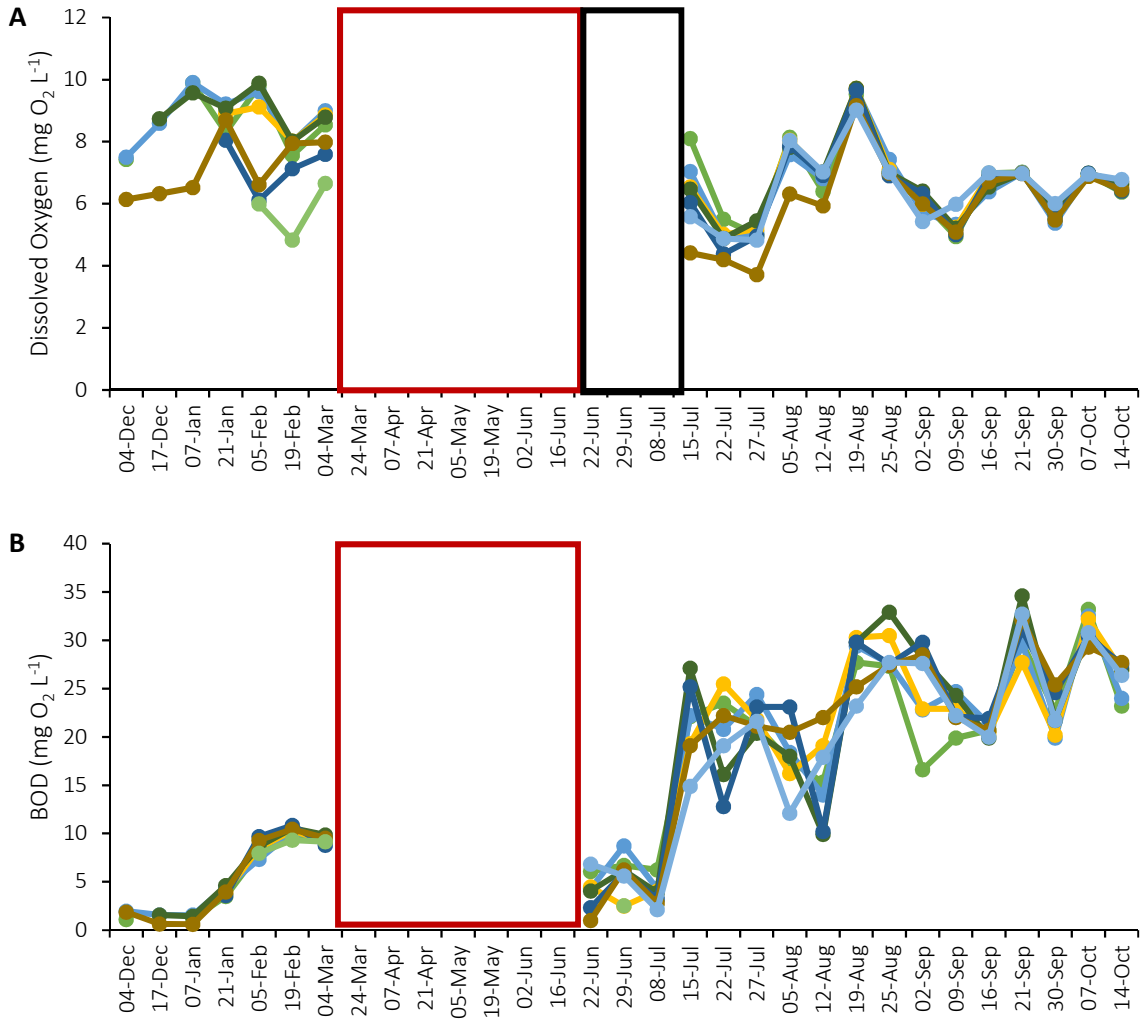


Figure 4.17: Levels of A) **dissolved oxygen** and B) **biochemical oxygen demand** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 3 and SD indicated (DO^- p = 0.1421, BOD p = 0.5464).

Fluxes in the levels of suspended solids (Figure 4.18A) were observed across all sampling points, whilst dissolved solid (Figure 4.18B) concentrations remained more consistent throughout the study. Statistical analysis conducted between all sampling points indicated no statistically significant differences (suspended solids p = 0.8604, dissolved solids p = 0.3172). The levels of suspended solids observed throughout the summer months (June, July, August) were well above the 25 mg L^{-1} (EPA, 2001) reaching highs of >120 mg L^{-1}. Levels observed in this novel system were then compared to the traditional aquaculture studies. Two studies on catfish aquaculture in the US demonstrated very high levels of up to 8119 mg L^{-1} (Stephens and Farris, 2004a, 2004b). Four studies observed similar suspended solids levels to those in this research (da Silva *et al.*, 2017; Guilpart *et al.*, 2012; Namin *et al.*, 2013; Ziemann *et al.*, 1992) most of which focused on rainbow trout. However, five studies investigated, which also mostly focused on rainbow trout research, observed suspended solids of no

greater than 9.40 mg L^{-1} (Boaventura *et al.*, 1997; Caramel *et al.*, 2014; Costanzo *et al.*, 2004; Lalonde *et al.*, 2014; Pulatsü *et al.*, 2004). Given that suspended solids can cause gill irritation, signs of which were observed in some of the fish, this was considered to be a major issue. It was also believed that this issue was linked to the issues with BOD previously mentioned. After filtration methods were applied to different areas of the farm, suspended solid levels dropped back to below the MAC level by September and remained so until the end of the study. Dissolved solid levels indicated no issues or cause for concern as all concentrations observed in the study were well below the WHO's suggested concentration of <300 mg L^{-1} for excellent water status (WHO, 2003).

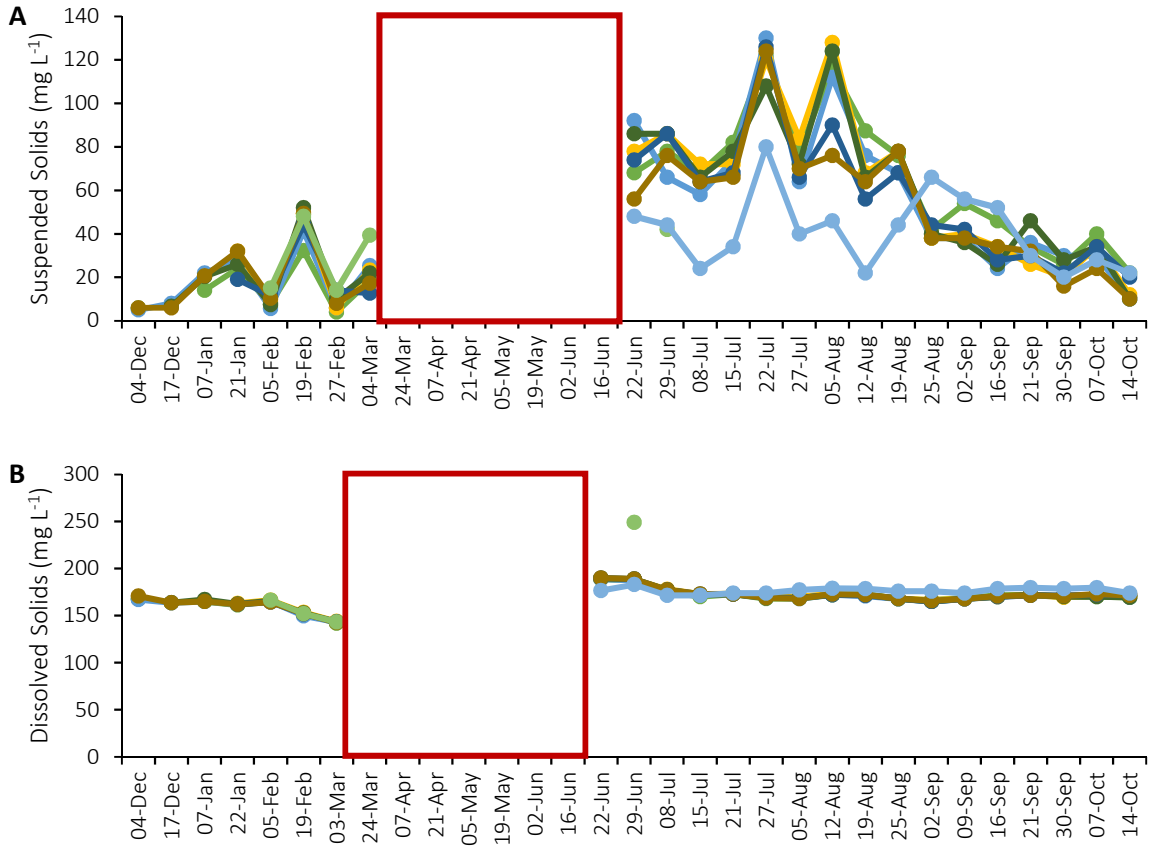


Figure 4.18: Levels of A) **suspended solids** and B) **dissolved solids** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 3 and SD indicated (SS^{-} p = 0.8604, DS p = 0.3172).

Fluctuations were indicated in the temperature range (Figure 4.19A) and the pH range (Figure 4.19B) observed across all of the sampling points. No significant differences were indicated across all results for either parameter (temperature p = 0.1671, pH p = 0.9952). The elevations in temperature were observed between June and September as would be expected given the season (summer). Although no specific guidance value for temperature could be established as all species of fish have a slightly different optimum temperature, any water released into an aquatic system must be <20°C (EPA,

2001). Temperatures were >20°C only once in mid-July. However, as water was not released from the system, this was not deemed to be an issue. Results from previous studies that focused on rainbow trout research displayed similar temperature fluctuations and levels to those observed in this study (Boaventura *et al.*, 1997; Camargo, 1994; Caramel *et al.*, 2014; Namin *et al.*, 2013; Noroozrajabi *et al.*, 2013; Pulatsü *et al.*, 2004; Živić *et al.*, 2009). Recommended pH levels of between pH 6 and pH 8 were suggested by the Freshwater Fish Directive and the SI's 272/2009 and 77/2019 (EPA, 2001; Irish Statutory Office, 2019, 2009). The pH levels remained within this range throughout the study. The pH levels were just below pH 8 however, levels dropped to just above pH 7 from August 2020 to September 2020. All previous rainbow trout studies reported a similar pH range to that observed across all sampling points within the system (Boaventura *et al.*, 1997; Camargo, 1994; Caramel *et al.*, 2014; Namin *et al.*, 2013; Noroozrajabi *et al.*, 2013; Pulatsü *et al.*, 2004). Although levels remained within the recommended range, the alteration in pH levels may have had an effect on the BOD issues observed in the farm. Alterations in pH can decrease the rate of organic removal rates thus affecting BOD measurements (Mukherjee *et al.*, 1968).

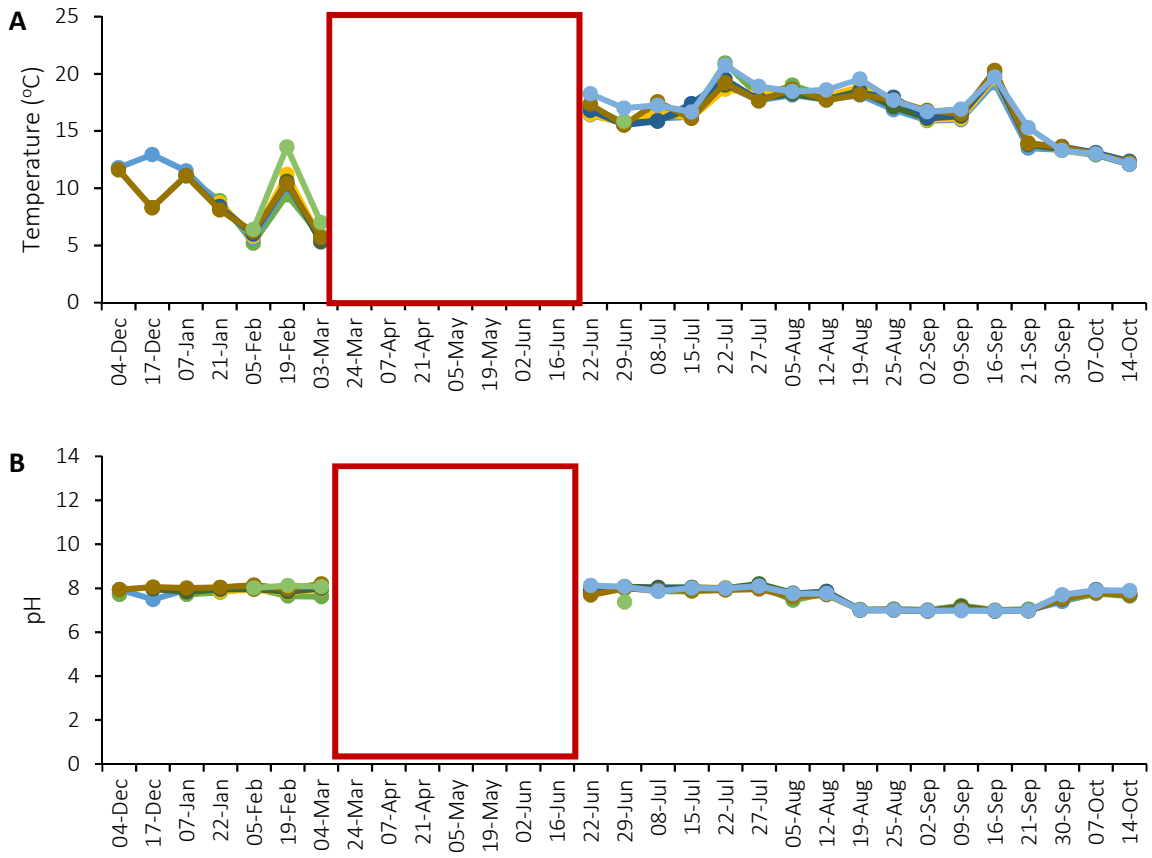


Figure 4.19: Levels of A) **temperature** and B) **pH** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 3 and SD indicated (T p = 0.1671, pH p = 0.9952).

$CaCO_3$ levels were measured in the eight sampling points in order to determine hardness (Figure 4.20A), alkalinity (Figure 4.20B) and total acidity (Figure 4.20C) levels. Statistical analysis was conducted for each parameter and no significant differences were observed (hardness $p = 0.5237$, alkalinity $p = 0.4806$, total acidity $p = 0.0769$). Hardness levels observed suggested that the water was slight to moderately hard. This correlated with water hardness maps of Ireland which demonstrated water in the midlands around Co. Offaly were also slight to moderately hard. Similar hardness levels were also observed in revised studies on rainbow trout, Atlantic salmon and catfish (Fadaeifard *et al.*, 2011; Lalonde *et al.*, 2014; Stephens and Farris, 2004b, 2004a). It has been suggested that fish prefer a minimum of 20 mg $CaCO_3$ L^{-1} alkalinity levels. Levels recorded within the farm remained above this optimum threshold throughout the study (Boyd and Tucker, 2015; EPA, 2001).

Alkalinity levels observed in this study demonstrated similar results to those reported by Stephens and Farris (2004b). At the request of the fish farm, total acidity was also included in the study from June 2020 to October 2020 for four of the sampling points where issues were being observed.

Total acidity expresses the waters capacity to neutralise a strong base up to a given pH and is an indicator of how corrosive that water is. CO_2 is the most common source of acidity. High levels of water acidity can affect aquatic ecosystems from biological processes to chemical reaction rates. Fish specifically, can only withstand a very narrow range of acidity before biological processes are affected and fatalities occur. Ideally, levels <200 mg $CaCO_3$ L^{-1} has been suggested as a threshold point where increased issues have been observed in fish (Chinedu *et al.*, 2015). Variations were observed across the four sampling points in June 2020. However, the variations stabilised in the total acidity readings recording and by October 2020 levels were similar across all sampling points. This also coincided when fish behaviour within the farm was found to improve.

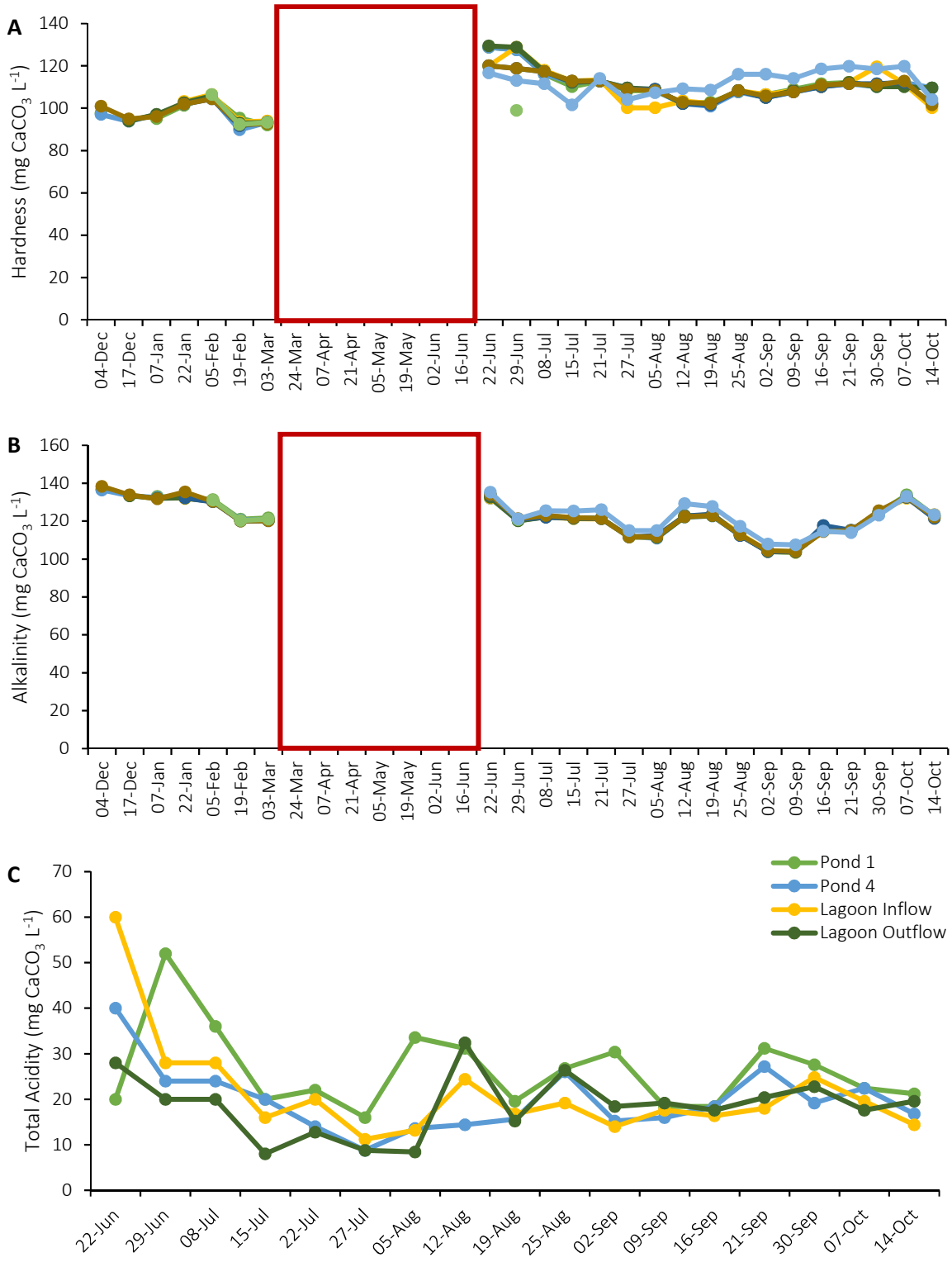


Figure 4.20: Levels of A) **hardness**, B) **alkalinity** and C) **total acidity** detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 3 and SD indicated (H p = 0.5237, A p = 0.4806, TA p = 0.0769).

Slight fluctuations were observed in the overall conductivity readings across the entire farm (Figure 4.21). No statistically significant differences were observed (p = 0.3172). Conductivity levels were consistent across all sampling points. With the exception of a slight drop just before the lockdown, very little variation was observed across the entire study. No issues or concerns were indicated in the findings as results remained well below the guidance value of <1000 µS cm^{-1}.

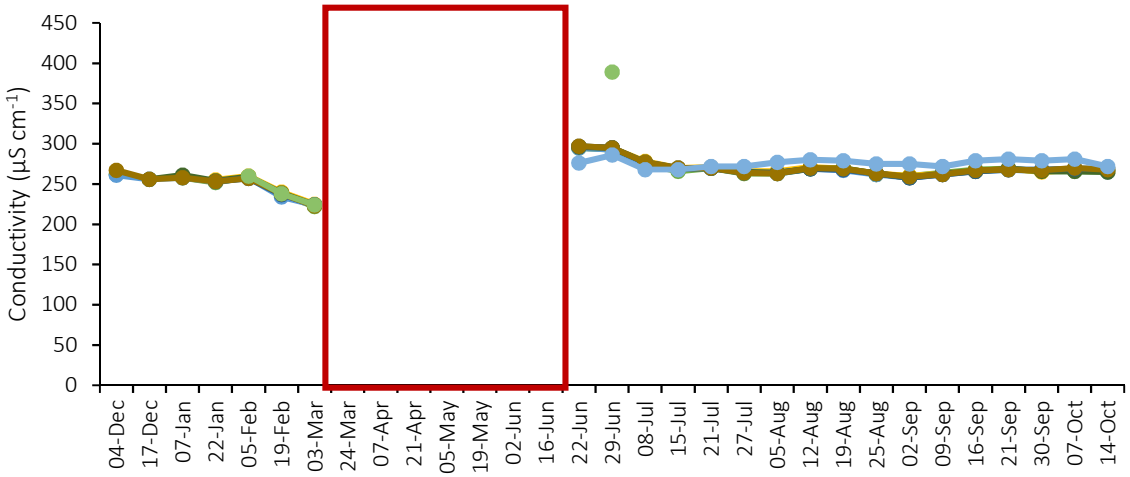


Figure 4.21: **Conductivity** levels detected in **Oasis** fish farm across **eight sampling points** from **December 2019 to October 2020**. Red section indicated when no sampling and analysis could be conducted as a result of the COVID-19 global pandemic and the resultant mandatory national lockdown and restrictions that were implemented. N = 3 and SD indicated (p = 0.3172).

Correlation studies were then conducted between all physicochemical parameters investigated. See Table A4.40 to Table A4.47 in Appendix 4. Correlationships were observed between the three N parameters (NH_4^+, NO_2^- and NO_3^-) as would be expected given their role in the nitrification process. A correlation between pH levels and both the alkalinity and total acidity readings. As pH is the overall ions concentration of hydrogen ions (acidity) and hydroxide ions (alkalinity), a correlationship between these three parameters was expected. Correlations were indicated between BOD levels and a range of parameters including pH, temperature, dissolved oxygen, alkalinity and suspended solids. This demonstrated the importance of maintaining high DO levels as oxygen is vital for the BOD process. Abnormal or irregular pH levels, which were observed for a time in the farm, can decrease the rate of removal of organic compounds which affect BOD levels. By proxy, changes in alkalinity will also have an impact (Chinedu *et al.*, 2015). Small amounts of all suspended solids are considered volatile suspended solids and exert greater pressures on the oxygen demand thus increasing BOD levels (Gerardi and Lytle, 2015). Finally, as temperatures increase so too does BOD removal rates as higher temperatures enhance microbes respiration rates (Lim *et al.*, 2001). The range of correlations with BOD has demonstrated how complex the process is and may be why issues were encountered in controlling the BOD levels within the farm.

ALGAE & CYANOBACTERIA ANALYSIS

To ensure the FCM was providing accurate cell counts, the FCM results were compared to the manual counts and no statistically significant differences were observed ($p = 0.5841$) suggesting both methods were producing similar readings. In order to develop a better understanding of the role algae may play in the novel peatland IMTA, enumeration was first conducted. (NOTE: Unlike that of the physicochemical parameters, some samples could be taken and preserved during the lockdown period as the required Lugols iodine was in the lab at AIT. Once restrictions eased, these samples could be preserved until the limited lab access was lifted.) With the exception of the reservoir between June and August, algal numbers demonstrated similar trends across all sampling points (Figure 4.22) with no statistically significant differences being observed. Lower levels of algae were observed during the winter months which is to be expected as temperatures are lower and less sunlight is experienced. The spring month displayed a rise in levels which corresponded with the increase in light and temperature. However, a drop in algal numbers were observed just prior to the first COVID-19 national lockdown that occurred in March 2020. This drop was most likely due to excessive levels of rain fall experienced during the month of February. This weather change and its impacts are further discussed in Chapter 5 which focuses on climate change. Algae numbers consistently remained between $1x10^5$ and $5x10^5$ cells mL^{-1} after the lockdown period until the end of the study. This suggested that stabilisation had occurred. Moderately strong correlationships were observed with most of the nitrogen nutrients (See Table A4.40 – Table A4.47 in Appendix 4) indicating that NH_4^+, NO_2^- and NO_3^- play a vital role in maintaining optimum algae levels in the novel IMTA process. Results also found that the higher and more stable the levels of NO_3^- present, the more stable the algae numbers. Given that NO_3^- is algae's preferred form of nutrient, and NH_4^+ and NO_2^- are necessary for the natural production of NO_3^- via the nitrification process, this result was expected.

Cyanobacteria levels were also monitored in parallel to the algal numbers, as shown in Figure 4.23. Although many species of cyanobacteria can provide beneficial elements (*e.g., Spirulina*) the presence of increased levels of cyanobacteria was found to have a negative impact on the novel IMTA system. Increased incidences of mortality were observed as cyanobacterial levels rose. Much like with freshwater bodies, cyanobacteria numbers were always present in the system. They remained below the level of algae being reported highlighting algae's ability to control cyanobacteria levels as both are competing for the nitrogen nutrient source. However, cyanobacteria levels were found to increase just before the lockdown period, demonstrating an inverse relationship with the algae. This suggested that the cyanobacteria were out competing the algae for nutrients. Again, this coincided with extreme weather conditions and is also further discussed in chapter 5. Once levels stabilised after the lockdown

period, they were once again consistently below the level of algae, remaining between $1x10^4$ and $1x10^5$ cells mL^{-1}. Reduction in mortality levels also coincided with the stabilisation of cyanobacteria levels.

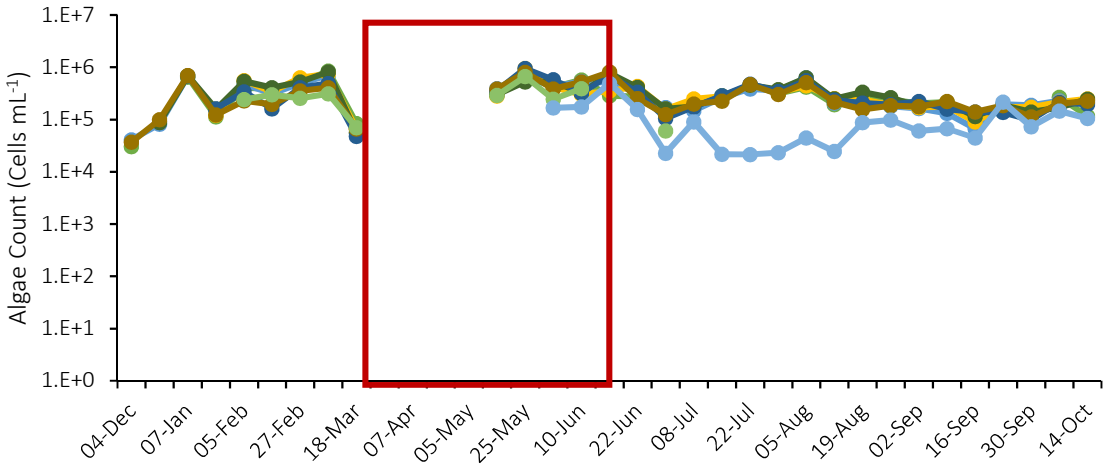


Figure 4.22: **Algae levels established from all sampling points at Oasis fish farm between December 2019 and October 2020**. The red box indicates when the COVID-19 lockdown period. Enumeration was conducted via FCM.

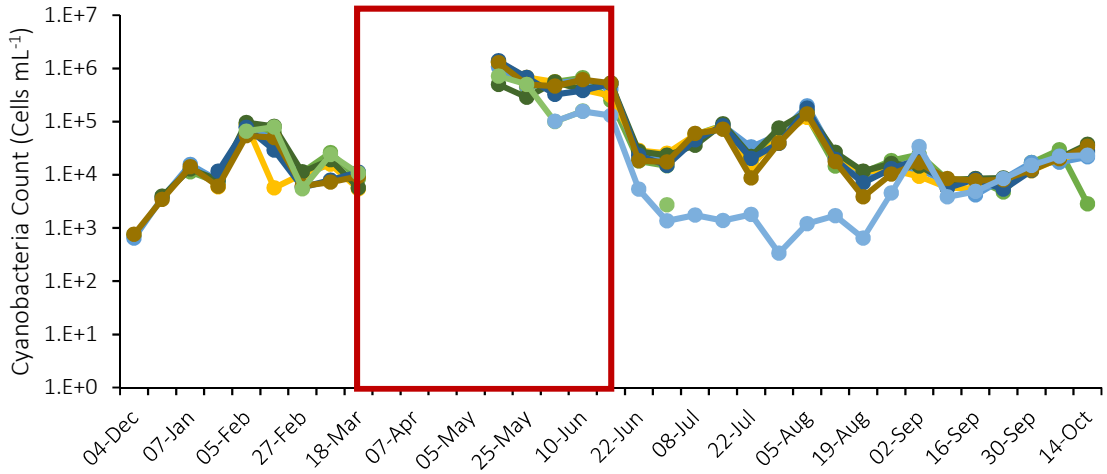


Figure 4.23: **Cyanobacteria levels established from all sampling points at Oasis fish farm between December 2019 and October 2020**. The red box indicates when the COVID-19 lockdown period. Enumeration was conducted via FCM

Correlation studies were conducted between the algae and cyanobacterial levels and all of the physicochemical parameters. See Table A4.40 to Table A4.47 in Appendix 4. In addition to a correlation observed between the algae and the cyanobacteria themselves, correlations were observed between both counts and a range of physicochemical parameters. A correlation was observed with the N parameters (NH_4^+, NO_2^- and NO_3^-) as well as with the P (PO_4^{3-}). As N and P are both necessary for algal growth this was expected. It also highlighted the need to ensure these nutrients were present in the

system to ensure the continued presence of algae that was necessary for the novel IMTA process to be effective. The correlation between the pH and algae was also expected as it is well known that although algae can tolerate small fluctuations in pH, increased and more frequent fluctuations can slow down growth rates (Dubinsky and Rotem, 1974).

In addition to establishing the levels of algae present in the novel system via FCM enumeration, identification also needed to be conducted in order to determine what species were present and were these species potentially hazardous to the system and the fish. Partial speciation was first conducted on all samples collected from Oasis. As similar findings were observed at all of the sampling points, results were grouped together as a whole. Approximately twenty genus of algae were identified using microscopy and classic identification keys (see Table 4.2 and Figure 4.24 to Figure 4.28).

Microscopic analysis indicated that at least four genus had multiple species present. However, due to the similarities and complexities of the algae, an indication of whether multiple species were present for all genus could not be established with this method. Additionally, similarities also prevented the determination of full speciation via microscopy. The most common type of algae present was green algae with a minimum of twelve genus identified. The majority of these genus have not known to cause any adverse effects on their ecosystems. These included *Scenedesmus, Monoraphidium, Micractinium, Chlorella, Chlamydomonas, Pediastrum, Dictyosphaerium, Closterium, Actinastrum,* and *Ankistrodesmus.* However, two species have been previously found to contribute to adverse effects. Some *Pandorina* species have been known to excrete toxic compounds that inhibit the growth of other algae and higher plant life (Patterson and Harris, 2007) whilst some species of *Oocystis* are well known to cause HAB's (Pal *et al.*, 2020). With the exception of *Peridinium*, which is also well known to be an instigator of HAB's (Hallegraeff *et al*., 2004; Ki and Han, 2007), the rest of the genus identified (*Cyclotella, Euglena, Mallomonas, Nitzschia, Rhodomonas, Stephanodiscus* and *Tabellaria*) in the samples are not known to cause negative effects on their environment. Although *Pandorina* can exhibit potentially negative effects by inhibiting beneficial algae and plants, it has been reported to be an inhibitor of *Peridinium,* making its presence in the system potentially advantageous (Patterson and Harris, 2007). This highlighted further that additional research needs to be conducted on the novel system.

Table 4.2: **Breakdown** of the **genus of algae** easily identifiable under microscopic examination. Breakdown includes all sampling points collected from **Oasis** fish farm between **December 2019 and October 2020**. The genus name, the individual algal groups to which they belong, the month the presence was recorded and the identification of multiple species have been included.

Genus Identified	Algae Group	Month Present	Multiple Species
Actinastrum	Green Alga	6 – 10	Unknown
Ankistrodesmus	Green Alga	3 - 9	Unknown
Chlamydomonas	Green Alga	1 – 4, 7 – 11	Unknown
Chlorella	Green Alga	1 – 11	Yes
Closterium	Green Alga	1 – 11	Unknown
Cyclotella	Diatom	1 – 4	Unknown
Dictyosphaerium	Green Alga	1 – 4, 6	Unknown
Euglena	'Naked' alga	3	Unknown
Mallomonas	Diatom	7	Unknown
Micractinium	Green Alga	1 – 11	Unknown
Monoraphidium	Green Alga	1 – 11	Yes
Nitzschia	Diatom	1 – 11	Yes
Oocystis	Green Alga	3 – 4, 6 – 8	Unknown
Pandorina	Green Alga	2 – 4, 6 – 8	Unknown
Pediastrum	Green Alga	2, 7 – 8, 10	Unknown
Peridinium	Yellow-Brown Alga	1 – 4, 6 – 7, 10	Unknown
Rhodomonas	Nearly Brown Alga	1 – 4, 6 - 8	Unknown
Scenedesmus	Green Alga	1 - 11	Yes
Stephanodiscus	Diatom	1 – 10	Unknown
Tabellaria	Diatom	6 – 7, 10	Unknown

1 = Dec, 2 = Jan, 3 = Feb, 4 = Mar, 5 = Apr, 6 = May, 7 = Jun, 8 = Jul, 9 = Aug, 10 = Sep & 11 = Oct.

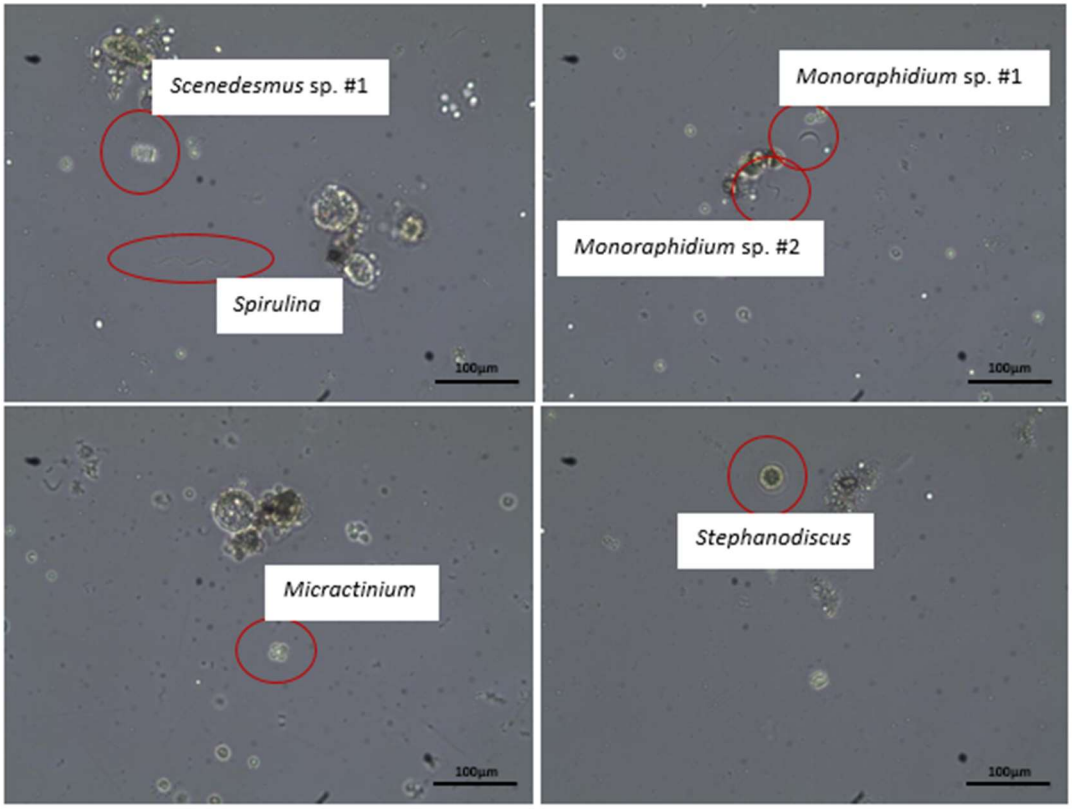


Figure 4.24: Imagery of the genus ***Scenedesmus***, ***Spirulina***, ***Micractinium***, ***Monoraphidium*** and ***Stephanodiscus*** identified in Oasis fish farm over the sampling period of December 2019 to October 2020.

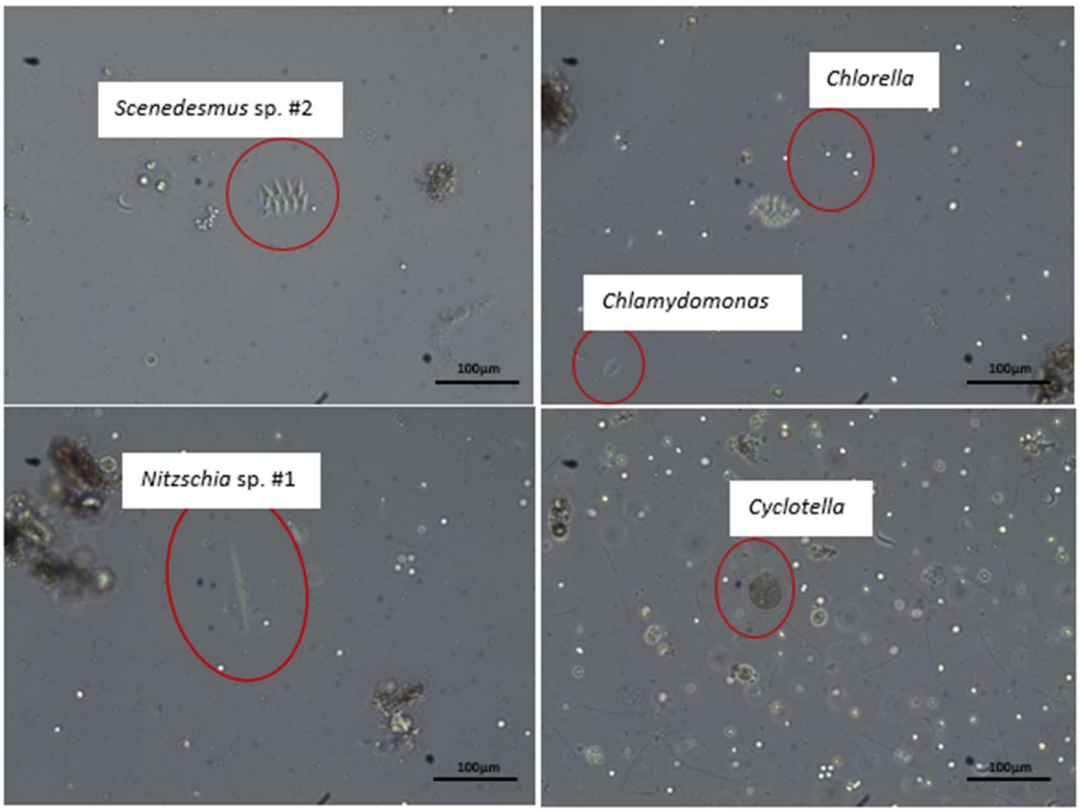


Figure 4.25: Imagery of the genus ***Scenedesmus***, ***Nitzschia***, ***Chlorella***, ***Chlamydomonas*** and ***Cyclotella*** identified in Oasis fish farm over the sampling period of December 2019 to October 2020.

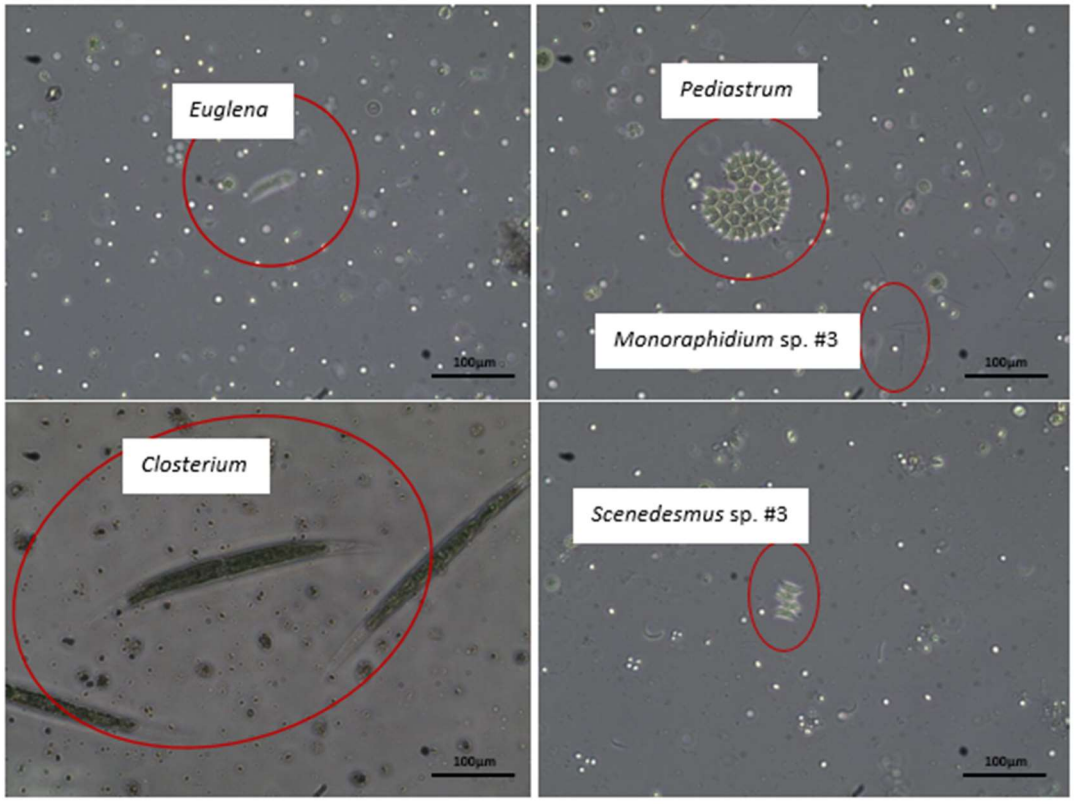


Figure 4.26: Imagery of the genus ***Euglena, Closterium***, ***Pediastrum***, ***Monoraphidium*** and ***Scenedesmus*** identified in Oasis fish farm over the sampling period of December 2019 to October 2020.

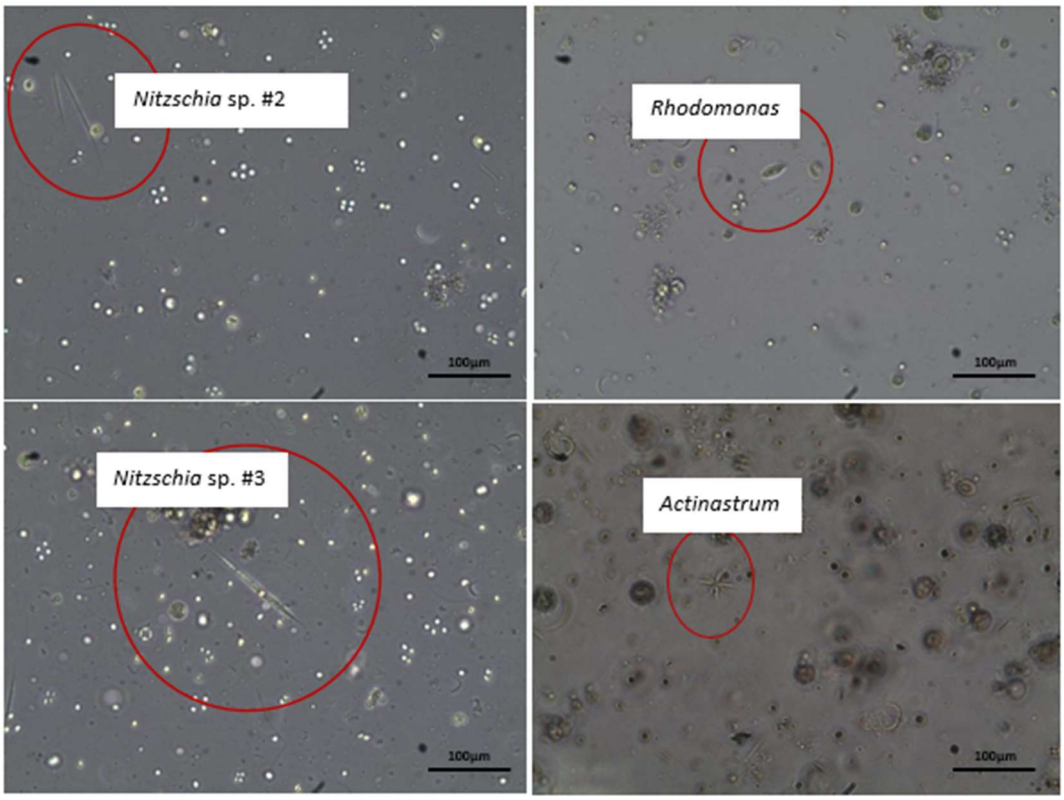


Figure 4.27: Imagery of the genus ***Nitzschia***, ***Rhodomonas***, and ***Actinastrum*** identified in Oasis fish farm over the sampling period of December 2019 to October 2020.

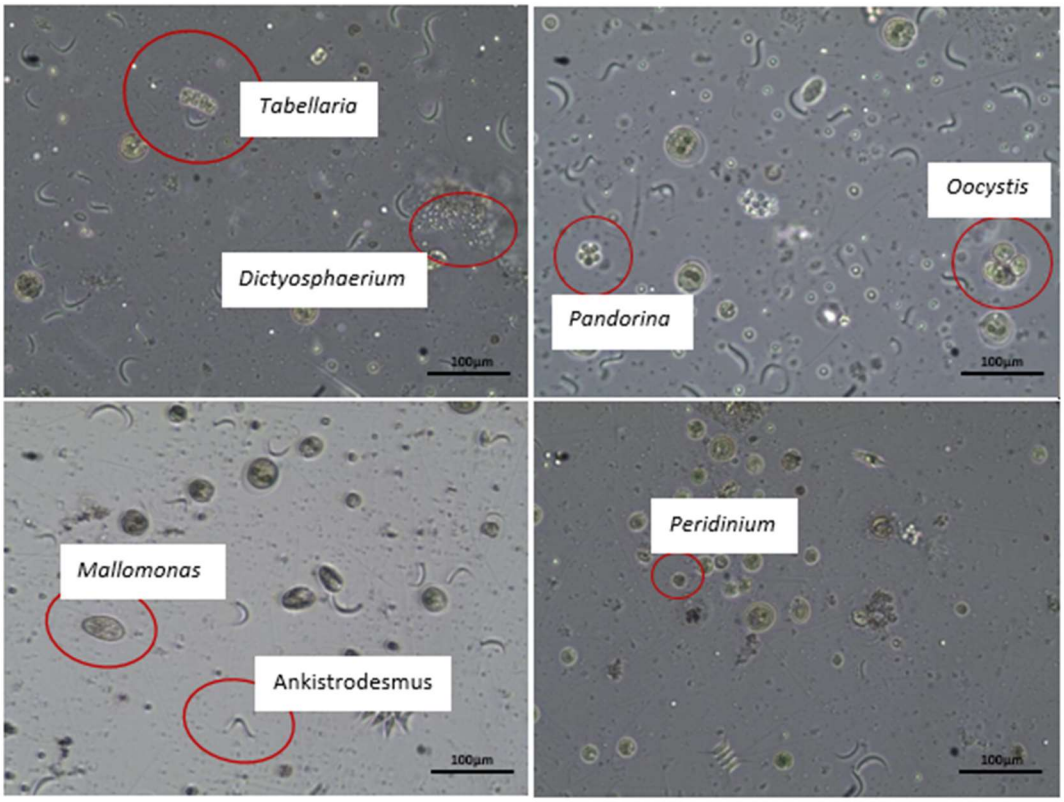


Figure 4.28: Imagery of the genus ***Tabellaria, Dictyosphaerium, Pandorina***, ***Oocystis***, ***Mallomonas***, ***Ankistrodesmus*** and ***Peridinium*** identified in Oasis fish farm over the sampling period of December 2019 to October 2020.

After partial speciation was conducted, DNA sequencing was then looked at in order to identify the species present in the novel IMTA system. This was done as many algae observed under the microscope could not be accurately identified *i.e.,* based on many of the morphological characteristics, several potential genus could be attached to the same algae. As time and resources were limited only one set of DNA samples could be sent for analysis. As the month of May provided the greatest variation based on microscopic examination, it was chosen to be sent for sequencing to Macrogen Bioinformatic Analysis. Price was the ultimate factor in deciding this company. After duplicates were removed 1864 species of algae were identified indicating an even greater range of diversity within the system. Of that 1864; 1551 species or sub-species of algae were identified across 210 genus, 60 were identified on family as opposed to genus or species, 42 were classified as uncultured species which indicates that the DNA had been entered into the database however complete identification has not been conducted as yet, and 31 “species like” algae. A phylogenetic tree was constructed using the phyloT V2 online software. This program was chosen because it generates the phylogenetic tree based on the NCBI taxonomy and BLAST database originally used to identify the DNA sequences. Inclusion of the phylogenetic tree would require the addition of a minimum of 50 pages and >100 pages to be legible. Therefore, given the sheer scale of species identified, the phylogenetic tree has been permanently placed online and a QR code generated for its access using the QRTY_MOBI online software (Figure 4.29). This method of presentation was opted for as it was deemed the environmentally friendliest. Additionally, the phylogenetic tree can also be used as a repository for future analysis.

Link to online document:
https://qrty.io/7IU9Sq

Figure 4.29: **QR Code** for the **phylogenetic tree** generated from **all algal species identified** in the novel peatland IMTA process during the month of May 2020 in Oasis.

A breakdown of the genus identified across both identification methods have been included in Table 4.3 and Table 4.4. Table 4.4 displays the 210 genus that were identified during the month of May. See the QR code in Figure 4.29 for a more detailed breakdown of the genus identified. Of the twenty that were identified during the microscopic analysis only four were not included; *Euglena, Closterium, Tabellaria* and *Pandorina* (Table 4.3). However, these four genera were not identified during microscopic analysis either. The presence of the remaining sixteen genus were confirmed after the DNA sequencing analysis. These genera that were identified using microscopic analysis provided the greatest variation of species. This is most likely why these sixteen genera were most prominent during the microscopic examination. A species count for these algae have been included in Table 4.3. *Chlorella* provided the greatest level of variation with 240 species identified, followed by *Scenedesmus* at 107 species. However, 87 additional species across four genera with very similar morphologically to *Scenedesmus* were also identified; *Acutodesmus, Desmodesmus, Pectinodesmus* and *Tetradesmus*. Figure 4.30 and Figure 4.31 demonstrate the vast complexity and similarities among the *Scenedesmus* genus itself as well as the four similar "*desmus*" genera. This highlights the high level of similarities between some species making identification with microscopy next to impossible to accurately conduct.

Table 4.3: **Species count** for the **sixteen genera identified** using microscopic examination of the Oasis fish farm samples collected in May 2020 that were later confirmed using DNA sequencing.

Genus Identified	Number of Species Identified
Actinastrum	2
Ankistrodesmus	24
Chlanydomonas	3
Chlorella	240
Cyclotella	22
Dictyosphaerium	57
Mallomonas	30
Micractinium	50
Monoraphidium	49
Nitzschia	3
Oocystis	15
Pediastrum	13
Peridinium	2
Rhodomonas	13
Scenedesmus	107 & 87 species across 4 related genera
Stephanodiscus	14

Table 4.4: List of all **genus identified** in the Oasis fish farm novel peatland IMTA process during the month of May 2020 using **DNA sequencing**. Colour designation indicates which class of algae each genus belongs to. Bold indicates all genera identified using both microscopinc and DNA analysis

Adriamonas	Roundia	Tessellaria	Densicystis	Tetradesmus	Planktosphaeria
Filos	Shionodiscus	**Mallomonas**	Droopiella	Pectodictyon	Schizochlamys
Paramonas	Thalassiosira	Synura	Echinocoleum	Pseudospongiococcum	Pseudomuriella
Siluania	Cyclostephanos	Heterogloea	Eremosphaera	Scotiellopsis	Pseudoschroederia
Bicosoeca	**Cyclotella**	Poterioochromonas	Euchlorocystis	Tetranephris	Tetraedron
Caecitellus	Discostella	**Actinastrum**	Franceia	Westella	Tumidella
Incisomonas	Lindavia	**Chlorella**	Granulocystis	Follicularia	Bracteacoccus
Cryptomonas	Planktoniella	Compactochlorella	Lagerheimia	Radiococcus	**Chlamydomonas**
Hemiselmis	Stephanopyxis	Carolibrandtia	Neglectella	Sphaerochloris	Ettlia
Katablepharis	Grammatophora	Chloroidium	Nephrocytium	**Ankistrodesmus**	Heterotetracystis
Leucocryptos	Hyalosynedra	Closteriopsis	Oocystella	Chlorolobion	Halochlorella
Baffinella	Pseudostaurosira	Dicloster	Oocystidium	Curvastrum	Chlorosarcinopsis
Chroomonas	Plagiogrammopsis	**Dictyosphaerium**	**Oocystis**	Drepanochloris	Tetraspora
Falcomonas	Trieres	Didymogenes	Planctonema	Kirchneriella	Thalassiosira
Komma	Apoikia	Graesiella	Planctonemopsis	Messastrum	Triposolenia
Geminigera	Chromophyton	Hegewaldia	Siderocystopsis	**Monoraphidium**	Apocalathium
Proteomonas	Hydrurus	Heynigia	Tetrachlorella	Nephrochlamys	Scrippsiella
Pyrenomonas	Chromulina	Hindakia	Elliptochloris	Ourococcus	Stoeckeria
Teleaulax	Ochromonas	Jaagichlorella	Parietochloris	Podohedriella	Azadinium
Rhodomonas	Pedospumella	Kalenjinia	Acutodesmus	Quadrigula	Chimonodinium
Storeatula	Spumella	Lewiniosphaera	Asterarcys	Raphidocelis	Heterocapsa
Goniomonas	Uroglena	Lobosphaeropsis	Chodatodesmus	Rhombocystis	Peridiniopsis
Hemiarma	Uroglenopsis	Marasphaerium	Coelastrella	Selenastrum	**Peridinium**
Achnanthidium	Urostipulosphaera	Marvania	Coelastropsis	Chlorotetraedron	Unruhdinium
Fragilariopsis	Chrysolepidomonas	Masaia	Coelastrum	Neochloris	Exuviaella
Nitzschia	Dinobryon	Meyerella	Comasiella	Polyedriopsis	Prorocentrum
Pseudo-nitzschia	Epipyxis	**Micractinium**	Coronastrum	Chromochloris	Gyrodinium
Bacterosira	Kephyrion	Mucidosphaerium	Crucigenia	Hydrodictyon	Karenia
Minidiscus	Poteriospumella	Muriella	Desmodesmus	Lacunastrum	Karlodinium
Stephanocyclus	Paraphysomonas	Planktochlorella	Didymocystis	Monactinus	Shimiella
Stephanodiscus	Chrysosaccus	Pleurastrosarcina	Enallax	Parapediastrum	Takayama
Chaetoceros	Chrysosphaera	Pseudochlorella	Hariotina	**Pediastrum**	Ptychodiscus
Navicula	Roombia	Quadricoccopsis	Neodesmus	Pseudopediastrum	Oxyrrhis
Conticribra	Undaria	Quadricoccus	Pectinodesmus	Sorastrum	Torodinium
Detonula	Chrysosphaerella	Amphikrikos	**Scenedesmus**	Stauridium	Kumanoa

Cryptophyceae, Bacillariophyceae, Chrysophyceae, Xanthophyceae, Chlorophyceae, Dinophyceae, Rhodophyceae.

Figure 4.30: Examples of the **morphological similarities** between **different species of *Scenedesmus*.** Source: Algal Base

Figure 4.31: Examples of the **morphological similarities between *Scenedesmus* and** the similar species identified in the Oasis IMTA system**.** A) **Acutodesmus,** B) **Desmodesmus,** C) **Pectinodesmus** and D) **Tetradesmus** Source: Algal Base

Results determined during the characterisation study of the novel peatland IMTA system were published in the technical report generated as part of the AquaAlgaePlus project for BIM, entitled "Investigation to elucidate role and relationship between algal and microbial communities in freshwater aquaculture". See Appendix 1.

4.4. ISSUES ENCOUNTERED

As it was deemed best to conduct the pilot study during times when the farm was stocked, only a four-month monitoring period could be conducted. However, despite its length, the study was accepted for publication in the Science of the Total Environment journal.

COVID-19 had a major impact on the characterisation study conducted in Oasis from December 2019 to October 2020. Due to the abrupt introduction of the first lockdown period, all sampling and subsequent analysis had to be halted for approximately six weeks. However, when restrictions eased in mid-May, samples were collected, preserved and stored until access to the laboratory was allowed. This was done in order to minimise the gap in data. Issues were also encountered during the lockdown period when increases in cyanobacteria and mortalities were observed. In order to help combat this continuous contact via email and telephone in conjunction with extensive research of available papers provided a means to reduce the negative impact these issues induced.

Changes in weather conditions had knock on effects within the farm. However, these issues provided insight into the impact climate change may have on aquaculture, as discussed in chapter 5.

4.5. CONCLUSION

The pilot study highlighted that microalgae can be exploited in this IMTA process for efficient assimilation of nutrients along with remediation and wastewater treatment (Han *et al.*, 2019). In addition to treating wastewater, microalgae could synthesize value-added components such as proteins, lipids and natural pigments for fish nutrition and disease mitigation, along with providing a high capacity for generating oxygen that could act like a bio-pump for aeration of aquaculture and positively adjust microbial communities (Han *et al*., 2019). Water deterioration in aquaculture is typically attributed to an excessive amount of aquaculture feed and wastes excreted by the consuming fish that are stocked to high density. However, the impact from the surrounding peatlands will also need to be taken into consideration. Nitrogen is one of the compositions of this waste and a high

concentration of ammonium in a water body can be toxic to fish. Common forms of nitrogen in wastewater include ammonium (NH_4^+-N), nitrate (NO_3^{--}-N) and nitrite (NO_2^--N). Ammonium can be absorbed by microalgae cells through active transport and directly utilized for amino acid synthesise, while nitrate and nitrite absorbed by microalgae through active transport have to be converted to ammonium by nitrate reductase and nitrite reductase before undergoing further assimilation (Han *et al.*, 2019; Sanz-Luque *et al.*, 2015). Exploiting this IMTA aquaculture process may also accelerate CO_2 fixation and promote oxygen release for farmed fish. However, future research that inform parameters of the C/N ratio, light intensity and quality, and carbon forms would be relevant in order to enhance carbon assimilation, further promoting nitrogen assimilation (Han *et al.,* 2019).

The analysis within Oasis fish farm was primarily conducted in order to be used as a tool to assist in the development and management of the novel system. A baseline of the physicochemical parameters and algal / cyanobacterial levels at different points of the year have been generated. However, the characterisation of the novel peatland IMTA demonstrated that the system is highly complex and will require additional research. For example; the N:P has suggested that the algal levels appear to improve the greater that ratio. However, the ratio of algae to cyanobacteria also influences N:P. Although this research has provided a much-needed leap forward in the sustainable development of the Irish aquaculture industry, much more still needs to be considered and researched in order for the industry to be capable of fully harnessing the potential of peatland bog aquaculture. See the additional research section in Chapter six for a more in-depth breakdown.

CHAPTER 5

FUTURE ISSUES IN THE IRISH FRESHWATER AQUACULTURE INDUSTRY

5.1 INTRODUCTION

"There's one issue that will define the contours of this century more dramatically than any other, and that is the urgent threat of a changing climate. Climate change is no longer some far-off problem; it is happening here, it is happening now" (Obama, 2015). Climate change is considered to be one of the most troubling, challenging and unrelenting scientific issues of our time (Bulkeley and Newell, 2015). Climate change, including extreme weather variances, is a complicated and increasingly problematic challenge leading to changes in rainfall and hydrology *e.g.,* extensive summer droughts caused by changes in rainfall (Paerl *et al.*, 2016; Paerl and Scott, 2010). In the last four years, Ireland has experienced these extreme weather variances first hand. According to Met Éireann, the Irish Meteorological Service, due to Ireland's proximity to the Atlantic Ocean it does not traditionally experience extreme temperatures when compared to other countries at a similar latitude. Additionally, snowfall in Ireland is infrequent and irregular. During occurrences, which usually only results in a maximum of several mm of snowfall, snow only tends to last on the ground for a day or two (Met Éireann, 2021). However, in 2018 both of these common facts about Ireland's weather and climate were contradicted. In March/April 2018, Ireland was subjected to what the media dubbed as "The Beast from the East" which saw Ireland covered in the largest level of snowfall it had seen in recent memory. Then, just a few short weeks later Ireland entered a sixteen week long, unbroken period of drought, which is unheard of in Ireland. The hottest recorded temperatures every reported in Ireland were also observed during this period. Finally, one of the most common features in Irish weather is its rainfall levels. However, this has also been altered in recent years with significant increases being observed *e.g.*, in February 2020 rainfall levels were reported to be 2.5 times greater than the monthly average due to the country being hit by two storm systems within a week of each other (Met Éireann, 2021b). Although addressing current pressure points within the Irish freshwater aquaculture system (delays in licensing, issues with environmental concerns and spatial limitations), as discussed in the previous chapters, is of vital importance for the sustainable growth of the industry, it is also important to be mindful of future issues and their potential impacts. For example; the ever changing and erratic weather conditions experienced throughout this entire research process, where a whole array of weather variances were all observed, have demonstrated the potentially devastating effects climate change may have on the aquaculture process and the surrounding environment due

these unprecedented, unpredictable and extreme weather variances adding additional stressors to the aquaculture systems.

The COVID-19 global pandemic has had a devastating effect on every aspect of human life as we know it and its overall impact is still unknown. As of the end of May 2020, there have been 170M cases worldwide and 3.54M deaths. Social and economic challenges have led to a food security crisis. According to the WHO, tens of millions of people are at risk of falling into extreme poverty, whilst the level of undernourished people could potentially increase by 132M to 822M people worldwide (Chriscaden, 2020). In Ireland, as of the end of May 2021, there have been 255K cases and just under 5K deaths. Since its first occurrence in Ireland, the country has faced three separate extended lockdown periods (March 2020 to May 2020, October 2020 to December 2020 and the current lockdown period, which is still ongoing, began in January 2021). This resulted in restrictions observed in all walks of life, including this research. In the interest of national health, during these lockdown periods little to no access was available to the AIT laboratory facilities. Although this limited research, it also highlighted the need to ensure essential *in-situ* technologies present in the aquaculture facilities were providing as accurate information as possible to mitigate and prevent issues within the respective aquaculture systems when access to wet laboratory facilities may become unexpectedly and abruptly unavailable for extended periods of time.

5.2 FINDINGS

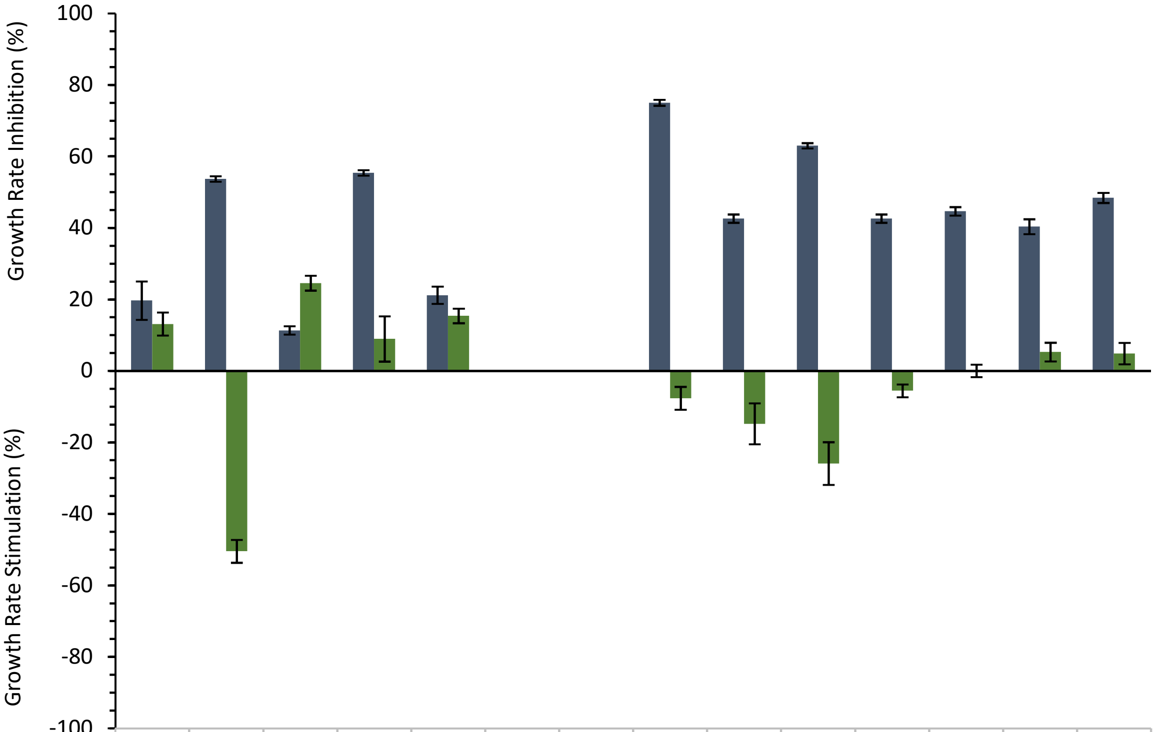
During this research process, algae demonstrated the potential to be used as an early warning indicator for highlighting issues associated with climate change, having experienced a range of different weather conditions including; a heat wave in 2018 where the highest recorded temperatures were observed throughout Ireland and subsequent drought conditions, and flooding conditions in 2020 where record levels of rain fell due to the development of a number of storm systems in close proximity to one another. Additionally, the global pandemic (COVID-19) in 2020 and 2021 resulted in limited to no access to wet-laboratory facilities for extended periods of time. This led to difficulties in monitoring algal and cyanobacterial levels which are a vital part of the novel peatland IMTA process at Oasis. The unforeseen and unavoidable circumstances demonstrated the importance of *in-situ* monitoring technologies such as the use of the Algae Torch® used to monitor cyanobacteria and chlorophyll levels in real time within the farm.

5.2.1 TRADITIONAL AQUACULTURE CASE STUDY- CLIMATE CHANGE

The observations of growth rate stimulation observed in the *P. subcapitata* algal bioassay during the pilot study in Keywater Fisheries from April 2018 to October 2018 (Figure 5.1), as previously reported and discussed in chapter three, led to a more in-depth analysis into its potential links to the uncommon weather conditions observed at the same time. Storm Emma, a snow storm which developed as a result of sudden stratospheric warming, hit Ireland from 28th February 2018 until 4th March 2018 and was known as "The Beast from the East". This storm system led to record levels of snowfall over several days leading to many parts of the country being "snowed in" for weeks *e.g.,* North Westmeath observed >9ft snow drifts that resulted it impassable roads for >10 days (Coleman *et al*., 2018). Then, just six weeks later just after the pilot study began in Keywater Fisheries, Ireland entered into a period of heat waves and drought conditions where the highest temperatures and driest summer ever were recorded in the >100 year record length (Met Éireann, 2018a).

Correlation studies were conducted (see Table A4.6 and Table A4.7 in Appendix 4) and a statistically significant, moderately strong negative correlationship was observed with the temperature ($r = -0.619$, $p = 0.032$) and suspended solids ($r = -0.727$, $p = 0.007$). In order to get a better understanding of these relationships and their links to the changing weather, Met Éireann metadata from the three closest weather stations (Markree, Knock and Mount Dillion) surrounding the farm were investigated (Figure 5.3). This data demonstrated increases in temperature and decreases in rainfall. Ireland's mean summer maxima temperature is traditionally between 18°C and 20°C (Met Éireann, 2018b; Walsh, 2012). In 2018, temperatures exceeded 30°C (Met Éireann, 2018b), as shown in Figure 5.2. An average total rainfall of only 61.9mm for the months of May, June and July 2018 compared to >88.9mm for the same three months in 2017 (Met Éireann, 2018c) as indicated in Figure 5.4, which subsequently led to a national hose pipe ban and water restrictions. Incidences of growth stimulation occurred in the farm just after the altered weather conditions were observed. The research suggested that the changes in temperatures that were observed during the heat wave and drought conditions experienced in the summer months of 2018 had a direct relationship with the increased levels of algal growth stimulation detected. With irregular weather patterns becoming more frequent, especially rises in mean temperatures, due to global warming, and changes to rainfall levels, further research into the effects of climate change on aquatic ecosystems, aquaculture output and the effects of output water on its receiving ecosystem needed to be conducted. Results observed during the drought conditions experienced in Keywater Fisheries during the pilot study of 2018 led to the publication of a research paper entitled "Novel use of the alga *Pseudokirchneriella subcapitata*, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish

farming as a case study" in the Science of the Total Environment Journal. See appendix 1 for a copy of the published paper.



	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	20-Sep	04-Oct
Intake	19.67	53.70	11.32	55.36	21.15			75.00	42.59	62.96	42.59	44.64	40.35	48.39
Output	13.11	-50.47	24.53	8.93	15.38			-7.69	-14.81	-25.93	-5.56	0.00	5.26	4.84

Figure 5.1: The **percentage growth rate inhibition** (dark blue), **and stimulation** (dark green), observed in ***P. subcapitata*** after exposure to the freshwater finfish aquaculture intake (dark grey) and output (light grey) water from Keywater Fisheries for 72 hours at 23^{o}C ± 2^{o}C under continuous illumination. Samples were collected and analysed from **April 2018 to October 2018** (n = 3, SD indicated).

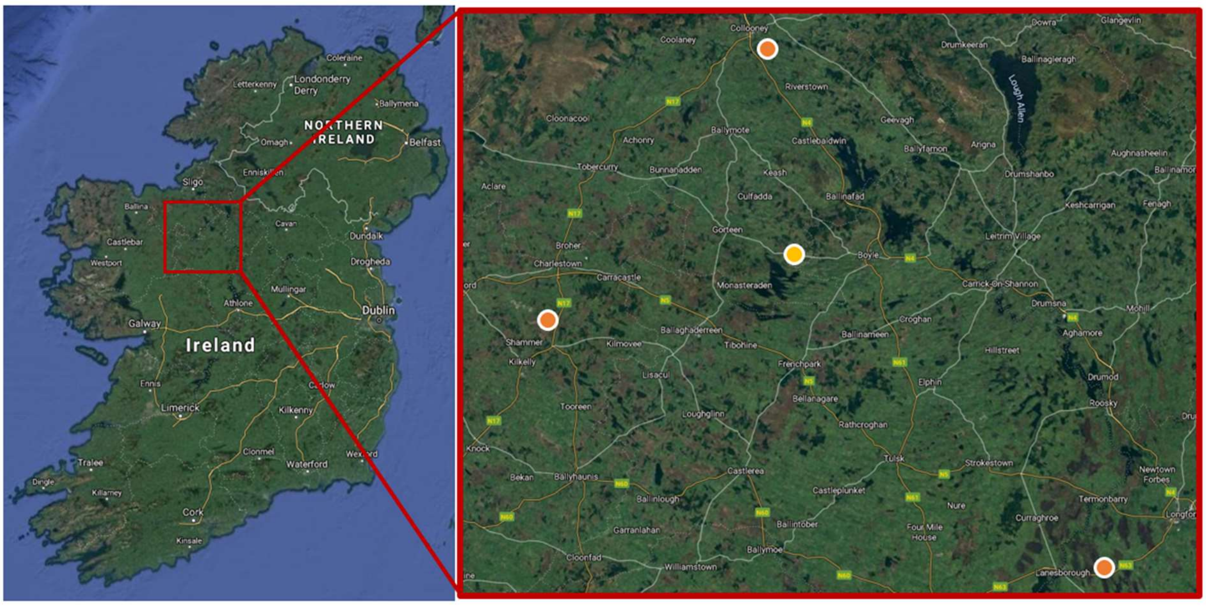


Figure 5.2: **Map of Ireland indicating** the approximate location of **Keywater Fisheries** (53^{o}58'16" N, 08^{o}24'44" W) indicated with yellow, and the three closest **Met Eireann weather stations** (Markree – 54^{o}10'30" N, 08^{o}27'20" W; Mount Dillon – 53^{o}43'37" N, 07^{o}58'51" W; Knock – 53^{o}54'22" N, 08^{o}49'4" W) surrounding the farm, indicated by orange.

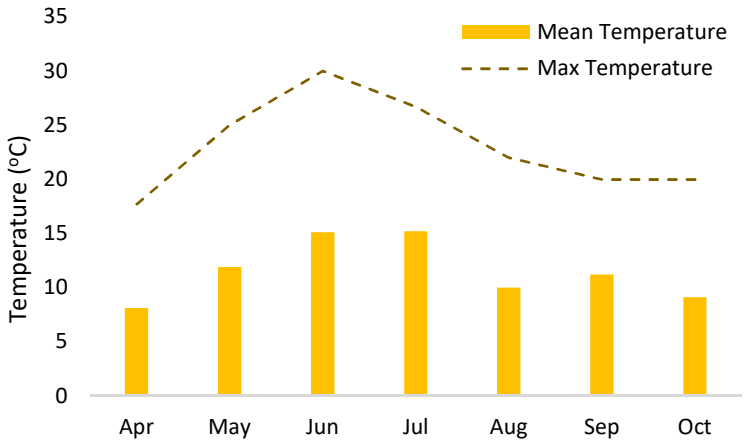


Figure 5.3: Mean temperatures (Bar Chart), and maximum temperatures (Line Chart), observed at three **Met Eireann weather stations** located closest to the fish farm, from April 2018 to October 2018. Data was based on all temperature readings collected at the three weather stations by Met Eireann.

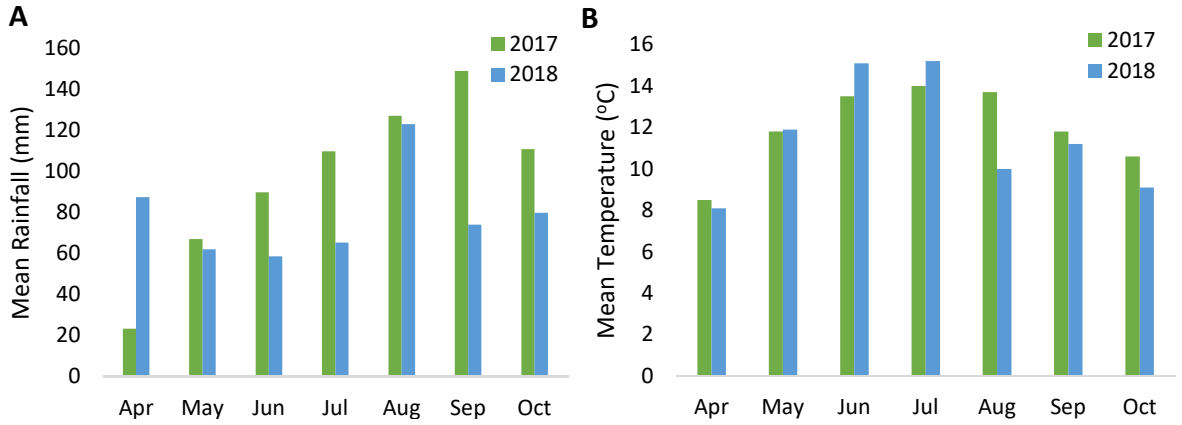


Figure 5.4: **Mean rainfall in mm** (A), **and temperature in °C** (B), collected from three **Met Eireann weather stations** located at; 1) Markree, Co. Sligo, 2) Knock, Co. Mayo, and 3) Mount Dillon, Co Roscommon. These stations were selected as they were located to the north-west, south-west and south-east of the fish farm investigated in this study. Data from April to October in 2017(green), and 2018 (blue), were examined.

The analysis of weather patterns were then conducted during the monitoring program that was ran from March 2019 to August 2019, as was previously discussed in chapter three. During 2019, Ireland experienced what was considered as normal or traditional weather conditions (Met Éireann, 2021a, 2019; Walsh, 2012). This provided an opportunity to develop a better understanding of the cause and effects the previous year's heat wave and drought conditions had on the farm, as well as greater evidence that climate change is and will continue to have an impact on freshwater aquaculture in Ireland.

In order to be as accurate as possible, only the data from April to August for both studies (pilot study of 2018 and monitoring program of 2019) were observed. The average and maximum temperatures (Figure 5.5 and Figure 5.6) and average rainfall levels (Figure 5.6) for the same period as that of the

drought were found to be in line with previous years *i.e.,* prior to 2018. Temperatures reached a maximum of just above 20°C in May 2019 and the average rainfall for the period of May to July 2019 was 84.3mm. A comparative study between the selected months for the pilot study and the monitoring program found that the only significant differences between both years existed with the algal results. A correlation study was performed once again and a moderately strong, negative correlationship between the algae and the temperature was observed in the intake water (r = -0.830, p =0.041) and a strong negative correlationship in the output water (r = -0.537, p = 0.042), both of which were statistically significant. This research suggested a potential toolbox that includes *P. subcapitata* may provide an early warning system for adverse effects as a result of climate change. These results led to the publication of the paper entitled "Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland" which was pubished in the Science fo the Total Environment journal.

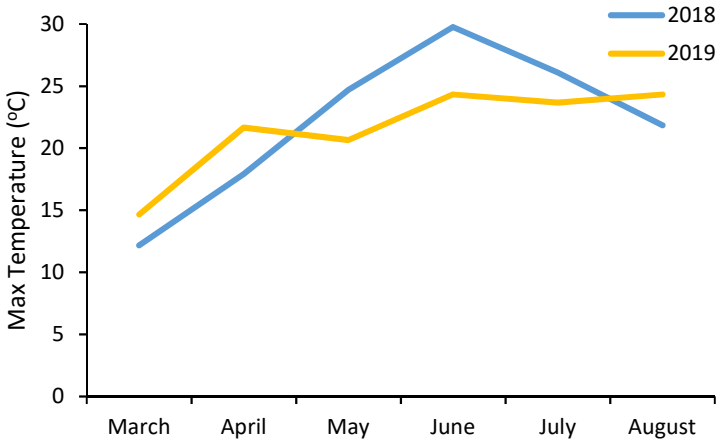


Figure 5.5: **Maximum temperatures** recorded for **2018** (blue) and **2019** (yellow) at three Met Eireann weather stations surrounding the freshwater fish farm during the sampling period of March 2019 to August 2019.

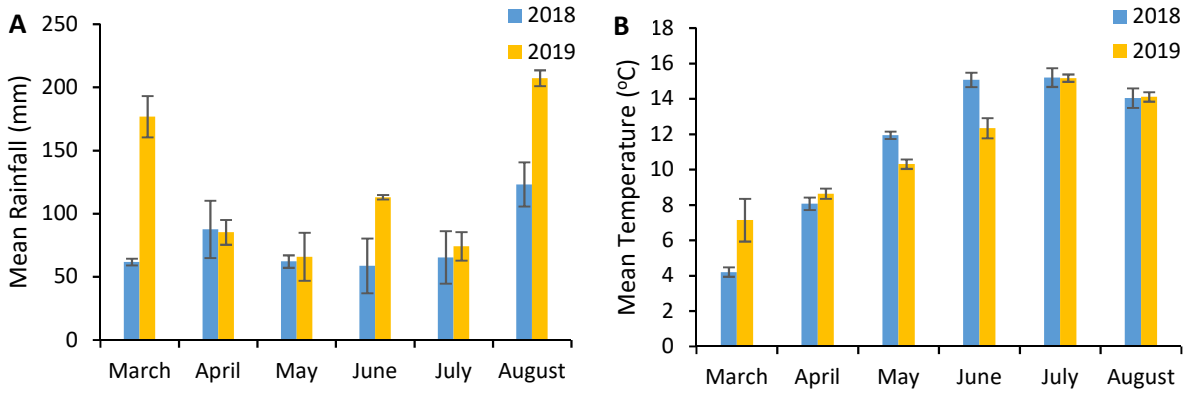


Figure 5.6: **Average** A) **rainfall** and B) **temperature** recorded for **2018** (blue) and **2019** (yellow) at three Met Eireann weather stations surrounding Keywater Fisheries during the sampling period of March 2019 to August 2019. Stations were located at 1) Markree, Co. Sligo, 2) Knock, Co. Mayo and 3) Mount Dillon, Co. Roscommon. Stations were located north-west, south-west and south-east of the fish farm, respectively.

5.2.2 *Novel Peatland IMTA Case Study – Climate Change*

February 2020 was one of the wettest on record. This high level of rainfall was as a result of two extratropical cyclone storms hitting Ireland in that month and in close proximity to one another. Storm Ciara (formed 7th February 2020, dissipated 16th February 2020) and Storm Dennis (formed 11th February 2020, dissipated 18th February) affected Ireland less than a week apart. Just after this weather event cyanobacteria levels began to rise within the farm, as shown in Figure 5.7, as well as fish mortalities (up to 44%) which had up until that point remained consistently low (˜3%). NOTE: Only the areas that were experiencing issues (culture ponds 1 and 4) have been included. Veterinary post-mortems found signs of hepatotoxicity (liver necrosis) and high instances of gill irritation. Unfortunately, the first lockdown period began in March 2020 and as a result no samples could be analysed after this point until May 2020. Continuous contact was kept with the Oasis team in order to be as of much help as possible. Literature searches were conducted remotely to find an appropriate action of reducing or removing the cyanobacterial levels without having additional consequences on the fish health. Both Iredale *et al*. (2012) and Rajabi *et al.* (2010) demonstrated successful cyanobacterial control and removal from freshwater bodies with the application of barley straw. This method was suggested and then subsequently applied to the culture ponds at which point the farm reported a reduction in mortalities and cyanobacterial levels.

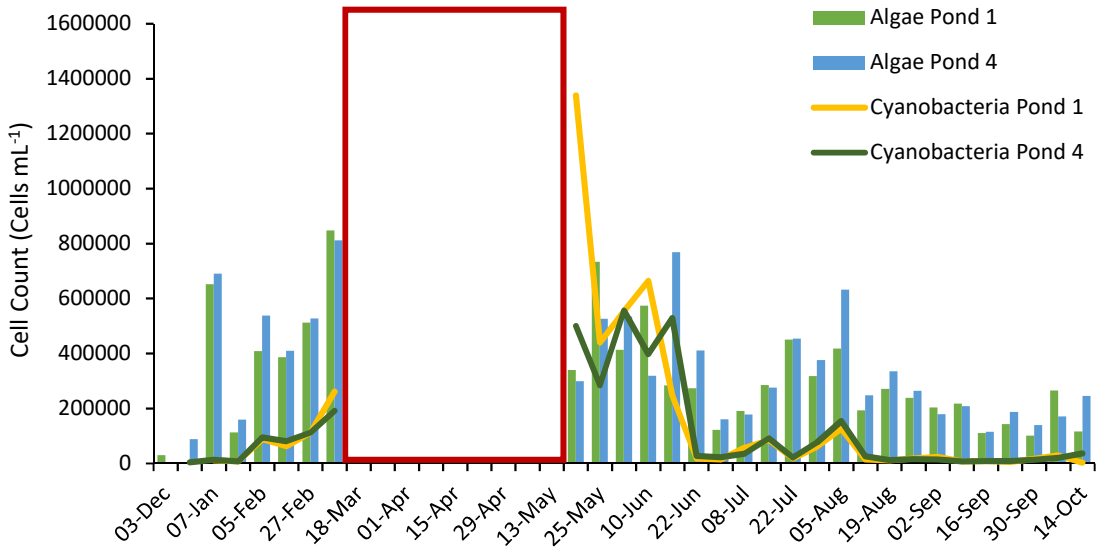


Figure 5.7: **Algae and cyanobacteria levels** recorded in **Oasis** fish farm from the problematic areas of the farm (**Pond 1 and Pond 4**) during the monitoring period of **December 2019 to October 2020**. Red box indicates when the first COVID-19 lockdown occurred and no samples could be analysed.

In order to determine whether the excessive levels of rainfall indirectly caused issues in February and the lack there of, caused issues in May, Met Éireann metadata was once again investigated. The three

closest weather stations surround Oasis fish farm at Mount Lucas were used (Mullingar, Co. Westmeath; Oak Park, Co. Carlow; and Gurteen, Co Tipperary), as indicated in Figure 5.8. The Irish midlands traditionally get an average of 70.3mm of rainfall for the month of February. However, according to Met Éireann, 197.7mm of rainfall fell for that month (Met Éireann, 2021b), as shown in Figure 5.9. This subsequently would have diluted down all nutrient levels and reduced algal / cyanobacterial numbers within the farm. As nutrients build back up, the ammonium, which is cyanobacteria's preferred source of nutrients, is used by the cyanobacteria before it has a chance to be converted to nitrate via the nitrification process (Herrero *et al.*, 2001) which is the algae's preferred form. This in turn allows the cyanobacteria to grow and out compete the algae. Normally the higher levels of algae and increased levels of nitrate control the levels of cyanobacteria. Leachate or run-off from the bog itself many also be contributing to the issues however no studies on this have been conduct to date. Cyanobacteria levels rose again in May with similar levels of mortality being observed again. However, there were no instances of rainfall during this instance (Figure 5.9). In fact, May 2020 was considered one of the driest in recent year's (Met Éireann, 2021b). No physicochemical analysis could be conducted at this time due to COVID-19 restrictions still being in place. Again, Veterinary post-mortems found signs of hepatotoxicity and gill irritation. The Barley straw had been removed from the ponds prior to this event and was therefore though to be the main cause for the problem. It may also be a seasonal event as cyanobacteria have been known to "bloom" during the spring and early summer months as temperatures increase. However, as this was the first study ever conducted on a peatland IMTA system additional research and analysis would need to be conducted.

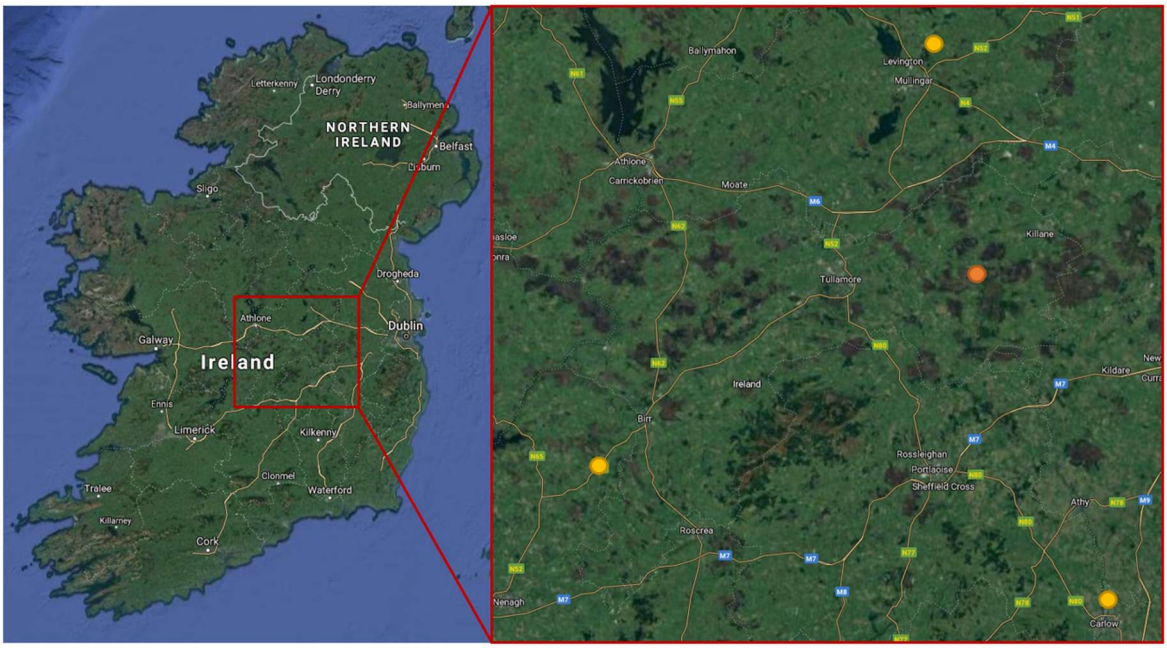


Figure 5.8: **Map of Ireland indicating** the approximate location of **Oasis fish farm** (53°17'03" N, 07°11'45" W) indicated with orange, and the three closest **Met Eireann weather stations** (Gurteen – 53°02'24" N, 08°00'36" W; Oak Park– 52°51'36" N, 06°55'36" W; Mullingar – 53°33'36" N, 07°20'24" W) surrounding the farm, indicated by yellow.

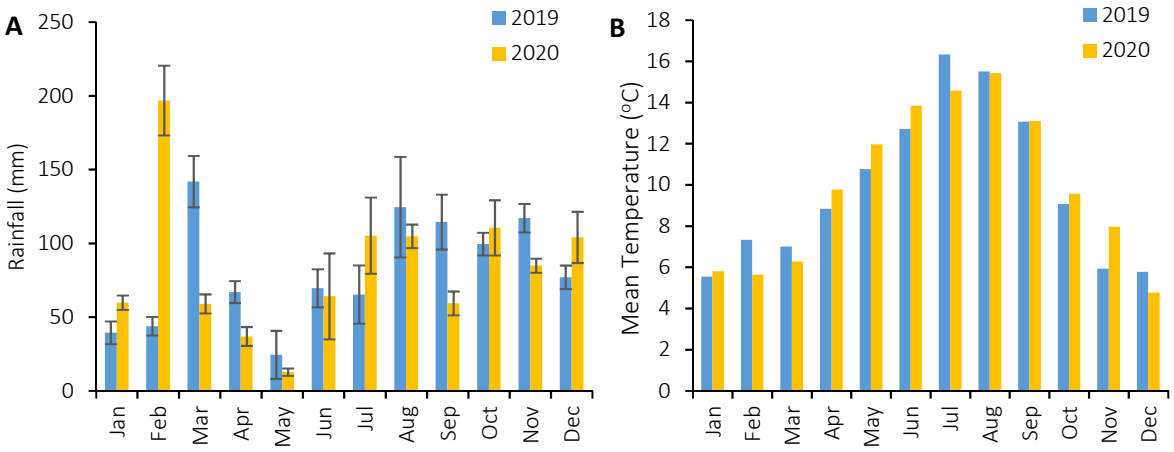


Figure 5.9: **Average** A) **rainfall** and B) **temperature** recorded for **2019** (blue) and **2020** (yellow) at three Met Eireann weather stations surrounding the Oasis fish farm during the sampling period of December 2019 to October 2020. Stations were located at 1) Mullingar, CO. Westmeath, 2) Oak Park, Co. Carlow and 3) Gurteen, Co Tipperary. Stations were located north, south-west and south-east of the fish farm, respectively.

5.2.3 NOVEL PEATLAND IMTA CASE STUDY - COVID-19

The restrictions set in place across Ireland during the COVID-19 lockdown period limiting any wet laboratory access and the coinciding issues observed within the Oasis fish farm demonstrated the assurances of reliable *in-situ* technologies needed for fish farmers. Especially as COVID-19 has also demonstrated that unforeseen and unpredictable challenges may happen at any moment.

Oasis fish farm use an AlgaeTorch® (Figure 5.10) to measure chlorophyll levels within the farm. The AlgaeTorch® is a hand-held measurement instrument designed for rapid deployment developed by bbe Moldaenke. Measurement is based on fluorescence and was developed in line with the Water Quality – Measurement of biochemical parameters-Spectrometric determination of the chlorophyll-a concentration (ISO 10260:1992) and the German standard methods for the examination of water, waste water and sludge; Test methods using water organisms (group L): Determination of chlorophyll a in surface water (DIN 38412-16). The AlgaeTorch® is used to determine algae content using fluorescence which is proportional to the chlorophyll present in microalgae and blue-green algae (cyanobacteria). As the fluorescence is measured in milliseconds, the results are generated in real-time (bbe Moldaenke, 2021).

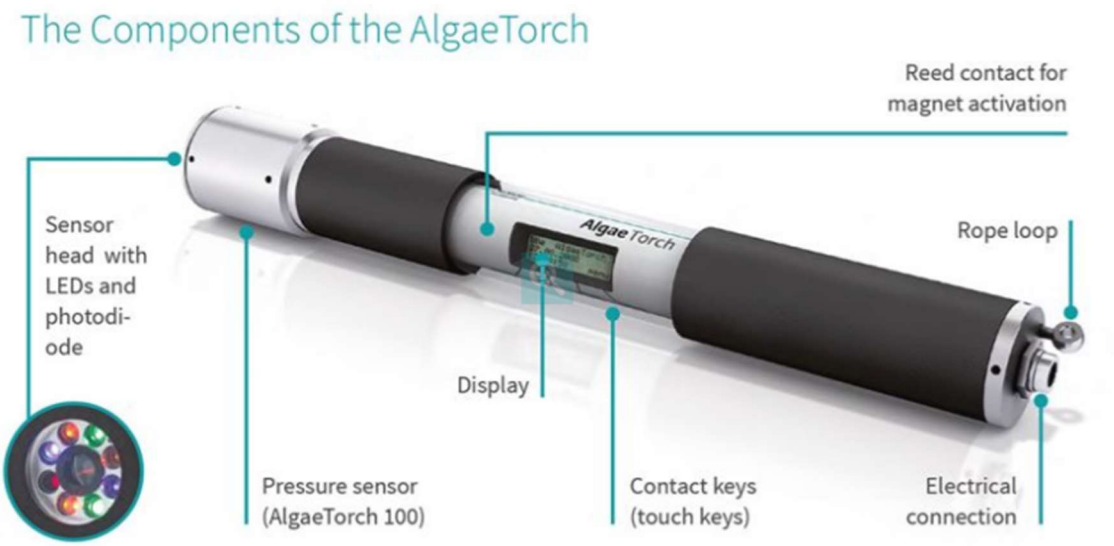


Figure 5.10: Schematic of the **AlgaeTorch®** which includes all of the main components of the device. Source: bbe Moldaenke.

Measurements from the AlgaeTorch® were compared directly to the algae counts generated from the FCM. This was conducted to confirm the accuracy of both the AlgaeTorch® and the Miltenyi Biotec MACSQuant® FCM. Only measurements that were taken from the same locations were included *i.e.,* AlgaeTorch® readings from all four of the culture ponds versus the culture pond FCM algal counts. The AlgaeTorch® recorded concentrations in µg L^{-1} whilst the FCM recorded cells mL^{-1}. As the AlgaeTorch® was used every day within the farm, only the days that sampling were conducted was used to ensure as accurate a comparison as possible. As no statistically significant differences were observed between the four culture pond readings for both instruments, results were averaged for ease of reporting, as shown in Figure 5.11. Similar trends were observed between both data sets. Correlation studies were then conducted (Figure 5.12). A strong positive correlationship was observed between both cyanobacteria data sets (r = 0.890) indicating that both instruments were accurately reporting on the cyanobacterial levels. A moderately strong positive correlationship was observed between the FCM algae count and the chlorophyll levels generated from the AlgaeTorch® (r = 0.570). The less strength in the correlationship is to be expected given that the AlgaeTorch® chlorophyll levels are a combined measurement of algae and cyanobacteria. However, this correlationship still indicated accuracy. The results have confirmed that both instruments are providing similar and accurate readings of algae and cyanobacteria levels within the farm thus providing assurances to the farmers should either instrument become unexpectedly unavailable (*e.g.*, should another lockdown prevent wet-lab analysis or should the AlgaeTorch® break down).

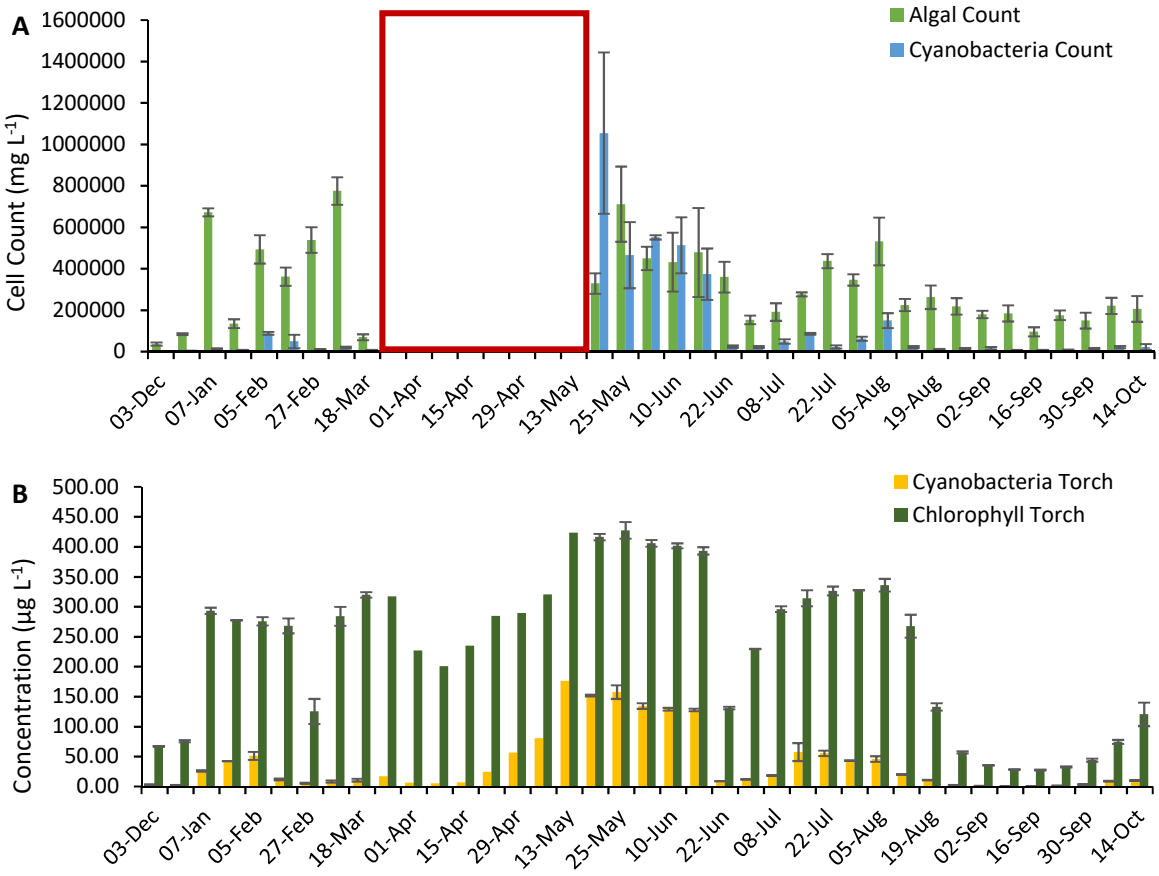


Figure 5.11: A) **algae and cyanobacteria cell counts** generated using **FCM** and B) **cyanobacteria and chlorophyll levels** generated using **AlgaeTorch®**. Data represents readings from all four culture ponds at **Oasis** fish farm from **December 2019 to October 2020**. Red box indicates the COVID-19 lockdown period when sampling could not be conducted.

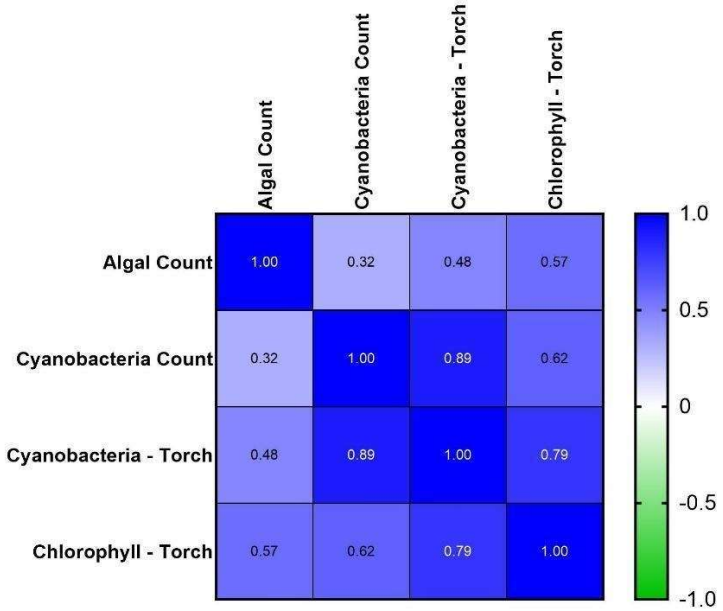


Figure 5.12: **Correlation matrix** established for **algal and cyanobacterial counts** determined using **FCM**, and **cyanobacterial and chlorophyll levels** detected using **AlgaeTorch®**. Blue indicates a positive relation (as one parameter rises, so too does the other parameter). Green indicates a negative/inverse relationship (as one parameter rises, the other parameter drops). 1.0 / -1.0 indicates a perfect linear relationship. 0 indicates no relationship. 0 to 0.5 / -0.5 indicates a low to moderate relationship. 0.5 / -0.5 to 1.0 / -1.0 indicates a moderate to strong relationship.

5.3 CONCLUSION

These findings have demonstrated the use of algae as an early warning indicator for issues associated with climate change in aquaculture. The need for additional research into climate change and its potential effects and impacts on the Irish aquaculture industry has also been demonstrated. This fact has been further highlighted by the new Agri-Food Strategy 2030, which is to be officially published later this year. The Agri-Food Strategy 2030 is the new initiative to be set out by the government of Ireland to update and replace the Food Wise 2025 strategy. Its official publication and subsequent application have been delayed as a result of the knock-on effects of COVID-19. However, its final draft form has recently been released into the public domain for public opinion. Once strong element within the document is the need for additional research into climate change and its effects and consequences on the Irish Agri-Food Industry (DAFM, 2021). This research has also led to the introduction of small weather stations within both of the fish farms making it easier for trends in weather patterns and changes in weather conditions to be monitored in the exact location required thus providing more accuracy. As this project was conducted during traditional and extreme weather conditions throughout the research, it provides the important advantage of being able to inform development and management during extreme weather variances. The tool developed in this research has also demonstrated its ability to adapt to climate change variances. As the new Agri-Food Strategy 2030 has highlighted much focus on climate change research, this work is ahead of the curve in meeting these new goals.

Findings have also provided reassurances on data being generated and fed back to the fish farms. By ensuring that the AlgaeTorch® and the FCS both are providing similar information, fish farmers will be more at ease knowing should unforeseen external challenges or issues within the aquaculture facility itself arise, their data generation and monitoring process will not be affected.

CHAPTER 6

CONCLUSION

6.1. MAIN FINDINGS & CONCLUSION

The three main core themes aud their respective objectives were successfully achieved. The first core theme focused on addressing issues surrounding environmental concerns and licencing delays in the freshwater Irish aquaculture industry. This was attained by analysing standardised algal species against the most common species found within Irish water and determining whether the standardised species, which are not commonly found in Ireland, was representative of the systems they would be applied to. The standardised species *P. subcapitata* was analysed against two of the most common species found in Irish freshwater bodies (*A. formosa* and *M. contortum*), where no statistically significant differences were observed demonstrating that despite its absence, *P. subcapitata* will still generate representative ecotoxicological effects in Irish systems. An ecotoxicological toolbox, which included the selected algal bioassays, was developed to provide a means to analyse and assess the water quality entering and exiting an aquaculture facility. Bioassays were run in parallel with the physicochemical parameters currently used to analyse water quality. A pilot study running from April 2018 to October 2018, and a monitoring program running from March 2019 to August 2019 were conducted on intake and output water in Keywater Fisheries traditional FTS to confirm the potential efficacy of the developed toolbox in providing an indication of both the current and future prediction of the condition of the water quality, and its environmental impacts. As environmental concerns are one of the main delay points in the licensing process, addressing this would speed up the process.

The second core theme sought to address issues with resource and spatial limitations within the Irish freshwater aquaculture industry. This was achieved by employing the developed ecotoxicological toolbox to determine the impact aquaculture may have on peatland environments. A short pilot study was conducted in Oasis fish farm between May 2019 and August 2019. The ecotoxicological toolbox, consisting of the bioassays and physicochemical parameters, were applied to the intake and output water. This toolbox not only demonstrated the unlikelihood of the novel IMTA process to induce adverse effect on the surrounding peatland but also demonstrated that the toolbox is applicable to both traditional and novel aquaculture processes. Then the role of algae in a novel, first of its kind, IMTA aquaculture process was investigated in order to inform its management and sustainable production efficiency. As this was an entirely new process, characterisation needed to be conducted. The physicochemical parameters were assessed from a variety of sampling locations within the farm

between December 2019 and October 2020 to determine a baseline for the water quality. Algae was incorporated into the novel system as the main source for wastewater assimilation therefore, monitoring of algal and cyanobacteria numbers for a baseline was also required. This was established alongside the physicochemical analysis. Inclusion of species identification also provided a more in-depth knowledge of whether beneficial / neutral algal species were present, as well as what potentially dangerous species were present. By applying the toolbox to the novel peatland IMTA and subsequently characterising the process, the use of peatlands has proven to be potentially successful locations to address the resource and spatial limitations in an environmentally sustainable fashion.

The final core theme focused on future issues that may arise in Irish freshwater aquaculture. These issues were highlighted throughout the generation of research conducted for the first two core theme. The overwhelming upcoming issue highlighted in this research was the potential effect climate change will have on the Irish aquaculture system. To further investigate this; the effects weather change had on water quality and algal growth were monitored in order to determine the potential application of algae as an early warning indicator for unforeseen issues associated with climate change. This was done so by the simple application of the ecotoxicological toolbox. Unlike monitoring physicochemical parameters alone, which only provide a snapshot in time of the current condition of the water, the inclusion of bioassays was able to provide a better means of predicting future issues associated with the water quality. Algal and cyanobacterial populations were also monitored in the novel IMTA process in order to provide a means to indicate any potential issues associated with negative algae and cyanobacterial blooms as a result of changing weather conditions. This was and will be of vital importance in the future given the fact that the novel peatland IMTA system is somewhat reliant on the use of algae for wastewater assimilation and also as the use of peatlands is proving to be a very promising location for future aquaculture facilities. By being able to predict and identify potential issues associated with the algae / cyanobacteria as a result of the ever changing and erratic weather, holistically sustainable mitigation measures can be applied before negative impacts are experienced. Finally, COVID-19 demonstrated the need to ensure fish farmers are reassured that their *in-situ* technology is providing as accurate a level of information as possible during times when access to traditional wet-lab facilities is not available. This was achieved by statistically comparing data from wet laboratory techniques (the FCM) with *in-situ* technologies for monitoring algal/cyanobacteria populations (the AlgaeTorch®) in order to ensure unforeseen issues were indicated early enough so that mitigation could be applied before major problems persisted or occur.

6.2. IMPLICATIONS OF RESEARCH

There was short-, medium- and long-term outcomes and implications of this work.

- Firstly, this research has led to the development of a sustainable quality control tool for fish farms by holistically allowing areas of concern to be flagged, as well as evaluating changes to improve output water. Its application as a secondary test to physicochemical analysis can provide a complementary approach to guide decision making on the farm. It can then be used further by confirming implemented changes applied to improve the farm processes have worked in terms of environmental protection thus ratifying the efficacy of the quality control check.
- It will allow for future proofing of the industry by providing smart tools that may be used for real-time processing within the industry. The developed tool can be used as a utility tool whereby baselines may be established and spot tests can be conducted in order to provide a simple, rapid and holistic approach for an early indication of a range of issues. It may also be applied to provide a screen to determine if more intensive research is required. Standard physicochemical analysis can vary. If there are issues within a system, the combination of physicochemical analysis with bioassays will allow to determine whether deeper analysis is required.
- The information generated will assist in harmonising traditional and novel processing applications within the industry. The tool may be used as an indicator to guide policy and has universal application so it is of most benefit to its targeted end-users *i.e.,* stakeholders, fish farmers, policy makers and regulators. This is of particular note as the Irish EPA are currently investigation the regulation of wastewater in aquaculture. This tool can also be used to inform and develop multi / triple helix hubs which is designed to bring academia, industry and authorities together to develop sustainability.
- This work has provided a means to inform efficacy innovation. The generated data will inform reliability and reproducibility of assessment techniques. The tool box also adapts to change making it a more robust means of analysis. It is flexible, dynamic and responds to variance / change. Therefore, it may allow for a greater degree of environmental sensitivity stressors.
- The data may be used for end to end / sensor use and may also be applied for use in a mobile application for real-time monitoring, a machine learning pre-step or for future artificial intelligence development.

The data generated from this research will also be the means of further developing and contributing to current knowledge by the publication of additional research papers. Five papers are in preparation for publication. These include;

1. The full development of the ecotoxicological toolbox as no work on a similar study in Ireland could be found in the available research.
2. The comparative study conducted between the standardised algae (*P. subcapitata*) and the species representative of Irish freshwater systems (*A. formosa* and *M. contortum*) as no comparative study focusing on the same combination of species has been found to date.
3. The characterisation of the novel peatland IMTA system as this is a first of its kind study.
4. A follow up paper further demonstrating the use of *P. subcapitata* as an early warning indicator for climate change ambiguity in Irish freshwater aquaculture.
5. The effects climate change may have on the novel peatland IMTA process.

6.3. Recommendations & Future Research

After reviewing the work generated in the research and its implications, the suggested recommendations and future research are as follows;

- Given the fact that the EPA are actively investigating the regulation of aquaculture wastewater and the beneficial application this research may provide in that process, it is recommended that advice be requested directly from the EPA so as to advise how best this research may be used for the benefit of its end-users.
- As previously mentioned in Chapter 4, the characterisation study on Oasis fish farm demonstrated the complexity of the novel peatland IMTA system. Therefore, it is recommended that additional research into this is required. This would include a more in-depth analysis into physicochemical combinations *e.g.*, are different physicochemical parameters influencing one another and producing greater (additive, synergistic) or inhibitory (subtractive) effects together than if they were present on their own? This would also include a more in-depth analysis into other biological organisms within the system *e.g.,* rotifers and ciliates, which are microscopic animalcules, were also observed in the system. However, the potential impact they may have had on physicochemical parameters as well as their interactions with the algae in the system are still unknown.
- If combinations of physicochemical parameters are influencing one another's effects then mitigating potential issues may not be straight forward, especially as the Oasis system is entirely natural and holistic. As such, it is recommended to elucidate strategies to offset physicochemical variances in the system that will allow the system to remain organic. Once such strategy may be the development of bioreactors for the growth of beneficial algae thus providing stocks that may be added when necessary.

- Expansion of research is also recommended for Oasis fish farm. This would include investigations into the surrounding peatland in order to determine the potential effects run-off and leachate from the peatland may have on the farm. Life Cycle Analysis will provide a better understanding of when the fish are most vulnerable within the system thus allowing for the opportunity to develop suitable holistic mitigation measures. Finally, once fully functional, the presence of increasing numbers of algae may provide additional bio-based products of value *e.g.,* biofuels, *etc*.
- Duckweed is also used as a means of wastewater assimilation in Oasis. However, issues in getting the duckweed to grow were continuously observed. Therefore, collaborative research on duckweed and its relationship with the algae and the system itself will inform its future management. Additionally, research into the potentially beneficial use and application of other species such as those that are native to the bog should also be considered. For example, McKeon-Bennett and Hodkinson (2021) have recently demonstrated that sphagnum moss, which is native to the peatland bogs of Ireland, may be used as a novel growth medium in sustainable indoor agriculture systems. The unique characteristics of sphagnum moss which include; pH control of its environment, water remediation and gas exchange, may be of great benefit to a system such as the novel peatland IMTA system at Oasis.
- The twenty first century is an age of technological advancements therefore, future aquaculture development could lie in future intensification of digitalisation also. The data (bioassay and physicochemical) and how it can be used for end users could provide an end-to-end digital tool which could help with policy and intervention. Therefore, a collaboration with software engineering is recommended where looking at and modelling of the data generated to go along with digitalisation will aid in the advancement of sustainable aquaculture. Pipeline researchers may also be used with digitalisation to build capacity for regional development.
- As the work conducted on Oasis fish farm has suggested great potential for sustainable aquaculture and the fact that there is still much research still to do, Oasis fish farm could be used as a future intended location for a national testing centre for aquaculture, especially peatland aquaculture development, and provide open innovation. This will also provide a building block and starting point to perform longitudinal studies across all of Ireland in regards to peatland aquaculture development which will be necessary in order for the novel peatland IMTA process to be applied to other areas.
- More research is recommended on how the use of the information in this tool can be used to inform and develop multi / triple helix hubs. The European Green Deal is a European initiative designed to make the European economy sustainable. The incorporation of academia, industry and

local / national authorities can make the transition to a low carbon economy more feasible by informing on appropriate policy, technology and social readiness (Rowan and Casey, 2021).

- Finally, greater integration across difference fields and subjects is recommended and encouraged. For example; combining STEM with social sciences could be used to help with behavioural changes needed for the freshwater aquaculture industry to grow thus alleviating barriers, misconceptions and attitudes by removing false negative opinions around / about aquaculture. Incorporating citizen science here will also help with this.

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APPENDIX 1

RESEARCH DISSEMINATION & KNOWLEDGE CONTRIBUTION

Journal Publications

O'Neill, E.A., Rowan, N.J. (2022). Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland. Sci. Total Environ. 802, 149800. (Impact Factor 7.963)

O'Neill, E.A., Stejskal, V., Clifford, E., Rowan, N.J., (2020). Novel use of peatlands as future locations for the sustainable intensification of freshwater aquaculture production – A case study from the Republic of Ireland. Sci. Total Environ. 706, 136044.

O'Neill, E.A., Rowan, N.J., Fogarty, A.M., (2019). Novel use of the alga *Pseudokirchneriella subcapitata*, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish farming as a case study. Sci. Total Environ. 692, 209–218.

Posters

O'Neill, E., Fehrenbach, G., Murphy, E., Pogue, R., Lynch, M., Rowan, N. (2020). Developing Sustainable Freshwater Aquaculture using Irish Peatlands during COVID-19 Crisis. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 19th June.

O'Neill, E., Murphy, E., Lynch, M., Rowan, R. (2019). Sustainable Intensification of Freshwater Aquaculture using Peatlands – Role of Algae. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 22nd November.

O'Neill, E., Rowan, N., Fogarty, A. (2019). Development of an Ecotoxicological Toolbox for Assessing Irish Freshwater Finfish Aquaculture Effluent. 29th Irish Environmental Researchers Colloquium (ENVIRON 2019). Carlow Institute of Technology, Carlow. 15th – 17th April

O'Neill, E., Fogarty, A., Donohoe, O., Rowan, N. (2018). Development of Ecotoxicological Toolbox for Assessing Freshwater Finfish Aquaculture Effluent. AIT Research Day. Athlone Institute of Technology, Co. Westmeath. 21st April & 28th Irish Environmental Researchers Colloquium (ENVIRON 2018). Cork Institute of Technology, Cork. 26th – 28th March.

Technical Reports

O'Neill. E., Fehrenbach, G., Murphy, E., Pogue, R., Lynch, M., Rowan, N. (2021). Investigation to elucidate role and relationship between algal and microbial communities in freshwater aquaculture. AquaAlgaePlus Final Report, June.

Kennedy, A., Tahar, A., Cooney, R., Naughton, S., O'Neill, E., Fogarty, A., Kavanagh, S., Rowan, N., Clifford, E. (2019). Supporting the sustainable development of the Irish freshwater aquaculture industry. EcoAqua Final Report, October.

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Novel use of the alga *Pseudokirchneriella subcapitata*, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish farming as a case study

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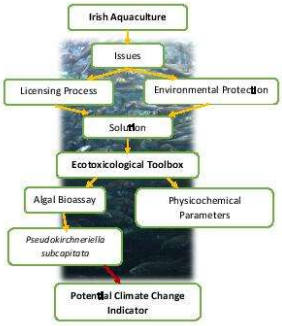
HIGHLIGHTS

- Fish farm effluent monitored using algal bioassay and physicochemical parameters.
- Alga (*Pseudokirchneriella subcapitata*), more responsive model than physicochemical parameters alone.
- Standard water quality parameters are not applicable to fish farm wastewater.
- Duckweed and constructed wetlands were under appreciated in wastewater treatment.
- Alga demonstrated potential use as early warning system for climate change.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquaculture is one of the fastest growing food producing industries in the world. This dramatic increase in growth has raised many environmental concerns. Evaluation of fish farm effluent is frequently assessed by physicochemical parameters. This approach indicates potential degradation caused by the effluent and not cumulative effects on aquatic ecosystems. This study investigated relationships between physicochemical parameters (temperature, pH, conductivity, nitrogen, phosphorus, oxygen and suspended solids), typically used to assess water quality with the *Pseudokirchneriella subcapitata* algal bioassay, which evaluated the potential ecotoxicological effects that freshwater fish farm effluent has on its receiving ecosystems and organisms. Influent and effluent samples were collected from a freshwater farm facility every two weeks from April 2018 to October 2018 in the Republic of Ireland. This monitoring period coincided with one of the warmest and driest periods recorded by meteorological stations in the Republic of Ireland. Physicochemical analyses were found to be similar to those in other farm studies. After exposure of algae to the effluent, stimulation of algal growth rates increased by >50%. This stimulation was observed during periods of increased temperatures which were as a result of heat wave and drought conditions experienced during monitoring. Correlation studies identified a moderately strong relationship between algal stimulation and temperature ($r = -0.619$). This study discovered that removal of *Lemna minor* (aquatic plant), impacted strongly on the freshwater farm pond-process to cope with nitrates.

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The constructed wetland system was unable to efficiently treat nitrates and phosphates during conditions of drought. These findings indicate that standard water quality parameters may not be applicable to inform appropriate suitability of fish farm effluent for discharge to receiving water. The research conducted in this study has suggested a potential toolbox that includes *P. subcapitata* may provide an early warning system for adverse effects as a result of climate change.

1. Introduction

Aquaculture is one of the fastest growing food producing industries in the world (Fečkaninová et al., 2017; Liu et al., 2017), and provides one of the most sustainable forms of edible protein (Liu et al., 2017; Yue and Wang, 2017). The dramatic increase in the growth of global aquaculture production indicate its importance in modern day food supply (Jegatheesan et al., 2011), by providing a means to meet the growth in global demand (Seoane et al., 2014). However, despite this and many other advantages (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014), there are many environmental concerns thought to be associated with aquaculture (Martinez-Porchas et al., 2014; Ngo et al., 2016; Troell et al., 2017), and in particular to the impacts fish farm effluent is thought to have on the receiving aquatic ecosystem (Jegatheesan et al., 2011).

In 2017, the Irish aquaculture sector produced 47,147 t of fish valued at €208.4 M which was an increase of almost €100 M since 2014 (Bord Iascaigh Mhara, 2018). The Irish aquaculture sector is primarily based in coastal areas but land-based recirculating aquaculture systems (RAS), and more traditional freshwater and land based systems are also used (Department of Agriculture Food and the Marine, 2015), and are projected to potentially grow rapidly over the next 10 years. The sector provides valuable employment on a year round basis and aids in the preservation of viable local and rural communities (Department of Agriculture Food and the Marine, 2015). The Irish aquaculture sector can be divided into shellfish and finfish culture with rope mussels, bottom grown mussels, Gigas oysters, salmon and trout the main species cultured. (Department of Agriculture Food and the Marine, 2015; Bord Iascaigh Mhara, 2018). Irish aquaculture also produces a wide range of novel species, both in marine and freshwater, such as Abalone, sea urchins, seaweed and perch (Bord Iascaigh Mhara, 2018).

Fish farm effluent typically contains nutrient rich waste products (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Sikder et al., 2016), which if released untreated into water bodies can lead to water pollution (Jegatheesan et al., 2011; Sikder et al., 2016). It may cause direct negative effects such as eutrophication (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Sikder et al., 2016; Troell et al., 2017), which is one of the greatest concerns (Ngo et al., 2016). Eutrophication is a process by which a water body receives large levels of nutrients and organic matter that can be taken in and biologically processed (Martinez-Porchas et al., 2014; Sikder et al., 2016), which in turn can result in increased levels of algal blooms and decreased levels of oxygen which can suffocate aquatic life in the water body (Jegatheesan et al., 2011; Chislock et al., 2013; Ngo et al., 2016). Fish farm effluent is commonly characterised by rich levels of nutrients, such as nitrogen and phosphorous, and organic matter (Jegatheesan et al., 2011; Ngo et al., 2016). These characteristics are as a result of uneaten or left over food and metabolic waste products such as faeces and urea (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Troell et al., 2017). In fact, the primary source of nitrogen, phosphorous and organic matter in the water is derived from the application of feed (Jegatheesan et al., 2011), which is necessary to maintain high production yields in order to meet demands (Kolarevic et al., 2014; Feucht and Zander, 2015). Another source of organic matter is the influent used to fill ponds or raceways and maintain the farms water levels (Jegatheesan et al., 2011).

The evaluation of fish farm effluent water quality is frequently assessed by the measurement of physicochemical parameters (da Silva et al., 2017), which include nitrogen, phosphorus, oxygen, suspended solids, temperature, pH, alkalinity, hardness and conductivity. Ammonium (NH_4^+) is highly toxic to aquatic life (Zhang et al., 2011), and requires treatment before its release into its receiving water body (Celik et al., 2001). Nitrite (NO_2^-), is very toxic to aquatic life (Pollice et al., 2002), but highly unstable and only remains in this form during for a short period of time during the transformation of NH_4^+ to nitrate (NO_3^-), (Durborow et al., 1997). Orthophosphate (PO_4^{3-}), a reactive form of phosphorus (Brogan et al., 2001), is one of the main causes of algal blooms and the hypoxic conditions which may occur in water bodies (Brogan et al., 2001; Barcellos et al., 2019). Biochemical oxygen demand (BOD), is the amount of oxygen used by bacteria in breaking down organic matter in the water (Lee and Nikraz, 2015). Chemical oxygen demand (COD), measures the stress a quantity of organic matter puts on a receiving water body (Lee and Nikraz, 2015). Suspended solids often consist of organic matter and elevated levels can be an indicator of eutrophic conditions (Bilotta and Brazier, 2008). Temperature is a critical environmental factor for fish farming due to its effect on growth, metabolism, survival, immune responses and oxygen consumption (Ferreira et al., 2011). Calcium carbonate ($CaCO_3$), improves conditions for benthic animals and microbial activity and increases CO_2 (Ferreira et al., 2011). The alkalinity is the buffering capacity of the water body and is related to important factors in fish farming and water hardness is the amount of dissolved calcium and/or magnesium present in the water (Ferreira et al., 2011). Investigation of these parameters alone only indicate the potential degradation caused by the effluent at a given time, not their effects on aquatic ecosystems and organisms (Stephens and Farris, 2004a, 2004b; da Silva et al., 2017). Thus, ecotoxicological bioassays are used in conjunction with physicochemical analysis however, there are few studies that assess the toxic effects of fish farm effluent on aquatic ecosystems and organisms (da Silva et al., 2017).

Planktonic microalgae are primary producers and are a key component in the food chains of aquatic ecosystems (Aruoja, 2011). Sphaeropleales are one of the most dominant groups of green microalgae in the world and contain species that are considered to be very important to freshwater ecosystems (Suzuki et al., 2018). According to Rodgher et al. (2012), these microalgae constitute an important group of highly sensitive photosynthetic organisms that are frequently used to assess aquatic ecosystems. One such species in this group is *Pseudokirchneriella subcapitata* (*P. subcapitata*), CCAP 278/4, also commonly known as *Raphidocelis subcapitata* or *Selenastrum capricornutum* (Aruoja, 2011; Rodgher et al., 2012; Yamagishi et al., 2017; Suzuki et al., 2018). *P. subcapitata* is a unicellular algae and is consider the most widely known and used bio indicator in ecotoxicological assessments of freshwater ecosystems due to its high growth rate, high sensitivity and high reproducibility (Suzuki et al., 2018). As such, *P. subcapitata* has become one of the species of choice for primary producer assessment in multi-trophic testing and is the main algae recommended by the ISO (6892:2012), guidelines (International Organisation for Standardisation, 2012).

Globally, aquaculture is dominated by freshwater farming and the bulk of the production is finfish (Wang et al., 2015). The aim of this

study was to conduct an ecotoxicological evaluation of Irish freshwater finfish farm effluent using the *P. subcapitata* algal bioassay ISO (8692:2012), in conjunction with traditional physicochemical analysis. Correlation studies were also conducted to determine any potential relationships between the algae and the physicochemical parameters.

2. Materials & methods

2.1. Sampling

Water samples were collected from a freshwater fish farm located in Boyle, Co. Roscommon (Fig. 1). The farm, which cultures perch (*Perca fluviatilis*), consisted of an RAS used for the hatchery and nursery, three culture ponds for the adult fish that uses a flow through system (FTS), a settlement pond and a constructed wetland for wastewater treatment. Samples were collected in 5 L octagonal carboy HDPE bottle (Lennox), and transported directly to the lab, 70 km away, via car. Samples were taken directly from the effluent source of the farm every two weeks from April 2018 to October 2018. Sampling could not be conducted in June due to unforeseen circumstances. Collection occurred on the same day (Thursdays), and at approximately the same time (10:30 a.m.). Influent samples were also collected and analysed so that any potential issues caused by works upstream of the fish farm and not as a result of works within the facility itself could be taken into consideration. Influent and effluent sampling points are shown in Fig. 2.

2.2. Physicochemical analysis

Water parameters – temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, dissolved oxygen (DO), BOD, COD, suspended solids (SS), hardness, alkalinity and conductivity – were investigated in the laboratory within 24 h of collection to prevent the need for preservation. Table 1 summarises the physicochemical methods employed in this study. Spectroquant® photometric kits were used to assess the NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and COD. Analysis was conducted as per the manufacturer's instructions. Temperature, pH and conductivity were analysed using a VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe and VWR CO11 conductivity probe. DO and BOD_{5day} were analysed using a Jenway 9500 DO_2 meter and probe. Suspended solids were analysed via filtration using a Buchner flask and funnel. Hardness was assessed via titration using pH 10 buffer, Erichrome black and EDTA.

Fig. 1. Map of Ireland indicating the location of the fish farm (yellow) and the closest weather stations (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

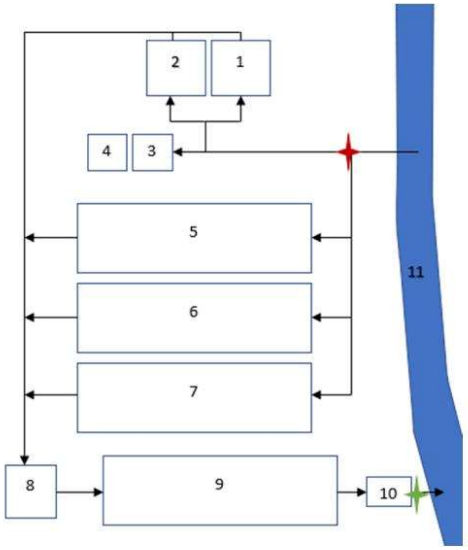


Fig. 2. Schematic of the fish farm layout. Location of the collection point for the influent (red X), and effluent (green X), are indicated. 1) Hatchery, 2) Nursery, 3–4) Mesocosms, 5–7) Culture ponds, 8) Settlement Pond, 9) Constructed Wetland, 10) Holding Tank, 11) River. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Methods, including detection limits, used for the analyses of water quality parameters in the influent and effluent. Figure in brackets indicate the standard method number.

Parameter/variable	Method	Detection limit ($mg\,L^{-1}$)
Temperature	Thermometer (2550-B)	–
pH	Membrane Electrode (2310-B)	–
Ammonium (NH_4^+)	Photometric (4500-NH_3-F)	0.013–3.86 2.6–193.0
Nitrite (NO_2^-)	Photometric (345–1)	0.007–3.28
Nitrate (NO_3^-)	Photometric (4500-NO_3)	0.4–110.7
Phosphate (PO_4^{3-})	Photometric (4500-P-C)	0.007–15.3 1.5–92.0
Dissolved oxygen	Membrane Electrode (4500-O G)	–
Biochemical Oxygen demand (BOD)	Membrane Electrode (5210-B)	–
Chemical oxygen Demand (COD)	Photometric (5220-D)	0–150 15–300
Suspended solids	Gravimetric (2540-D)	–
Hardness	Titrimetric (2340-C)	–
Alkalinity	Titrimetric (2320-B)	–
Conductivity	Electrical Conductivity (2510-A)	–

Alkalinity was analysed by titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid.

2.3. Toxicity testing

The freshwater unicellular green algae *P. subcapitata*, was used in the toxicity test. A starter culture of the *P. subcapitata* (*Raphidocelis subcapitata*), was obtained from The Culture Collection of Algae and Protozoa (CCAP 278/4; SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.), and grown in standard Jarworski's Medium under controlled conditions of 23 °C ± 2 °C exposed to continuous illumination (lux 6000–10,000). Sub-culturing was conducted every two to three days to ensure the growth rate remained in the exponential phase. Toxicity testing was conducted as per the Water quality – Fresh water algal growth inhibition test with unicellular green algae ISO (8692:2012), guidelines. The *P. subcapitata* was exposed to the influent and effluent samples for 72 h under static conditions at 23 °C ± 2 °C exposed to the continuous illumination. The algae growth rate inhibition and stimulation, in percent, was calculated by comparing the samples to a negative control containing just the Jarworski's medium. Calculations were conducted as follows;

$$\text{Algal cells mL}^{-1} = \frac{n}{0.02}\ x\ 10^{3.}$$

where n = number of cells counted using a haemocytometer

$$\text{Average specific growth rate } (\mu), = \frac{\ln X_n - \ln X_o}{T_n - T_o}$$

where ln = natural log of
X_n = Algae cells mL^{-1} at 72 h
X_o = Algae cells mL^{-1} at 0 h
T_n = Duration of test
T_0 = Time zero

$$\text{Percent inhibition in growth rate} = \frac{C\mu - T\mu}{C\mu}\ x\ 100$$

where Cμ = Average specific growth rate for control
Tμ = Average specific growth rate for treatment

2.4. Statistical analysis

All statistical analyses were conducted using GRAPHPAD PRISM 8 and MINITAB 18. The data generated were grouped and subject to normality test (Anderson-Darling). *t*-tests and one-way ANOVA with Tukey were used to identify significant differences in the variables. Pearson's correlation was used to assess any correlations between the algae and/or the physicochemical parameters.

3. Results

3.1. Physicochemical analysis

Mean concentrations determined for the physicochemical parameters investigated on Irish freshwater fish farm influent and effluent samples over the entire testing period are summarised in Table 2 and Fig. 3 provides a monthly breakdown of the results. With the exception of the pH, increases in concentrations between the influent and effluent were observed in all parameters. When the influent and effluent were compared via statistical analysis, a significant difference was observed in the NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and DO levels ($p < 0.05$).

Table 2
Physicochemical parameters investigated on Irish freshwater finfish aquaculture influent and effluent samples from April 2018 to October 2018. Results are presented as means ± S.D. *Significant differences, where $p < 0.05$, are indicated (n = 12).

Parameter	Influent	Effluent	*P* value
Temperature (°C)	14.76 ± 2.53	15.53 ± 2.66	0.854
pH	7.76 ± 0.19	7.11 ± 0.18	0.709
Ammonium (mg NH_4^+ L^{-1})	0.16 ± 0.18	1.16 ± 0.64	0.001*
Nitrite (mg NO_2^- L^{-1})	0.02 ± 0.01	0.32 ± 0.38	<0.001*
Nitrate (mg NO_3^- L^{-1})	3.62 ± 1.60	5.29 ± 5.56	0.006*
Orthophosphate (mg PO_4^{3-} L^{-1})	1.76 ± 0.84	3.78 ± 2.00	0.013*
DO (mg O_2 L^{-1})	10.31 ± 0.87	5.10 ± 2.85	<0.001*
BOD (mg O_2 L^{-1})	2.27 ± 1.47	3.24 ± 1.95	0.185
COD (mg O_2 L^{-1})	45.91 ± 40.81	76.44 ± 59.06	0.230
Suspended Solids (mg L^{-1})	40.17 ± 79.08	83.67 ± 144.33	0.073
Hardness (mg $CaCO_3$ L^{-1})	100.49 ± 9.22	116.03 ± 16.80	0.092
Alkalinity (mg $CaCO_3$ L^{-1})	122.55 ± 17.71	128.91 ± 18.19	0.915
Conductivity (μS cm^{-1})	247.18 ± 57.82	298.17 ± 57.12	0.241

DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand.

3.2. Algal bioassay

The *P. subcapitata* algal bioassay ISO (8692:2012), was performed on influent and effluent samples from the Irish freshwater fish farm every two weeks to determine whether or not the rate of growth inhibition or stimulation were observed as a result of exposure to either sample. The growth rate inhibition and stimulation observed in the influent and effluent can be found in Fig. 4. Statistical analysis was conducted between the influent and effluent and a significant difference was observed ($p \leq 0.0001$ where <0.05 indicates a significant difference).

3.3. Correlation studies

The Pearson's correlation test (Table 3), demonstrated that in the effluent, the algae (*P. subcapitata*), was negatively correlated with temperature and suspended solids and positively correlated with alkalinity. Nitrite was positively correlated with orthophosphate. Temperature was negatively correlated with dissolved oxygen and positively correlated with conductivity. The parameters hardness and alkalinity were negatively correlated with orthophosphate. Dissolved oxygen was negatively correlated with conductivity and positively correlated with nitrate. Hardness was positively correlated with alkalinity and a negative correlation with nitrite. The pH had a negative correlation with conductivity.

3.4. Weather conditions

The Republic of Ireland experienced one of its hottest summers on record in 2018 that coincided with the sampling period in the study (Met Eireann, 2018b). Drought conditions and a national hose pipe ban were put in place for most of the country up to the end of August 2018 (Irish Water, 2018). As a result of these unusual weather conditions mean rainfall and temperature data collected at three Met Eireann weather stations surrounding and closest to the fish farm in the Republic of Ireland (Fig. 1), were observed. These stations were located in; Markree, Knock and Mount Dillon. Decreases in the average monthly rainfall and increases in temperature were observed across the weather stations (Fig. 5). Statistical analysis suggested that moderately strong inverse relationship existed between the algae and the rainfall ($r = -0.562$), and a weak relationship between the algae and the temperature ($r = 0.276$). Fig. 6 compares the average temperatures observed across the stations and the average maximum temperatures experienced. When the maximum temperatures were taken into account, a moderately strong relationship between the algae and temperature was observed ($r = 0.505$).

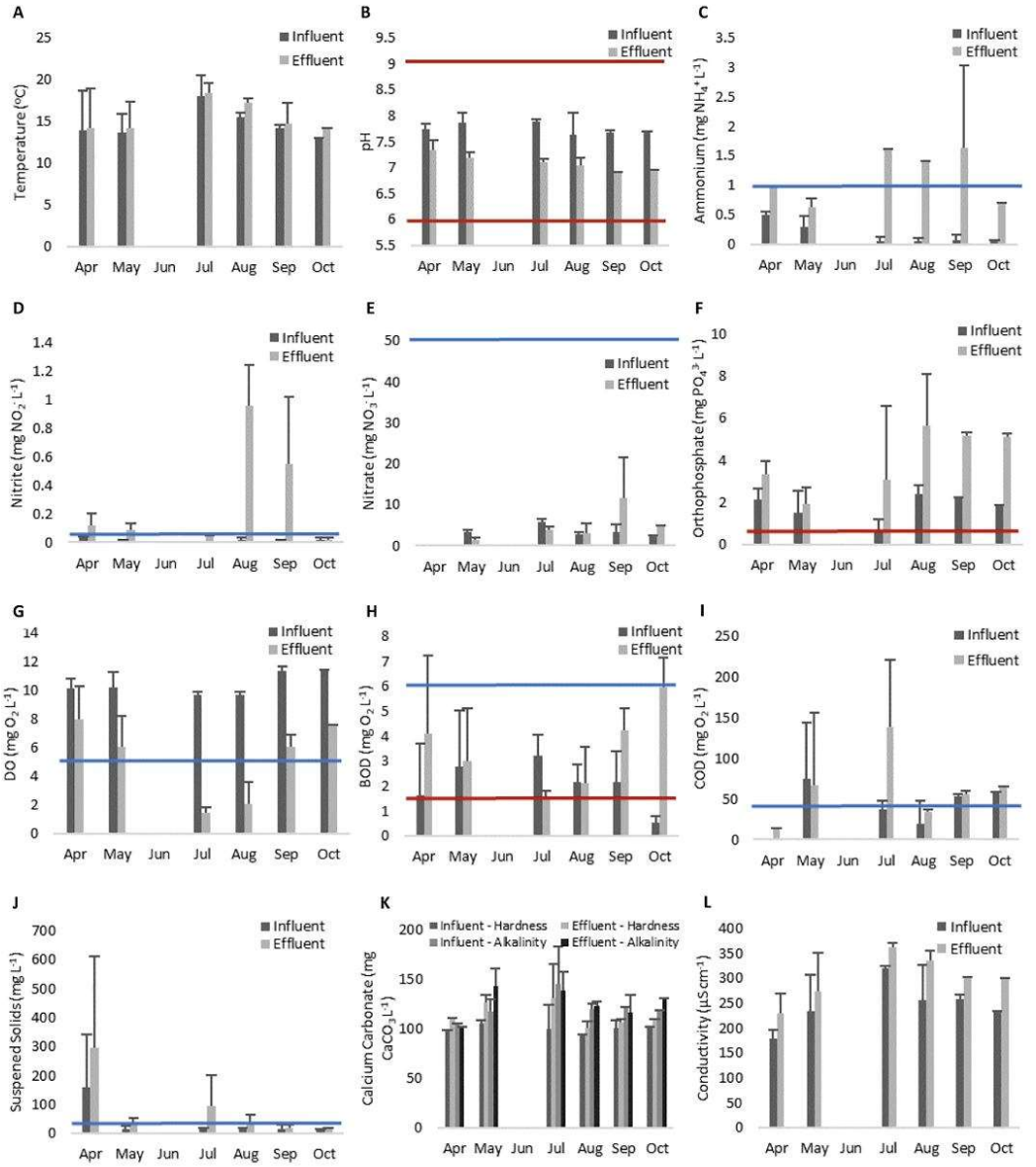
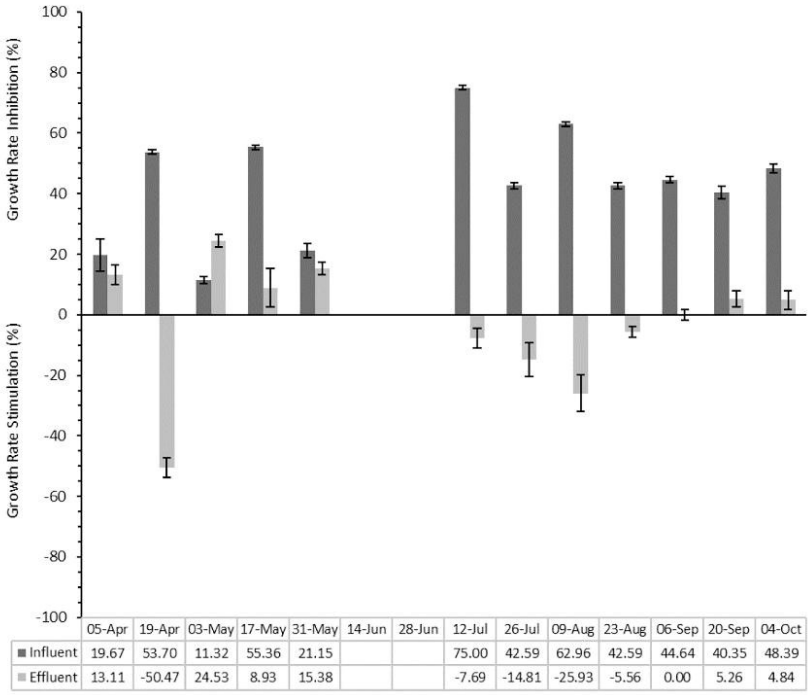


Fig. 3. Bar charts displaying monthly means for the physicochemical parameters investigated on Irish freshwater finfish aquaculture influent and effluent from April 2018 to October 2018. Parameters investigated were A) temperature, B) pH, C) NH_4^+, D) NO_2^-, E) NO_3^-, F) PO_4^{3-}, G) dissolved oxygen, H) Biochemical Oxygen Demand, I) Chemical Oxygen Demand, J) suspended solids, K) hardness and alkalinity via $CaCO_3$ content, and L) conductivity. S.D. indicated, $n = 12$. Red lines indicate levels set out by S.I. 272 of 2009, which is the European Communities Environmental Objective (Surface Waters) Regulations of 2009. Blue lines indicated levels set out by the Irish EPAs Parameters for water quality, which are based on Freshwater Fish Directive [78/659/EEC], Irish Salmonid Waters Regulations [1988], and Irish Surface Water Regulations [1989]. NOTE: The dilution factor of the receiving water body has not been taken into consideration. Lines do not appear on temperature, $CaCO_3$ content and conductivity as no limits were indicated and/or the limit is well above the range of the graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	20-Sep	04-Oct
Influent	19.67	53.70	11.32	55.36	21.15			75.00	42.59	62.96	42.59	44.64	40.35	48.39
Effluent	13.11	-50.47	24.53	8.93	15.38			-7.69	-14.81	-25.93	-5.56	0.00	5.26	4.84

Fig. 4. The percentage growth rate inhibition (positive y scale), and stimulation (negative y scale), observed in *Pseudokirchneriella subcapitata* after exposure to the freshwater finfish aquaculture influent and effluent for 72 h at 23 °C ± 2 °C under continuous illumination. Samples were collected and analysed from April 2018 to October 2018. Dates of collection appear at the bottom of each pair of results. Individual results appear in the table. Positive figures indicate inhibition and negative figures indicate stimulation. ($n = 3$, SD indicated).

4. Discussion

4.1. Physicochemical evaluation

As far as the authors are aware, water quality parameters specific for fish farm effluent in Irish water are not currently available and the Irish EPA are currently investigating the regulation of Irish freshwater fish farm effluent. Recommended water quality parameters set out by the Statutory Instrument (S.I.), 272/2009 (European Communities Environmental Objectives – Surface Waters – Regulations 2009), and the Irish Environmental Protection Agency's (EPA), parameters of water quality were therefore used as guidance (Environmental Protection Agency, 2001; Irish Stationery Office, 2009a, 2009b). The parameters set out in the above-mentioned Irish EPA document are based on the Freshwater Fish Directive [78/659/EEC] and/or Irish Surface Water Regulations [1989].

Presence of the highly toxic NH_4^+ (Zhang et al., 2011), is an indicator of recent pollution. When comparing both samples, the concentration of

Table 3
Correlation matrix for the algae (*Pseudokirchneriella subcapitata*), and physicochemical parameters investigated on the Irish freshwater finfish aquaculture effluent. Bold figures indicate where significant differences ($p < 0.05$), have been observed. Breakdown of correlation figures are indicated in box. Breakdown of correlation values is based on Ratner (2009), and his work correlation coefficient values.

	ALGAE	PH	TEMP	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	HARD.	ALK.
ALGAE	1							***0 = No relationship***					
PH	0.112	1						***>0–0.3 = Weak relationship***					
TEMP	**−0.619**	−0.480	1					***0.3–0.5 = Moderately weak relationship***					
NH_4^+	−0.285	−0.437	0.414	1				***0.5–0.7 = Moderately strong relationship***					
NO_2^-	−0.307	−0.389	0.213	**0.554**	1			***0.7 - < 1 = Strong relationship***					
NO_3^-	0.164	−0.468	−0.475	−0.346	−0.100	1		***1 = Perfect linear relationship***					
PO_4^{3-}	−0.393	−0.435	0.209	0.308	**0.651**	0.320	1						
DO	0.356	0.408	**−0.846**	−0.492	−0.298	**0.578**	−0.028	1					
BOD	−0.197	−0.307	0.008	−0.065	0.048	0.251	0.310	0.422	1				
COD	0.270	0.310	−0.216	−0.083	−0.430	−0.003	−0.088	0.004	−0.495	1			
SS	**−0.727**	0.228	0.244	−0.064	−0.191	−0.121	−0.139	0.069	0.399	−0.345	1		
HARD.	0.343	0.393	−0.021	−0.095	**−0.580**	−0.335	**−0.885**	−0.156	−0.199	0.085	0.115	1	
ALK.	**0.607**	0.224	0.034	−0.408	−0.493	−0.228	**−0.657**	−0.240	−0.359	0.098	−0.368	**0.701**	1
COND.	−0.292	**−0.547**	**0.841**	0.394	0.251	−0.485	0.138	**−0.851**	−0.227	−0.083	−0.174	0.101	0.344

Temp = Temperature, DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity.

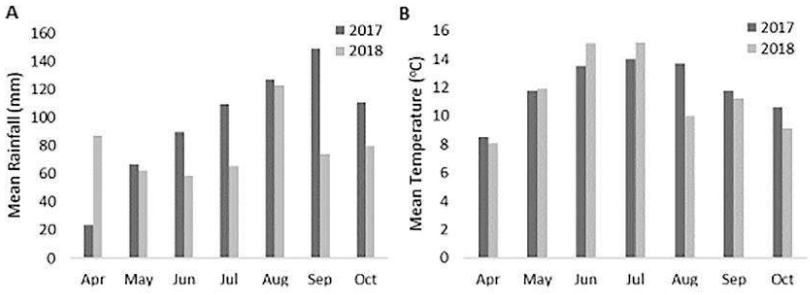


Fig. 5. Mean rainfall in mm (A), and temperature in °C (B), collected from three Met Eireann weather stations located at; 1) Markree, Co. Sligo, 2) Knock, Co. Mayo, and 3) Mount Dillon, Co Roscommon. These stations were selected as they were located to the north-west, south-west and south-east of the fish farm investigated in this study. Data from April to October in 2017 (dark grey), and 2018 (light grey), were examined.

NH_4^+ present in the influent is lower than that of the effluent. This suggested that the levels of NH_4^+ detected are being generated within the farm itself. However, the small amount detected in the influent also suggests some form of pollution is being generated upstream of the farm. The concentrations observed in the effluent were more than five times greater than the 1 mg L^{-1} suggested by the Irish EPA (Environmental Protection Agency, 2001). NO_2^- levels detected were very low mainly due to its transient nature and quick transformation from NH_4^+ to NO_3^- (Durborow et al., 1997). Similar with NH_4^+, the presence of NO_2^- is an indication of recent pollution. When comparing the NO_2^- levels in both samples, levels in the effluent were much higher than that of the influent. The small amount detected in the influent suggests some form of pollution is being generated upstream of the farm. The higher levels detected in the effluent are generated within the farm itself. The concentrations detected in the effluent were one log dose greater than the 0.03 mg L^{-1} for cyprinid waters, as per the Irish EPAs suggested water quality parameters (Environmental Protection Agency, 2001). NO_3^- levels observed in the influent indicated that NO_3^- may have been entering the river upstream of the fish farm as well as its expected generation within the farm. The aquatic plant *Lemna minor* (duckweed), was present in the farm and can use NO_3^- as a source of nutrients (Stewart and Rhodes, 1976). A spike in the effluent at the end of September 2018 was observed and may have been due to the fact that the *Lemna minor* within the farm had been removed. NO_3^- levels were well below the guidance value of 50 mg L^{-1} suggested by the Irish EPA (Environmental Protection Agency, 2001). *Lemna minor*'s ability to use NO_3^- as a nutrient source and the spikes observed in the farm after its removal highlighted that the duckweed holds great potential as a wastewater treatment option for fish farms and further research is currently being conducted.

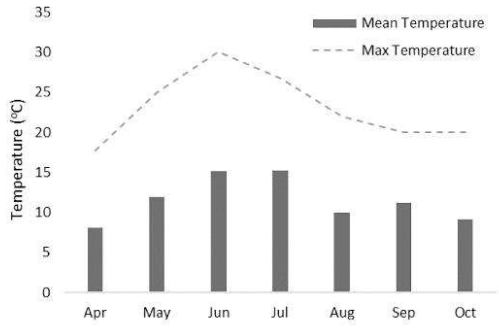


Fig. 6. Mean temperatures (Bar Chart), and maximum temperatures (Line Chart), observed at three Met Eireann weather stations located closest to the fish farm, from April 2018 to October 2018. Data was based on all temperature readings collected at the three weather stations by Met Eireann (Met Eireann, 2018a).

PO_4^{3-} was detected in both the influent and effluent. The levels observed in the influent were less than that in the effluent. This suggested that phosphorus pollution is entering the river upstream of the fish farm, as well as being generated within the farm itself. Levels in both the influent and effluent may be cause for concern due to its ability to cause algal blooms and the hypoxic conditions which may occur thereafter in water bodies. Concentrations detected were just over two log doses greater than the recommended value of 0.035 mg L^{-1} set out by the S.I. 272/2009 for good water status (Irish Stationery Office, 2009b). The farm uses a constructed wetland pond for treatment of nitrates, phosphates and so forth, before being released. Further research is currently being conducted in the efficacy of the constructed wetland by way of expansion of treatment to address culture area. This has been the subject of previous research (Jegatheesan et al., 2011; Sharrer et al., 2016), however it still remains unclear as to the governing factors underpinning the effective ratio for culture treatment of fish farm effluent.

The recommended DO concentration in salmonid waters should be ≥9 mg L^{-1} and cyprinid waters (e.g. perch) should be ≥7 mg O_2 L^{-1} (Environmental Protection Agency, 2001). There are no issues with the DO levels present in the influent however there may be cause for concern with levels observed in the effluent as they were well below the recommended concentration of ≥7 mg O_2 L^{-1}. Levels below this concentration were only observed during the heat wave and drought conditions, and the unusual weather conditions may have played a role. Conditions began to improve once weather conditions had returned normal. Alam et al. (2007), and da Silva et al. (2017), have suggested that oxygen concentrations of ≥4 mg L^{-1} are sufficient for maintenance of aquatic life. S.I. 272/2009 recommend a mean BOD concentration of 1.3 mg L^{-1} for high water status and 1.5 mg L^{-1} for good water status. However, the Irish EPA has suggested ≤3 mg L^{-1} and ≤ 5 mg L^{-1} for salmonid and cyprinid waters, respectively (Environmental Protection Agency, 2001). The current BOD levels detected in the influent suggested no issues. The concentration of BOD detected in the effluent may be cause for concern. Although the level was below that suggested by the Irish EPA for cyprinid water, it was greater than that suggested in the S.I. 272 of 2009. COD was detected in both the influent and effluent. The levels observed in both sets of samples may be cause for concern. The mean concentration was almost double the suggested 40 mg L^{-1} set out by the Irish EPA (Environmental Protection Agency, 2001).

Two concentrations of suspended solids have been suggested by the Irish EPA (Environmental Protection Agency, 2001). Fifty mg L^{-1} as per the Surface Water Regulations [1989], and 25 mg L^{-1} as per the Freshwater Fish Directive [78/659/EEC], and Salmonid Waters Regulations

[1988]. Suspended solids can increase gill irritation and blanket the benthos (Bilotta and Brazier, 2008), therefore the lower concentration of 25 mg L^{-1} was taken as the maximum allowable concentration (MAC), in this study of an Irish freshwater finfish farm. The average levels detected in both the influent and effluent may be cause for concern as they were above the 25 mg L^{-1}.

With growing concerns associated with climate change and global warming, increases in temperatures may become more frequent. Fluctuations in temperature were observed in both sets of samples. These rises in temperatures were only observed during the elevated temperatures experienced in Ireland in 2018. The results for the pH indicated that the influent was slightly more alkaline than the effluent, which the pH just above neutral (pH 7). The slight difference in pH levels in the samples may have been due to the alkalinity levels observed in the samples. The effluent had a higher level of $CaCO_3$ and thus a better buffering capacity. The recommended pH levels should be between 6 and 9. Levels in both the influent and effluent are well within this level and therefore are present no issues.

Alkalinity has been measured as $CaCO_3$. For hardness, $CaCO_3$ levels were also measured. Results suggested that the water is slight to moderately hard. This correlates with water hardness maps of Ireland which demonstrated water around Boyle, Co. Roscommon was slightly too moderately hard.

Some of the results observed (NH_4^+, NO_2^-, PO_4^{3-}, COD and suspended solids), suggested potential issues however, the dilution factor of the receiving river has not been included and needs to be taken into consideration. Equally, the condition of the influent needs to be considered. Additionally, as this research only focused on one fish farm, the results observed in this study were compared to previous studies conducted on a range of different farm effluents including; brown and rainbow trout, Atlantic salmon, catfish, shrimp and prawns. All results obtained were similar to other research studies conducted (Ziemann et al., 1992; Camargo, 1994; Boaventura et al., 1997; Mcintosh and Fitzsimmons, 2003; Biao et al., 2004; Pulatsü et al., 2004; Stephens and Farris, 2004a, 2004b; Costanzo et al., 2004; Živić et al., 2009; Moreira et al., 2010; Guilpart et al., 2012; Namin et al., 2013; Noroozrajabi et al., 2013; Herbeck et al., 2013; Caramel et al., 2014; Lalonde et al., 2014; Ferreira et al., 2015; da Silva et al., 2017), with the exception of BOD. Although not many studies included BOD, those reviewed demonstrated higher levels than the concentrations detected in this study (Boaventura et al., 1997; Mcintosh and Fitzsimmons, 2003; Ansah et al., 2012; Miashiro et al., 2012).

4.2. Ecotoxicological bioassay evaluation

Growth stimulation was observed in the effluent. Stimulation occurred in mid-April and then again from July to September. This coincided with the elevated temperatures and drought conditions experienced in the Republic of Ireland in the summer of 2018. Ireland's mean summer maxima temperature is between 18 °C and 20 °C (Walsh, 2012). In 2018, temperatures exceeded 30 °C (Met Eireann, 2018b). This resulted in low rainfall levels, e.g. the three weather stations (Markree, Knock and Mount Dillion), measured an average total rainfall of only 61.9 mm for the months of May, June and July 2018 compared to 88.9 mm for the same three months in 2017 (Met Eireann, 2018a), leading to a national hose pipe ban and water restrictions (Irish Water, 2018). The ability of the effluent to cause growth stimulation suggested that the possibility of algal blooms (resulting in eutrophication), downstream of the fish farm are more likely to occur. This may result in loss of biodiversity, habitat and submerged aquatic vegetation, disruption of the ecosystems functionality, deficiencies in oxygen and modifications in food webs (Rabalais, 2002).

A higher level of growth inhibition was observed in the influent compared to the effluent. This suggested that the influent would seem unlikely to cause issues such as algal blooms. However, the high level of growth inhibition in the influent also indicated toxicity and may result in losses to the biodiversity of the receiving water body (Rabalais, 2002). This toxicity may result in the loss of primary producers (e.g. algae), in the aquatic ecosystem. This may subsequently cause indirect adverse effects on the aquatic food chain, e.g. microcrustaceans feed on algae and fish in turn, feed on the microcrustaceans. Loss of the algae removes the food source for the microcrustaceans, resulting in their potential loss. This in turn, could result in the removal of a valuable food source for the fish. It should be noted that this toxic effect does not occur within the fish farm itself and suggests potential issues upstream of the farm. This issue may also affect the health and welfare of the fish within the farm. This is being closely monitored by the owner and no adverse effects have been observed with the fish. Further investigation into the water quality entering the fish farm has also been conducted. An old school is situated just upstream of the fish farm that has an older waste water treatment system which may have resulted in some waste entering the river. As a result of this, water is now piped from upstream of the school directly into the farm in order to assist in determining the exact cause for the poor water quality.

Most of the available research involving *P. subcapitata* focused on inhibition of growth (Guéguen et al., 2004; Ivanova and Groudeva, 2006; Ma et al., 2006). One study involving *P. subcapitata* and fish farm effluent was published by Miashiro et al. (2012), demonstrated similar results to this study, i.e. growth stimulation instead of inhibition was observed. Miashiro et al. (2012), suggested that the stimulated algal growth may have been due to the high concentration of nutrients that were observed. High levels of nutrients, were also observed in this study. The lack of available research suggested an under use of *P. subcapitata* as an early indicator of potential issues in fish farming. Stimulation of *P. subcapitata* in the effluent may be indicative of potential eutrophication downstream of the fish farm.

4.3. Climate change

"There's one issue that will define the contours of this century more dramatically than any other, and that is the urgent threat of a changing climate. Climate change is no longer some far-off problem; it is happening here, it is happening now" (Obama, 2015). Climate change is considered to be one of the most troubling, challenging and unrelenting scientific issues of our time (Bulkeley and Newell, 2015). Climate change, including global warming, is a complicated and increasingly problematic challenge causing changes to rainfall and hydrology, e.g. extensive summer droughts caused by changes in rainfall (Paerl and Scott, 2010; Paerl et al., 2016).

The results observed in this study have demonstrated that climate change may have a direct impact on fish farming, as suggested by the moderately strong relationship ($r = -0.619$), observed between the increases in temperature and the stimulation of algal growth rates, which could lead to increased instances of eutrophication downstream of the fish farm. *P. subcapitata* should be investigated further for its ability to be utilised as a potential early warning indicator of climate change.

5. Conclusion

The use of *P. subcapitata* as an early warning indicator to potential environmental issues associated with fish farms has been demonstrated as a more responsive model than physicochemical parameters alone. Evaluation of farm effluent could include ecotoxicological bioassays in order to determine any potential effects the effluent may have on the receiving aquatic ecosystem. Inclusion of the *P. subcapitata* Algal Bioassay ISO (8692:2012), in this study has demonstrated the potential eutrophication implications as a result of releasing untreated effluent from fish farms. It should be noted that this research focused on only one fish farm and therefore additional research is currently being conducted. Other bioassays that focus on different trophic levels should also be

considered in order to develop a broader picture of the potential effect's fish farm effluent poses on its receiving ecosystems.

Water quality parameters specific for fish farm effluent has not yet been established in Ireland. The Irish EPA has begun the process of regulating fish farm effluent. Results observed in this study have demonstrated that water quality parameters suggested by S.I. 272/2009 and the Irish EPA may not be applicable to fish farm effluent. The dilution factor of the receiving aquatic ecosystem is important and therefore also needs to be taken into consideration when these water quality parameters are to be determined. Results have also indicated that influent water quality is also as important when assessing fish farm effluent as it may indicate potential environmental issues as a result of activities upstream and not that which is occurring within the farm.

The study has highlighted that on review of the performance of the constructed wetland, it is envisaged that it needs to be increased in order to be effective. Also highlighted is the importance and potential use of *Lemna minor* as a waste water treatment system within fish farming facilities.

Although this study focused on the ecotoxicological effects freshwater fish farm effluent may induce on its receiving aquatic ecosystem, the research suggested that the changes in temperatures that were observed during the heat wave and drought conditions experienced in the summer months of 2018 had a direct relationship with the increased levels of algal growth stimulation detected. With irregular weather patterns becoming more frequent, especially rises in mean temperatures due to global warming, further research into the effects of climate change on aquatic ecosystems, fish farm effluent and the effects of effluents on its receiving ecosystem will need to be conducted and is currently being investigated. The research conducted in this study has suggested a potential toolbox that includes *P. subcapitata* may provide an early warning system for adverse effects as a result of climate change.

CRediT authorship contribution statement

Emer A. O'Neill: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Neil J. Rowan:**Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Andrew M. Fogarty:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

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Novel use of peatlands as future locations for the sustainable intensification of freshwater aquaculture production – A case study from the Republic of Ireland

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HIGHLIGHTS

- Peatlands offer a new sustainable means for integrated multitrophic aquaculture.
- Trial farm in peatland revealed favourable intake and holding tank water quality.
- Use of algae and duckweed show promise as natural means of treating water.
- Ecotoxicology study showed no differences between intake and holding tank water.
- Water quality findings were similar to previously reported aquaculture processes.

GRAPHICAL ABSTRACT

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ABSTRACT

There has been an increasing interest in enhancing freshwater aquaculture processes without hindering the progress of the Water Framework Directive. This constitutes the first study to describe a new concept in integrated multitrophic aquaculture (IMTA) that uses cutaway peatlands (bogs) to farm rainbow trout and Eurasian perch with associated organic status that is powered by wind energy and utilizes algae and duckweed to treat rearing water. Approximately 5% of Ireland comprises bogs that support natural ecosystems where there is a pressing need to develop alternative innovation to that of burning peat in order to reduce Ireland's carbon emissions. Specifically, this study evaluates water quality from this new IMTA where intake and terminal holding tank samples were evaluated from May to August 2019. Physicochemical parameters (temperature, pH, nitrogen, phosphorus, oxygen, suspended solids, hardness and alkalinity), and ecotoxicological bioassays (*Pseudokirchneriella subcapitata* and *Daphnia pulex*), were used to investigate the potential effects that introducing aquaculture processes may have on peatlands. Nitrite ($P < 0.001$), nitrate ($P = 0.016$), and chemical oxygen demand ($P = 0.011$), were the only physicochemical parameters that differed significantly between the intake and holding tank water indicating that water quality for the most part remained unchanged. Low levels of toxicity were observed between the bioassays suggested the introduction of the processes into the bog were unlikely to

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cause adverse effects on the ecosystem and the organisms therein. Observations were similar to or lower than those reported previously by other researchers for intensive flow-through aquaculture processes that discharge to receiving water. Findings from this study support the use of peatlands as future locations for integrated aquaculture processes.

1. Introduction

Aquaculture is the fastest growing food producing industry globally (Fečkaninová et al., 2017; Liu et al., 2017; O'Neill et al., 2019). The rapid expansion of aquaculture has arisen due to increasing global population and commensurate demand for more food (Seoane et al., 2014). Aquaculture now accounts for ~50% of fishery products (Liu et al., 2017), which is estimated to reach 62% by 2030 (Fredricks et al., 2015). The Irish aquaculture industry was valued at €208.4 M in 2017 (Bord Iascaigh Mhara, 2018) and agri-food exports that includes aquaculture estimated to reach €19 Bn by 2025. However, issues associated with the aquaculture licensing process and the potential environmental impacts caused by aquaculture effluent have hampered expansion of the Irish aquaculture industry (Department of Agriculture Food and the Marine, 2015a).There is also a commensurate interest in exploiting low-cost environmental-friendly 'natural' processes in aquaculture (Han et al., 2019). These issues have led to an increased research focus on developing integrated multitrophic aquaculture systems or IMTA (Granada et al., 2016) along with eco-innovation and monitoring of processes (Tahar et al., 2018a, 2018b; Rowan, 2019). Advances in aquaculture must also be balanced by the need to meet commitments as set out by the Water Framework Directive (WFD), which aims to achieve good water status in all waters across all European Union (EU) member countries (Voulvoulis et al., 2017; WFD Ireland, 2018). As part of Ireland's Strategic Plan for Sustainable Aquaculture Development, Bord Iascaigh Mhara (BIM) undertook a feasibility study to assess the novel use of peatlands (bogs) for aquaculture diversification (Department of Agriculture Food and the Marine, 2015b). Peatlands (bogs) are wetland environments characterised by the build-up of organic matter (peat) derived from dead plant material over thousands of years, which slowly decompose under wet conditions (Ward et al., 2019). Over time, poor drainage and the build-up of decaying plants have resulted in the raised bogs that are only fed by rainfall. Bord Na Móna, a state company that was originally developed to establish Irish peat resources for economic benefit, owns or controls approximately 80,000 ha of bog. The urgent threat of climate change, in addition to some of these bogs now being listed as important habitats under the EU's Birds and Habitats Directives due to their scarcity, have resulted in dramatic changes in the peat industry including conversion of peatland-usage to wind energy, forestry, biodiversity, amenity and waste management (Bord na Mona, 2019; Irish Peatland Conservation Council, 2019; O'Neill et al., 2019; Toner, 2018; Ward et al., 2019). Recently, Bord Na Mona, in conjunction with BIM, has further expanded use of these cutaway bogs to develop Ireland's first IMTA adhering to organic principles. This IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*Lemna minor*) and gibbous duckweed (*Lemna gibba*) and exploits use of microalgae for waste removal (Bord na Mona, 2019). This IMTA process differs from traditional aquaculture practices that use water from rivers and lakes where latter traditional systems must consider potential pollutants from agricultural runoff, industry and waste-water treatments plants (Rowan, 2011; Hayes et al., 2013; Barrett et al., 2016; Tahar et al., 2017; Tahar et al., 2018c; Tiedeken et al., 2017).

Aquaculture discharge typically contains nutrient rich waste that requires treatment prior to release (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; O'Neill et al., 2019; Sikder et al., 2016). If discharged untreated, this waste can lead to water pollution in the environment (Jegatheesan et al., 2011; O'Neill et al., 2019; Sikder et al., 2016). Water quality is generally assessed by measuring physicochemical parameters (da Silva et al., 2017), such as temperature, pH, phosphorus, nitrogen, oxygen, suspended solids, hardness and alkalinity. Temperature and dissolved oxygen affects the growth and survival of fish and are therefore critical environmental factors (Ferreira et al., 2011). Ammonium (NH_4^+) and nitrite (NO_2^-) are toxic to aquatic life and require treatment by way of conversion to nitrate (NO_3^-) before being released from fish farms (Celik et al., 2001; Durborow et al., 1997; Pollice et al., 2002; Zhang et al., 2011). Orthophosphate (PO_4^{3-}) is a reactive form of phosphorus and is one of the main causes of algal blooms and hypoxic conditions that blooms ensue (Barcellos et al., 2019; Brogan et al., 2001; O'Neill et al., 2019). The biochemical oxygen demand (BOD) is the amount of oxygen bacteria use to break down organic matter, while the chemical oxygen demand (COD) measures the level of stress the organic matter puts on the receiving water system (Lee and Nikraz, 2015). Analysis of these parameters alone, although important, will only indicate potential adverse effects at a given time and not the effects on the ecosystem or organisms therein (da Silva et al., 2017; O'Neill et al., 2019; Stephens and Farris, 2004a). Therefore, ecotoxicological bioassays are used in conjunction with the physicochemical parameters (da Silva et al., 2017). This may be very important as ultimately the WFD is not concerned with exact emission levels as such, but rather what can be emitted such that harmful effects can be avoided, and good or excellent status can be both achieved and maintained (WFD Ireland, 2001). Primary producers (e.g. algae) and primary consumers (e.g. micro-crustaceans) are key components in aquatic food chains (Aruoja, 2011). The microalgae *Pseudokirchneriella subcapitata* (*P. subcapitata*) is commonly used for ecotoxicological assessment of primary producers in multi-trophic testing due to its high sensitivity, reproducibility and growth rate (International Organisation for Standardisation, 2012a; Suzuki et al., 2018). *Daphnia* are freshwater micro-crustaceans commonly used for ecotoxicological assessment of primary consumers in multi-trophic testing. Similarly with *P. subcapitata*, these were also chosen due to their reproducibility and ease of culture (International Organisation for Standardisation, 2012b).

The hypothesis of this study is that use of natural eco-innovations underpinning this IMTA peatland process efficiently treats wastewater to quality level suitable for recycling within the system. Therefore, the aim of this timely research was to introduce the concept of integrated multitrophic aquaculture production using cut-away peatlands (bog) as an Irish case study, and to determine if any adverse environmental effects may occur to this natural habitat arising from developing this novel approach.

2. Methods

2.1. Novel fish farm process and sampling

The multitrophic aquaculture system consists of four split (pill) ponds connected with duckweed lagoon with 16 channels serves as a treatment system (Fig. 1A). Fish are kept at a density that does not exceed the organic farming standard (<20 $kg \cdot m^{-3}$ for perch), using screens at the D-ends of each split pond (Fig. 1B). The space between two D-end fish culture areas is also used to treat waste with free living algae and bacteria in suspension. Flow in each split pond is generated and water is circulated using an airlift. Each D-end fish culture area is equipped with oxygen and temperature probes connected to paddlewheels to provide extra oxygen when necessary. The farm is

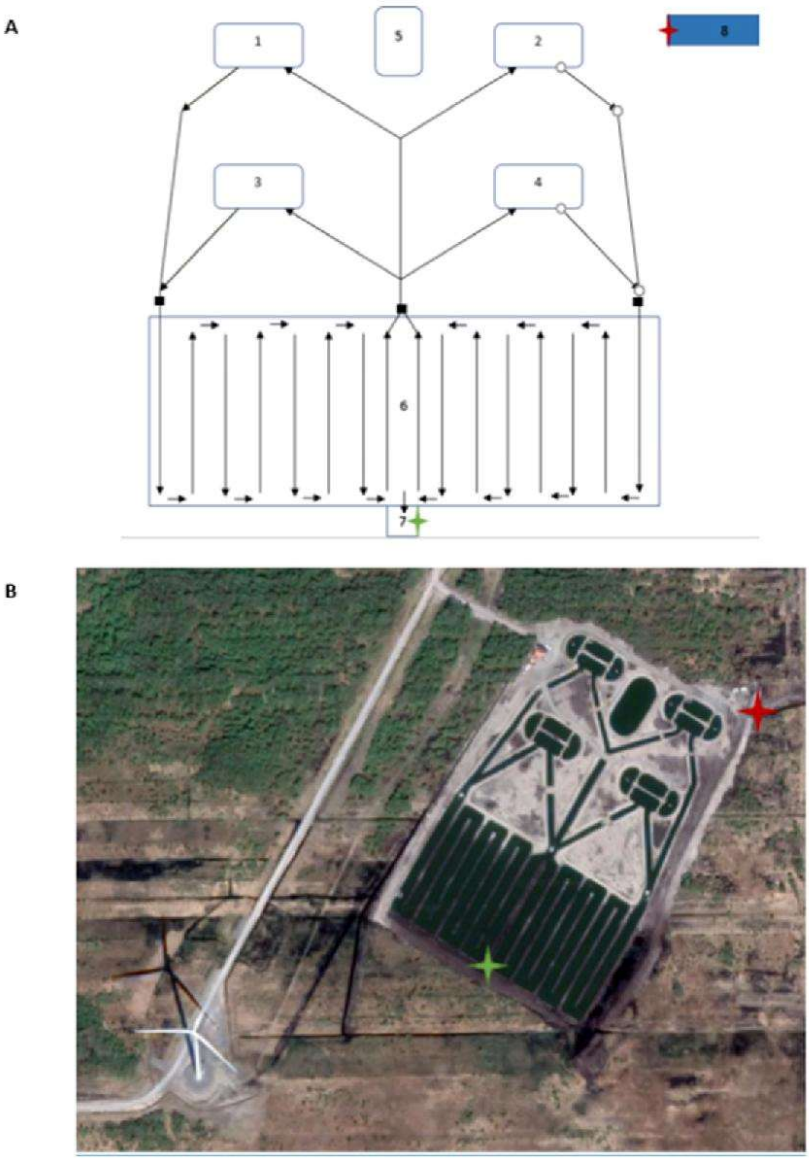

Fig. 1. A) Schematic of trial fish farm, located in Mount Lucas, Co. Offaly (53° 16′ 36″ N, 7° 12′ 34″ W). 1, 2, 3 & 4) Split Ponds for culturing, 5) Water Reservoir, 6) Algae & Duckweed Treatment Channels, 7) Holding Tank, 8) Bog River (intake source). Black lines indicate the direction of the flow of water. Black boxes indicate the locations of the paddle wheels. NOTE: Schematic is not to scale. B) Aerial view of the trial fish farm within the peatlands. The wind turbine used to provide all electrical needs for the farm is included. The D-ends of each split pond can be seen. The red stars indicate the location of the intake sampling point at the peat bog drain. The green stars indicate the location of the holding tank sampling point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Source: Google Maps (2019).

designed to hold a maximum of 32,000 kg of fish. Holdings reached just over 11,000 kg in August (Table 1). The fish farm is powered by electricity from one the wind turbines within the wind farm (Fig. 1B).

Water samples were collected from the trial fish farm located in Mount Lucas Wind Farm, Co Offaly in the Republic of Ireland. The farm, which cultures perch (*Perca fluviatilis*) and rainbow trout

Table 1
Summary of production characteristics on AQUAMÔNA fish farm from May to August 2019.

Month	Actual biomass (kg)	Biomass gain (kg)	Feed applied (kg)	FCR	N applied	P applied	Duckweed area (m^2)	Duckweed area (%)
May	6282	2128	2288	1.08	148	41	182	1.67
June	8410	2373	3144	1.33	206	57	662	6.07
July	10,783	783	3083	3.94	207	58	2999	27.49
August	11,565	1643	3146	1.91	213	59	4982	45.66

FCR = Feed Conversion Ratio, N Applied = Protein % in feed, P Applied = Phosphorus % in feed.

(*Oncorhynchus mykiss*), utilizes a multi trophic approach, and reuses up to 100% of its water. A large algae and duckweed lagoon used to assist in treating wastewater (Fig. 1). The bog is undergoing restoration and is in the process of returning to its natural state before peat extraction begun. The farm is adjacent to a large natural wetland. Grab samples were collected from the trial fish farm located at Mount Lucas Wind Farm (Co. Offaly, Republic of Ireland) in 5 L octagonal carboy HDPE bottles (Lennox) and transported directly for laboratory analysis. Samples were obtained directly from the intake and holding tank sources of the farm once a month, from May to August 2019. The intake samples consisted of water taken directly from the bog. The terminal holding take samples contained water that had been used to culture fish in the split ponds, and had then passed through the algae and duckweed channels for treatment. This water continues on after the holding tanks and returns to the culture ponds to be reused, thus is recirculated. As discharge only occurs during times of high rainfall, resulting in overflow, the samples were taken directly from the holding tank at the discharge point. Discharge did not occur during the sampling period. As shown in Fig. 2, heavy rain was observed in August at three Met Éireann (Irish Meteorological Services) weather stations (Met Éireann, 2019) surrounding the farm however rainfall did not occur on the day of sample collection. Collection occurred on the week day and at approximately the same time. Sampling points are shown in Fig. 1. Due to issues in collecting individual and composite samples, triplicate samples were taken from the single 5 L grab sample from each monitoring point and analysed separately. All samples were tested in triplicate.

2.2. Physicochemical analysis

In order to assess the quality of the water, recommended water quality parameters set out by the Statutory Instrument (S.I.), 77/2019 (European Communities Environmental Objectives – Surface Waters-Regulations 2019), and the Irish Environmental Protection Agency's (EPA), water quality parameters were used as a guidance (Environmental Protection Agency, 2001; Irish Stationery Office, 2009). This was done so as, as far as the authors are aware, water quality parameters for aquaculture holding tank are currently unavailable in Ireland as the Irish EPA are actively investigating the regulation of freshwater aquaculture discharge (O'Neill et al., 2019). Discharge licensing is currently assessed on an individual basis. It should be noted that the guidance values used (S.I. 77/2019, Freshwater Fish Directive [78/659/EEC], and/or the Irish Surface Water Regulations [1989]), are based on freshwater systems and not peatland environments. These freshwater parameters were included as the bogs freshwater receiving system is the Daingean/Philipstown River, 3.8 Km away from the farm. Emission levels set out in the fish farm's current discharge licence were not applicable here as composite sampling over 24 h could not be conducted due to limitations. Also, the system is quite new and full operation only commenced in April.

Water parameters, including; temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, DO, BOD, COD, suspended solids, hardness and alkalinity were analysed in the laboratory within 24 h of collection to eliminate the need for preservation. Spectroquant® photometric kits (Sigma-Aldrich) were used to assess the NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and COD. Analysis was performed as per the manufacturer's instructions. Temperature and pH were analysed using a VWR pHenomenal™ MU 6100 L meter and VWR 111 662-1157 pH probe. DO and BOD_{5day} were analysed using a Jenway 9500 Dissolved O_2 meter and probe. Suspended solids were analysed via filtration using 7 cm 0.45 μm filter paper. Hardness was assessed via titration using pH 10 buffer, Erichrome black and EDTA. Alkalinity was analysed by titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid. See Table 2 for a summary of all standard methods and their corresponding method numbers used in this research.

2.3. Ecotoxicity testing

The freshwater unicellular green algae *P. subcapitata* (also known as *Raphidocelis subcapitata*) and the freshwater crustacean *Daphnia pulex* (*D. pulex*) were used in the ecotoxicity test. A starter culture of the *P. subcapitata* was obtained from The Culture Collection of Algae and Protozoa (CCAP 278/4; SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.), and grown in standard Jarworski's medium under controlled conditions of 23 °C ± 2 °C exposed to continuous illumination (lux 6000–10,000). Sub-culturing was conducted every two to three days to ensure the growth rate remained in the exponential phase. Ecotoxicity testing was conducted as per the Water quality – Fresh water algal growth inhibition test with unicellular green algae ISO (8692:2012) guidelines. The *P. subcapitata* was exposed to the intake and holding tank samples for 72 h under static conditions at 23 °C ± 2 °C, and exposed to the continuous illumination. Inhibition of the algae growth rate, in percent, was calculated by comparing the samples to a negative control containing just the Jarworski's medium. Eqs. (1), (2) and (3) were taken directly from the ISO (8692:2012) guidelines (International Organisation for Standardisation, 2012a) and calculations were conducted as follows;

$$\text{Algal cells mL}^{-1} = \frac{n}{0.02} \times 10^3 \quad (1)$$

where

n = number of cells counted using a haemocytometer

$$\text{Average specific growth rate } (\mu), = \frac{1nX_n - 1nX_0}{T_n - T_o} \quad (2)$$

where

ln = natural log of
X_n = Algae cells mL^{-1} at 72 h
X_0 = Algae cells mL^{-1} at 0 h
T_n = Duration of test
T_0 = Time zero

$$\text{Percent inhibition in growth rate} = \frac{C\mu\text{-}T\mu}{C\mu} \times 100 \quad (3)$$

where

Cμ = Average specific growth rate for control

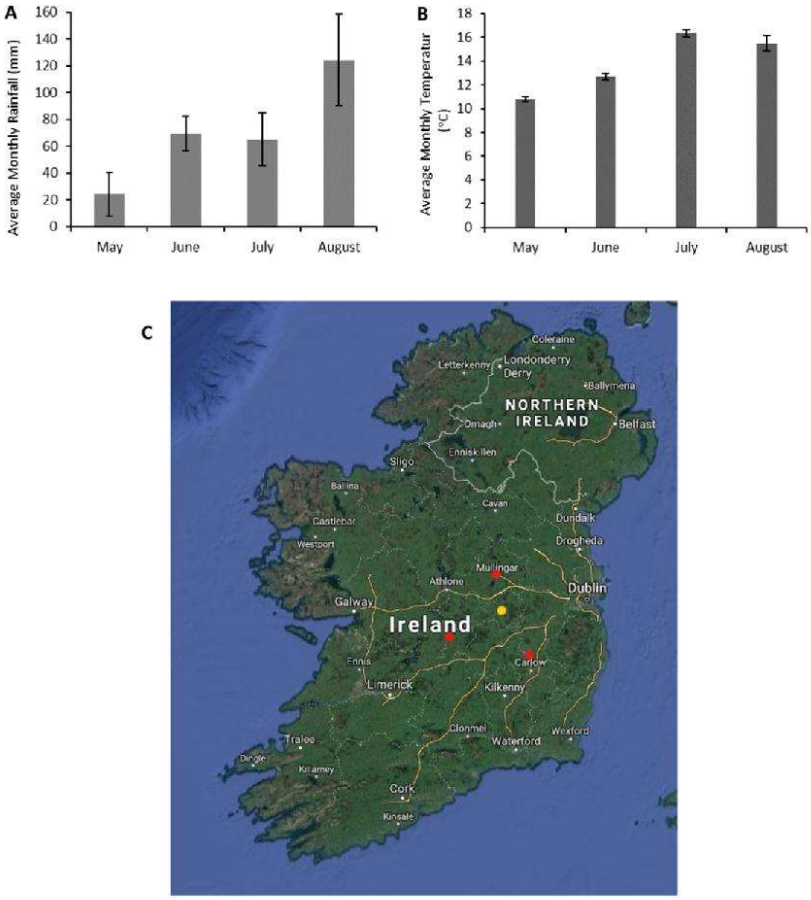

Fig. 2. (A) Average monthly rainfall and (B) average monthly temperature recorded at three of Met Eireann's weather stations surrounding Mount Lucas Wind Farm during the sampling period of May 2019 to August 2019. (C) Location of the three Met Eireann weather stations (Red) surrounding the Mount Lucas Wind Farm (Yellow). The three stations observed were Mullingar, Gurteen and Oak Park. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of methods used for the analysis of water quality in the trial fish farm intake and holding tank water samples. The standard water and wastewater analysis methods and their corresponding number are listed. Detection limits for all kits have been included.

Parameter	Standard method & number	Detection limit (mg L^{-1})
Alkalinity	Titrimetric (2320-B)	–
BOD	Membrane electrode (5210-B)	–
COD	Photometric (5220-D)	0–150 15–300
DO	Membrane electrode (4500-O G)	–
Hardness	Titrimetric (2340-C)	–
NH_4^+	Photometric (4500-NH_3-F)	0.013–3.86 2.6–193.0
NO_2^-	Photometric (345-1)	0.007–3.28
NO_3^-	Photometric (4500-NO_3)	0.4–110.7
pH	Membrane electrode (2310-B)	–
PO_4^{3-}	Photometric (4500-P-C)	0.007–15.3 1.5–92.0
Suspended solids	Gravimetric (2540-D)	–
Temperature	Thermometer (2550-B)	–

$T\mu$ = Average specific growth rate for treatment

An in-house stock of the freshwater crustacean *D. pulex* was maintained in aerated spring water. Sub-culturing was conducted twice a week to ensure the culture conditions of the crustacean remained at optimum levels. Ecotoxicity testing was conducted as per the Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test ISO (6341:2012) guidelines. *D. pulex* were used over the more common *Daphnia magna,* as the former species are more commonly found in Irish water systems. The *D. pulex* was exposed to the intake and holding tank samples for 24 h under static conditions at 20 °C ± 2 °C, and exposed to a photoperiod of 16 h bright and 8 h dark. Immobilisation, in percent, was calculated by comparing the samples to a negative control containing just the aerated spring water. *D. pulex* was deemed immobile when no movement was observed after 15 s under gentle agitation. The percent immobilisation equation was taken directly from the ISO (6341,2012) guidelines (International

Organisation for Standardisation, 2012b) and calculations were conducted as follows;

$$\text{Percent immobilisation} = \left(1 - \frac{T}{C}\right) \times 100$$

where

T = Total number of mobile *D. pulex* in the treatment
C = Total number of mobile *D. pulex* in the control

2.4. Statistical analysis and hypothesis

All statistical analyses were conducted using GRAPHPAD PRISM 8 and MINITAB 18. The data generated were grouped and subject to normality tests (Anderson-Darling). *t*-Tests were used to identify significant differences in the variables. $P < 0.05$ indicated a statistically significant difference. Pearson's correlation was used to assess any correlations between the parameters investigated.

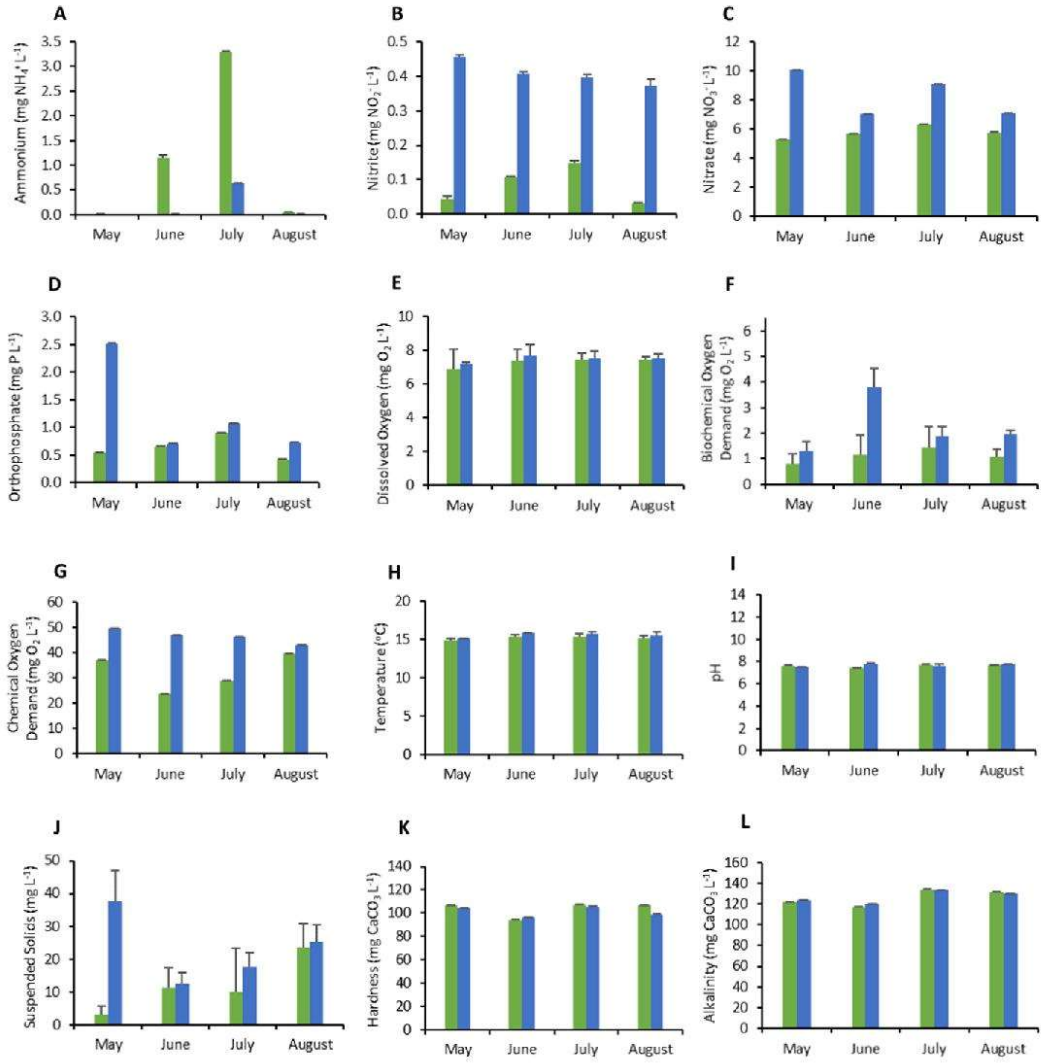

Fig. 3. Bar charts indicating the physicochemical results determined for the trial fish farm intake samples (green), and holding tank samples (blue), from May 2019 to August 2019. Parameters investigated were A) NH_4^+, B) NO_2^-, C) NO_3^-, D) PO_4^{3-}, E) dissolved oxygen, F) Biochemical Oxygen Demand, G) Chemical Oxygen Demand, H) temperature, I) pH, J) suspended solids, K) hardness and L) alkalinity. S.D. indicated, n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Physicochemical parameters

The results determined for the physicochemical parameters investigated on the trial fish farm intake and holding tank samples are displayed in Fig. 3. Increases in concentrations from the intake to the holding tank were observed in NO_2^-, NO_3^-, PO_4^{3-}, BOD, COD and suspended solids. Similar results between the samples were observed in DO, temperature, pH, hardness and alkalinity. The only parameter to display a decrease in concentration between the intake and holding tank was NH_4^+. When the intake and holding tank samples were compared via statistical analysis (*t*-tests), a significant difference was observed in the NO_2^- ($P \leq 0.001$), NO_3^- ($P = 0.016$), and COD levels ($P = 0.011$).

3.2. Ecotoxicological bioassays

The levels of growth rate inhibition observed in the intake and holding tank samples can be found in Fig. 4A. With the exception of the July sample, growth rate inhibition decreased from the intake to the holding tank water. Statistical analysis (*t*-test) was conducted between the intake and holding tank samples and no significant differences were observed ($P = 0.402$).

The levels of immobility observed in the intake and holding tank water can be found in Fig. 4B. Increased immobilisation was observed from the intake to the holding tank samples during May and July. Immobility remained unchanged in the month of June. August displayed a decrease. Statistical analysis (*t*-test) was conducted between the intake and holding tank samples, and no significant differences were observed ($P = 0.390$).

3.3. Correlation studies

Positive correlations between two parameters indicate that as one parameter increases or decreases, so too does the other. Negative correlations between two parameters indicate that as one parameter increases or decreases, the opposite occurs in the other. The Pearson's correlation matrix (Table 3) demonstrated that in the intake water, the *P. subcapitata* was negatively correlated with the pH and hardness. The pH was positively correlated with the hardness. The orthophosphate was positively correlated with the ammonium and nitrite. The nitrate was positively correlated with the BOD. In the holding tank water (Table 3), the *P. subcapitata* was positively correlated with the ammonium. The *D. pulex* was negatively correlated with the pH, and positively correlated with the nitrate and orthophosphate. Suspended solids were negatively correlated with temperature and dissolved oxygen. Nitrite was positively correlated with the COD.

4. Discussion

4.1. Physicochemical analysis

In order to determine the potential effects and alterations aquaculture processes may have on the water quality of Irish peatlands, intake and terminal holding tank water samples were compared to one another. Nitrification is the process by which NH_4^+ is firstly converted to NO_2^- and then to NO_3^- via enzymatic oxidation (Subba Rao et al., 2017). The presence of NH_4^+ in freshwater systems is an indication of recent pollution (Zhang et al., 2011). The NH_4^+ levels detected in the terminal holding tank water were lower than that of the intake water. This suggested that the fish farms and its treatment processes were reducing the levels of NH_4^+ present in the bog water. The NO_2^- levels in the holding tank water were significantly greater than the intake. Two very important points need to be highlighted when interpreting these results; 1) grab samples not composite samples were collected and analysed, and 2) water in the holding tank is only discharged from the farm when overflow occurs as a result of very heavy rainfall. Therefore, the concentration being released would be considerable reduced as the rain water would dilute the levels present. The results were similar to levels detected in previous aquaculture studies (Table 4), and therefore no issues were foreseen. Results show that almost complete nitrification had taken place (i.e. NO_2^- concentrations were low in the holding tank relative to NO_3^- concentrations). The NO_2^- concentrations measured could be further reduced if water was present in the microalgae and duckweed treatment bed for long enough. Also, as this is a new process, the combined microalgae and duckweed pond was not yet working to its maximum capacity (Table 1). Therefore, additional research would need to be conducted to establish the exact cause of the increase as the lack of ammonium could indicate that a low retention time may be unlikely. NO_3^- levels detected in the holding tank were also significantly greater, than that of the intake. The increases in the concentrations of these compounds were due to the rise in the feed applied, as a result of increased stocking densities (Table 1). The bog is undergoing restoration and is in the process of returning to its natural state before peat extraction begun. The release of these nitrogen compounds may assist in the restoration of the recovering bog, as plants require nitrogen for growth and development (Xu et al., 2012). However, as peatlands are normally nutrient poor, additional research would need to be conducted to fully assess its effects.

High levels of phosphorus in freshwater systems can result in algal blooms and hypoxic conditions (O'Neill et al., 2019). However, phosphorus is also an essential element for plant growth (Vance et al.,

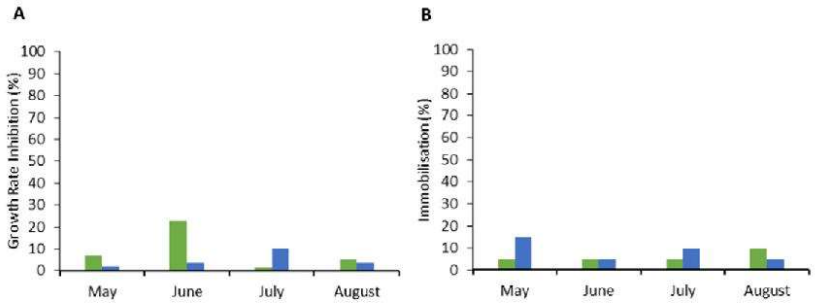

Fig. 4. Bar charts displaying the results determined from the A) *Pseudokirchneriella subcapitata* bioassay and B) *Daphnia pulex* bioassay investigated on the trial fish farm intake samples (green), and holding tanks samples (blue), from May 2019 to August 2019. S.D. indicated, n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Correlation matrix for the parameters investigated on the Irish trial peatland fish farm intake and holding tank water. Bold figures indicate where significant differences ($P < 0.05$) have been observed. Breakdown of correlation figures are indicated in the box.

	P. sub	*D. pul*	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H
Intake													
D. pul	−0.288							**0 = No relationship**					
T	0.271	−0.041						**>0–0.3 = Weak relationship**					
pH	**−0.985**	0.437	−0.199					**0.3–0.5 = Moderately weak relationship**					
NH_4^+	−0.211	−0.465	0.699	0.172				**0.5–0.7 = Moderately strong relationship**					
NO_2^-	0.105	−0.610	0.764	−0.150	0.948			**0.7–<1 = Strong relationship**					
NO_3^-	−0.349	0.008	0.798	0.388	0.881	0.757		**1 = Perfect linear relationship**					
PO_4^{3-}	−0.128	−0.687	0.554	0.046	**0.961**	**0.951**	0.716						
DO	0.093	0.359	0.916	0.031	0.495	0.484	0.777	0.268					
BOD	−0.158	−0.069	0.895	0.199	0.891	0.829	**0.980**	0.740	0.829				
COD	−0.628	0.662	−0.722	0.661	−0.624	−0.839	−0.383	−0.681	−0.397	−0.543			
SS	−0.093	0.905	0.383	0.263	−0.171	−0.259	0.305	−0.432	0.714	0.285	0.289		
H	**−0.971**	0.333	−0.485	**0.951**	−0.014	−0.320	0.117	−0.067	−0.280	−0.081	0.776	0.052	
A	−0.874	0.487	0.176	0.927	0.389	0.091	0.673	0.196	0.400	0.520	0.422	0.469	0.765
Holding tank													
D. pul	−0.025												
T	0.599	−0.740											
pH	−0.059	**−0.990**	0.723										
NH_4^+	**0.980**	0.174	0.450	−0.253									
NO_2^-	−0.358	0.837	−0.671	−0.751	−0.178								
NO_3^-	0.149	**0.985**	−0.634	**−0.991**	0.342	0.758							
PO_4^{3-}	−0.335	**0.950**	−0.889	−0.916	−0.142	0.895	0.882						
DO	0.251	−0.899	0.925	0.913	0.076	−0.669	−0.852	−0.930					
BOD	−0.099	−0.707	0.691	0.790	−0.225	−0.253	−0.730	−0.644	0.870				
COD	−0.217	0.764	−0.473	−0.670	−0.050	**0.971**	0.707	0.780	−0.502	−0.090			
SS	−0.483	0.737	**−0.981**	−0.746	−0.338	0.562	0.654	0.851	**−0.952**	−0.809	0.353		
H	0.505	0.817	−0.379	−0.880	0.656	0.404	0.900	0.615	−0.703	−0.805	0.374	0.474	
A	0.754	0.000	0.217	−0.138	0.730	−0.537	0.143	−0.227	−0.073	−0.546	−0.553	−0.028	0.552

P. sub = *P. subcapitata*, *D. pul* = *D. pulex*, T = Temperature, SS = Suspended Solids, H = Hardness, A = Alkalinity.

2003). The PO_4^{3-} levels detected in holding tank were greater that than of the intake samples. This rise was due to the increased application of feed (Table 1). Once again, this concentration would be greatly decreased when diluted by rain water. Concentrations in the holding tank were greater than that of the intake however the difference was not statistically significant suggesting that the introduction of aquaculture processes did not appear to have altered the quality of the water. Additional research may need to be conducted in order to determine its effects on the receiving natural wetland, as well as to develop improved treatment methods if required. It should also be noted that the algae and duckweed channels used for treatment were not working to their maximum capacity (Table 1).

Similar DO levels were observed in both samples. The use of the and inclusion of paddle wheels in the farm allows workers to maintain full

Table 4
Summary of previous studies conducted on aquaculture discharge. Location, culture species and physicochemical parameters used have been listed. Many studies included additional parameters however, for the purpose of this paper, only the same physicochemical parameters investigated in this study have been included.

Culture species	Location	Physicochemical Parameters	Reference
Rainbow Trout (*Oncorhynchus mykiss*)	Portugal	DO, BOD, A, NH_4-N, PO_4-P, SS, pH, H, NO_3-N, NO_2-N, T	Boaventura et al. (1997)
Rainbow Trout (*Oncorhynchus mykiss*) & Brown Trout (*Salmo trutta*)	Spain	H, pH, DO, T, NO_3^-	Camargo (1994)
Range of Marine & Freshwater Aquaculture	China	NH_4-H, COD, BOD, SS	Cao et al. (2007)
Rainbow Trout (*Oncorhynchus mykiss*)	Brazil	PO_4-P, NH_4-N, NO_2-N, NO_3-N, pH, SS, DO, T	Caramel et al. (2014)
Banana Shrimp (*Penaeus merguiensis*)	Australia	T, pH, SS, NH_4^+, NO_3^-	Costanzo et al. (2004)
Shrimp (*Litopenaeus vannamei*)	Brazil	pH, DO, SS, NO_2^-, NO_3^-, PO_4^{3-}, COD	da Silva et al. (2017)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	DO, BOD, PO_4-P, NO_3^-, NO_2^-, H, pH, SS	Fadaeifard et al. (2011)
Rainbow Trout (*Oncorhynchus mykiss*)	France	NH_4^+, NO_3^-, PO_4^{3-}, SS	Guilpart et al. (2012)
Atlantic Salmon (*Salmo salar*)	Canada	SS, NO_3^-, pH, A, H, PO_4^{3-}, BOD	Lalonde et al. (2014)
Nile Tilapia (*Oreochromis niloticus*)	Brazil	NH_4-N, PO_4^{3-}, pH, DO, T	Miashiro et al. (2012)
Freshwater Prawn (*Macrobrachium rosenbergii*)	Brazil	T, pH, NH_4^+, NO_2^-, NO_3^-, DO, BOD, H	Moreira et al. (2010)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	DO, pH, SS, T	Namin et al. (2013)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	T, pH, DO, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}	Ghorbani et al. (2013)
European Perch (*Perca fluviatilis*)	Ireland	T, pH, DO, BOD, COD, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, A, H, SS	O'Neill et al. (2019)
Rainbow Trout (*Oncorhynchus mykiss*)	Turkey	T, DO, pH, BOD, SS, NO_2^-, NO_3^-	Pulatsü et al. (2004)
Channel Catfish (*Ictalurus punctatus*)	United States of America	pH, DO, T, A, H, SS, PO_4^{3-}, NO_2^-, NO_3^-, NH_4-N	Stephens and Farris (2004a)
Channel Catfish (*Ictalurus punctatus*)	United States of America	NO_3^-, NO_2^-, SS, PO_4^{3-},	Stephens and Farris (2004b)
Rainbow Trout (*Oncorhynchus mykiss*)	Ireland	BOD, NO_2-N, PO_4-P, SS, NH_4-N, T, DO, pH	Tahar et al. (2018a)
Range of Marine & Freshwater Fish & Shrimp	Hawaii	PO_4^{3-}, SS, NH_4-N	Ziemann et al. (1992)
Rainbow Trout (*Oncorhynchus mykiss*)	Serbia	PO_4^{3-}, NO_2^-, NO_3^-, DO, T, pH, NH_4^+	Živić et al. (2009)

T = temperature, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, NH_4-N/NH_4^+ = ammonium, NO_2-N/NO_2^- = nitrite, NO_3-N/NO_3^- = nitrate, PO_4-P/PO_4^{3-} = orthophosphate, A = alkalinity, H = hardness, SS = suspended solids.

control of oxygen levels within the farm ensuring optimum levels are sustained. Alam et al. (2007), da Silva et al. (2017) and O'Neill et al. (2019) have suggested that concentrations of ≥4 mg L^{-1} are sufficient to maintain aquatic life, therefore current DO levels were not considered to be any cause for concern. Similar BOD levels were observed in both samples. As the water quality remained unchanged, no issues were foreseen. COD levels detected in the holding tank were significantly greater than that of the intake. This may have highlighted a potential issue. However, the levels detected in this study were lower than those conducted on previous traditional aquaculture studies (Cao et al., 2007; da Silva et al., 2017; O'Neill et al., 2019). Additionally, this level of COD would not be released as dilution would occur after the heavy levels of rainfall that would result in its release.

Suspended solids can cause increased gill irritation. With the exception of a spike observed in May that may have resulted after recent rainfall, suspended solids were similar in both samples. Water temperatures remained consistent in both samples, as too did the pH values. Water may not be released from facilities if temperatures above 20 °C are observed, and the temperature of the samples may have risen during its transportation to the laboratory. Therefore, there were no problems with the temperature. The pH values were well within the recommended value of between pH 6 to pH 9. Calcium carbonate ($CaCO_3$) improves conditions for benthic animals and microbial activity and increases CO_2. The alkalinity is the buffering capacity of the water body and is related to important factors in fish farming. Water hardness is the amount of dissolved calcium and/or magnesium present in the water (Ferreira et al., 2011; O'Neill et al., 2019). Both alkalinity and hardness were measured as $CaCO_3$. The consistent measurement of alkalinity ensured the pH also remained consistent. Results for hardness suggested that water was slightly too moderately hard.

Some of the results observed (NO_2^-, NO_3^-, PO_4^{3-} and COD), suggested potential problems. No research conducted on the use of peatlands for aquaculture could be found in the available literature. Additionally, this research only focused on one fish farm in a peatland setting. As a result, all physicochemical results were then compared to a range of studies conducted on traditional fish farms. All results obtained were similar to or below the concentrations determined in the previous aquaculture studies investigated, including NO_2^-, NO_3^-, PO_4^{3-} and COD (Table 4). This indicated that there appeared to be no observable differences between the water quality after traditional aquaculture settings, and that of bog-land settings.

The aforementioned highlight that duckweed and microalgae can be exploited in this IMTA process for efficient assimilation of nutrients along remediation and wastewater treatment (Han et al., 2019). In addition to treating wastewater, microalgae could synthesize value-added components such as proteins, lipids and natural pigments for fish nutrition and disease mitigation, along with providing a high capacity for generating oxygen that could act like a bio-pump for aeration of aquaculture and positively adjust microbial communities (Han et al., 2019). Water deterioration in aquaculture is typically attributed to an excessive amount of aquaculture feed and wastes excreted by consuming fish that are stocked to high density. Nitrogen is one of the compositions of this waste and a high concentration of ammonium in a water body can be toxic to fish. Common forms of nitrogen in wastewater including ammonium (NH_4^+-N), nitrate (NO_3^--N) and nitrite (NO_2^--N). Ammonium can be absorbed by microalgae cells through active transport and directly utilized for amino acid synthesize, while nitrate and nitrite absorbed by microalgae through active transport have to be converted to ammonium by nitrate reductase and nitrite reductase before undergoing further assimilation (Sanz-Luque et al., 2015; Han et al., 2019). Exploiting this IMTA aquaculture process may also accelerate CO_2 fixation and promote oxygen release for farmed fish. However, future research that inform parameters of the C/N ratio, light intensity and quality, and carbon forms would be relevant in order to enhance carbon assimilation, further promoting nitrogen assimilation (Han et al., 2019).

4.2. Ecotoxicological analysis

Low levels of inhibition in the growth rate of the *P. subcapitata* were observed in both the intake and terminal holding tank water. This suggested that the quality of water in the intake samples and the holding tank water, in terms of its effect on the algae, remained unchanged and that the water within the bog was unlike to cause growth inhibition, therefore the recovery of the bog would not be affected. Most of the previous research using *P. subcapitata* in assessing water were based on polluted river systems and drainage water (Guéguen et al., 2004; Ivanova and Groudeva, 2006). These studies displayed higher growth rate inhibition levels than those observed in this study. Only two previous studies could be found in the available literature that focused on the use of the algae in the context of aquaculture. The research conducted by O'Neill et al. (2019) found considerably higher inhibition levels than those reported in this study. Miashiro et al. (2012) and O'Neill et al. (2019) also reported stimulation of growth in the algae which could results in eutrophic conditions. As no stimulation of the algae was observed in this study, issues with eutrophic would seem highly unlikely. Flow levels of immobilisation in the *D. pulex* were observed in both samples. This suggested that the water quality seemed unlikely to cause any adverse effects on any of the aquatic organisms present in the bog. However, no studies in the available research focused on the use of *D. pulex* to assess aquaculture discharge. Despite the low levels of toxicity observed in this short study, further ecotoxicological tests must be conducted in order to fully determine the effects of the aquaculture process on the receiving natural wetland. Equally important is the potential effects the quality of the bog water may have on fish themselves. Therefore, additional ecotoxicological assessments also need to be conducted in order to ascertain any possible health risks to the fish.

5. Conclusion

Although sampling in this case study was limited to only four months, no differences were observed between the physicochemical parameters of the intake and holding tank samples, with the exception of NO_2^-, NO_3^- and COD, indicating that aquaculture processes did not adversely alter the quality of the water. The sub-lethal levels of toxicity observed in the ecotoxicological bioassays have also demonstrated that the introduction of aquaculture processes into peatlands may be unlikely to cause any major adverse effects on the ecosystem and organisms within it. This suggested that the use of cutaway bog as future locations for integrated multitrophic aquaculture (IMTA) processes holds promising potential. This study demonstrates that this new IMTA peatland process, that exploits use of microalgae and duckweed using organic principles for wastewater remediation, has a positive influence of water quality control. This IMTA process also offers an alternative low-cost approach to traditional technologies that require high energy consumption such as aeration, filtration, and anaerobic-anoxic-oxic systems, which may not fully utilize and recycle nutrients including nitrogen, phosphorous and carbon in wastewater. The use of bogs may aid in achieving the aims set on in the Food Wise 2025 initiative to increase Irish aquaculture production to 81,700 by 2023, assisting in the enhancing freshwater aquaculture processes. This constitutes the first study to report on sustainable development of new fish farms in cutaway bogs that were powered by wind turbine and used algae and duckweed as natural processes for treating water adopting organic principles. IMTA may also assist in providing an economically sound alternative for sustainable land use for stakeholders once all peat harvesting processes cease. However, while results so far have indicated positivity, additional research needs to be conducted in order to fully appreciate all parameters governing large scale up of this peatland aquaculture process to ensure commercially viability that includes fine tuning hydronamics, microbial ecology and probiotic benefits of microalgal, bacterial and duckweed interactions.

Declaration of competing interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

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Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland

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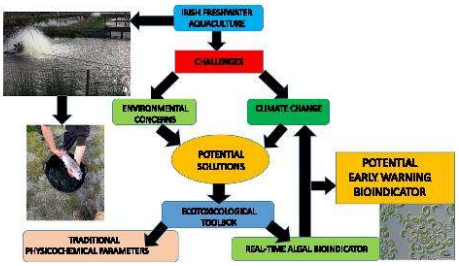
HIGHLIGHTS

- Traditional monitoring of aquaculture outputs using physicochemical parameters has limited efficacy.
- Complementary use of algae supports environmental monitoring of aquaculture.
- Duckweed supports and improves efficacy of aquaculture wastewater treatment.
- Algae are a potentially rapid and sensitive bioindicator of aquaculture water quality.
- Algae are a potential early warning tool for assessing impacts of climate change in aquaculture.

GRAPHICAL ABSTRACT



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ABSTRACT

Aquaculture is one of the fastest growing food producing industries globally, providing ~50% of fish for human consumption. However, the rapid growth of aquaculture presents a range of challenges including balancing environmental impact that can be influenced by variations in climatic conditions. Monitoring of physicochemical parameters is traditionally used to evaluate aquaculture output quality; however, this approach does not indicate the cumulative ecotoxicological effects on receiving waters. Specifically, this case study investigated the relationship between measuring traditional physicochemical parameters and the health of the alga *Pseudokirchneriella subcapitata* in order to evaluate the potential ecotoxicological effects of freshwater aquaculture on the receiving aquatic ecosystem in the Irish midlands. This constituted the first 2-year longitudinal study conducted in 2018 and 2019 that reports on the efficacy of using algae as a natural bioindicator to monitor and assess freshwater aquaculture wastewater from a traditional flow-through fish farm producing Eurasian Perch (*Perca fluviatilis*); monitoring was compared over a same six-month period in the same location each year. Findings demonstrated significant differences between the two monitoring periods when using *P. subcapitata* for assessing the quality of aquaculture intake ($P = 0.030$) and output ($P = 0.039$). No stimulatory effects were observed during 2019 unlike >50% rates experienced the previous year. These observations coincided with changes in climatic conditions whereby the 2018 period experienced extended levels of drought; whereas non-drought conditions were observed during 2019. Findings suggest that reliance upon traditional monitoring techniques may not provide

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sufficient robustness or versatility to address emerging issues, such as extremes in climate variance, which may influence the future intensive sustainability of freshwater aquaculture. This research supports the complementary use of *P. subcapitata* as a rapid and simple early-warning bioindicator for measuring aquaculture output quality on receiving aquatic ecosystems.

1. Introduction

The depletion of wild capture fishery practices has resulted in the rapid development of aquaculture (Han et al., 2019) making it the fastest growing food producing industry worldwide (Ottinger et al., 2016; O'Neill et al., 2019, 2020). According to the FAO (2018), aquaculture now accounts for ~50% of fish produced for human consumption; this figure is expected to rise to ~62% by 2030 (Fredricks et al., 2015; Liu et al., 2017). The dramatic increase in aquaculture production is attributed to over exploitation of wild fisheries that are now at their maximum sustainable yields, along with increased consumer demand for fish (Tahar et al., 2018a, 2018b). Farmed fish is rich in protein and is also a more efficient protein utilisation and feed conversion source than other animals destined for protein production (Tschirner and Kloas, 2017). However, despite its numerous advantages, the rapid increase in aquaculture production has resulted in the emergence of several issues within the industry that include limitations in water and space, increased incidences of disease and increased environmental concerns (Ngo et al., 2016; Troell et al., 2017; Han et al., 2019; O'Neill et al., 2019). Stenevik and Sundby (2007) have also indicated that variations in climatic conditions have demonstrated substantial effects on increases as well as decreases in stocking densities; therefore, the success of fish stock assessment depends to a large extent on the ability to predict impacts climate change has on the dynamics of aquatic ecosystems. These treats have hindered the sustainable development and expansion of the industry (Han et al., 2019).

The ecological importance of algae have received consideration in studies focusing on natural approaches to wastewater remediation in freshwater aquaculture (Naughton et al., 2020) including potential influence of climate variance on process performance (O'Neill et al., 2019). Previous researchers have also noted the potential of algal communities to exhibit many attributes as biological indicators of spatial and temporal environmental change (Omar, 2010); additionally, microalgae have been reported as potentially useful monitoring quality of water bodies (Zaghloul et al., 2020; Parus and Karbowska, 2020; Kadam et al., 2020; Tsarenko et al., 2021). Parus and Karbowska (2020) recently reported on the possibility of using the algae *Ulva* and *Cystoseira* as natural indicators of environmental cleanliness given that these species where shown to accumulate metals. Parmar and Rawtani (2016) described several potential advantages for use of bioindicators, namely (1) biological impacts can be determined; (2) potential synergetic and antagonistic impacts of various combined pollutants on ecosystems can be exhibited; (3) early stage diagnosis of putative harmful effects of toxins on human and animal health can be monitored; and (4) can be considered as a potentially viable economic alternative to use of conventional sophisticated methods.

According to Rindi (2014), terrestrial algae (green algae and diatoms) are more directly affected by climate change and can therefore respond in a more immediate way. This is attributed in part to the fact that algae have short generations, fast turnovers and respond quickly to changes in environmental conditions. Sarmaja-Korjonen et al. (2006) demonstrated that algae appeared to be comparatively good indicators of environmental conditions by representing productivity disparities during changing climatic conditions. Hallegraeff (2010) has also indicated that changes in algal communities can putatively provide a sensitive early warning for climate-driven uncertainties in aquatic ecosystems. There has been increased interest in alternative uses for microalgae within aquaculture to assist with sustainability, in addition to enabling ecotoxicological assessment and water quality control (Han et al., 2019; O'Neill et al., 2019). According to Han et al. (2019), microalgae can also be utilised in aquaculture for wastewater assimilation, oxygen production and partial feed replacement. The microalgae *Pseudokirchneriella subcapitata* (*P. subcapitata*) has previously been suggested as a potential early warning indicator for altering issues associated with in aquaculture processing due to environmental variances, including climate change (O'Neill et al., 2019).

Fish farm wastewater is traditionally high in nutrient rich products (Ngo et al., 2016; Sikder et al., 2016). Nitrogen, phosphorus and organic matter are characteristic of this nutrient rich waste which is normally as a result of metabolic waste products and left over food (Jegatheesan et al., 2011; O'Neill et al., 2019). If this is released into a water body untreated, water pollution will develop leading to issues that may include eutrophication in that aquatic system (Martinez-Porchas et al., 2014). Eutrophication occurs when a water body is put under pressure with large levels of organic matter and nutrient waste that is taken in and biologically processed which in turn leads to algal blooms (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Sikder et al., 2016). Algal blooms in turn can lead to decreases in light and oxygen production, which can suffocate aquatic life (Jegatheesan et al., 2011; Chislock et al., 2013; O'Neill et al., 2019). Organic matter and nutrient waste is typically as a result of the application of artificial feed supplementation which is necessary in order to increase and maintain yields to meet the increased demands (Kolarevic et al., 2014; Feucht and Zander, 2015; O'Neill et al., 2019).

Water quality is typically assessed to determine the potential effects it may have in its receiving system; this is traditionally conducted by means of physicochemical analysis (da Silva et al., 2017). The use of these parameters alone will only provide a limited window in time of the water quality for a system (O'Neill et al., 2019; O'Neill et al., 2020). Inclusion of bioassays to assess the potential effects on aquatic ecosystems and the organisms therein will provide a broader scope on the quality of water. Microalgae are primary producers and are keystones in aquatic food chains. They represent an imperative group of highly sensitive photosynthetic organisms frequently used to assess aquatic systems (Rodgher et al., 2012). *Pseudokirchneriella subcapitata* (*P. subcapitata*) is unicellular green algae most commonly used and recommended for ecotoxicological assessment due to its being inexpensive, and both highly reliable and reproducible (ISO, 2012).

The hypothesis of this study is that algal traditionally used in ecotoxicology bioassays can be further utilised for the real-time sustainable enhancement of aquaculture as it provides a potential means of monitoring the influence of adverse environmental effects caused by extreme weather events attributed to variances in climate. Thus, the aim of this research is to determine the robustness of *P. subcapitata* as a putative early warning bio-indicator for monitoring impact of climate variance using an Irish freshwater aquaculture farm as a case study.

2. Materials & methods

2.1. Sampling

Intake and output water samples were collected from a freshwater fish farm located in Boyle, Co. Sligo (Fig. 1). The farm cultures European perch (*Perca fluviatilis*) and consists of three culture ponds that use a flow through system, a hatchery and nursery that use a recirculating aquaculture system (RAS) and a constructed wetland that is used for culture water treatment. Grab samples were collected

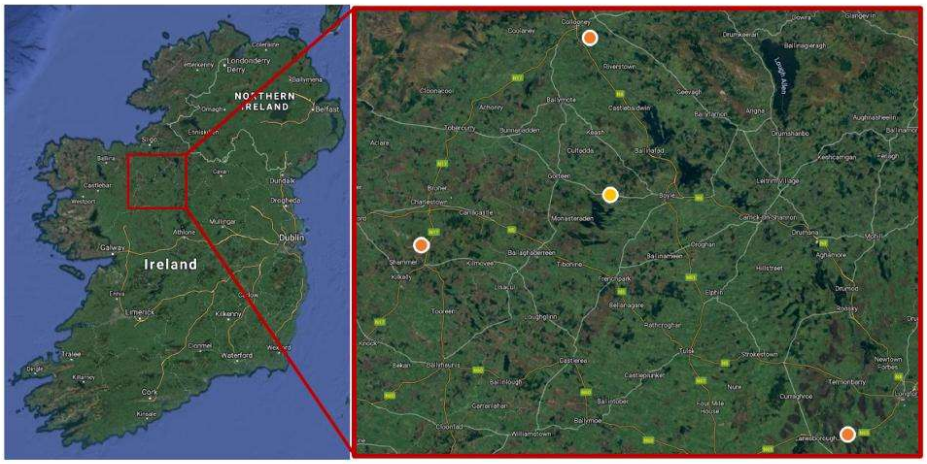

Fig. 1. Map of Ireland indication the approximate location of the freshwater fish farm (53°58′16″ N, 08°24′44″ W) indicated with yellow, and the three closest Met Eireann weather stations (Markree – 54°10′30″ N, 08°27′20″ W; Mount Dillon – 53°43′37″ N, 07°58′51″ W; Knock – 53°54′22″ N, 08°49′4″ W) surrounding the farm, indicated by orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in 5 L octagonal carboy HDPE bottles (Lennox) and transported directly to the lab via car approximately 70 km away. Samples were collected directly from the intake and output sources once a month from March 2019 to August 2019 as this was the time period analysed during the previous study at same freshwater aquaculture farm reported in O'Neill et al. (2019). Samples from the settlement pond were also collected for analysis in order to determine the treatment efficacy of the constructed wetland, which was not fully operational until June 2019 reflecting period of maintenance. Wastewater collection occurred on the same day, at approximately the same time, during each month of monitoring where sampling points are displayed in Fig. 2. Triplicate samples were analysed from the same 5 L grab sample.

2.2. Physicochemical analysis

The Statutory Instrument (S.I.) 77/2019, S.I. 272/2009, and the Irish Environmental Protection Agency's (EPA) water quality parameters (Environmental Protection Agency, 2001; Irish Statutory Office, 2009; Irish Statutory Office, 2019) were followed to measure water quality parameters. Discharge licensing in Ireland is currently based on an individual basis. Grab samples collected represented 30 min of the 24 h period; composite sampling was not possible. To compensate for the latter, results complied in this study were also compared to previous research studies conducted on a range of aquaculture facilities (Table 7 in the Supplementary data).

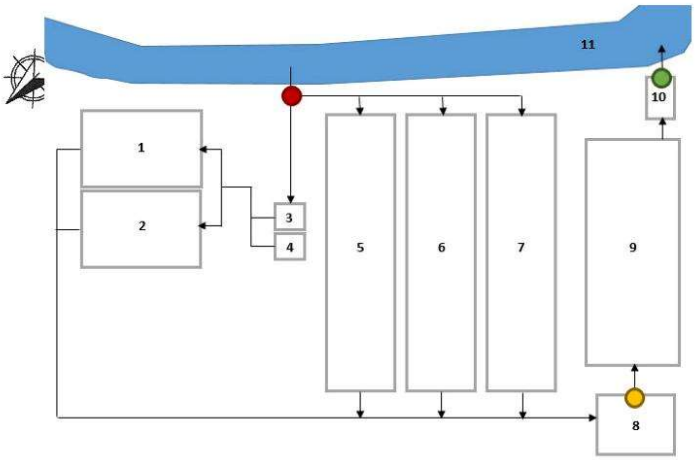

Fig. 2. Schematic of the Irish freshwater fish farm layout indicating the locations of the collection points for the intake (red), output (green) and settlement pond (yellow) water samples. 1) hatchery, 2) nursery, 3-4) mesocosms, 5-7) culture ponds, 8) settlement pond, 9) constructed wetland, 10) holding tank, 11) river. Black arrows indicate flow of water. Note: Schematic is not to scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of the methods used to assess the physicochemical parameters investigated on the Irish freshwater aquaculture intake, output and settlement pond water samples. The method employed, detection limit for all kits used and standard water and wastewater analysis methods numbers have been included.

Physicochemical parameter	Method	Detection limit (mg L^{-1})	Standard method number
Alkalinity	Titrimetric	–	2320-B
BOD	Membrane electrode	–	5210-B
COD	Photometric	0-150 15-300	5220-D
DO	Membrane electrode	–	4500-O G
Hardness	Titrimetric	–	2340-C
NH_4^+	Photometric	0.013-3.86 2.6-193.0	4500-NH_3-F
NO_2^-	Photometric	0.007-3.28	345-1
NO_3^-	Photometric	0.4-110.7	4500-NO_3
pH	Membrane electrode	–	2310-B
PO_4^{3-}	Photometric	0.007-15.3 1.5-92.0	4500-P-C
Suspended solids	Gravimetric	–	2540-D
Temperature	Thermometer	–	2550-B

NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand.

Physicochemical parameters – temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, DO, BOD, COD, suspended solids, hardness and alkalinity were analysed within 24 h of collection to remove the need for preservation. Spectroquant® kits (Sigma Aldrich) were used as per the manufacturer's instructions to assess NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and COD levels. Temperature and pH were analysed using the VWR pHenomenal™ MU 6100 L meter and VWR 111662-1157 pH probe. DO and BOD_{5day} were assessed using the Jenway 9500 DO_2 meter and probe. The suspended solids were analysed by filtration using a Buchner flask and funnel. Alkalinity was assessed via titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid. Hardness was analysed via titration using pH 10 buffer, erichrome black and EDTA. A summary of all physicochemical methods employed in this study, including each standard method number, are shown in Table 1.

2.3. Ecotoxicity analysis

The unicellular freshwater green algae *P. subcapitata* was used to determine the quality of the water. A culture was obtained from The Culture Collection of Algae and Protozoa (CCAP 278/4; SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.) and grown in standard Jarworski's culture medium at 23 °C ± 2 °C exposed to continuous illumination (lux 6000-10,000). Additionally, starter cultures of *Asterionella formosa* (CCAP 1005/9) and *Monoraphidium contortum* (CCAP 245/2) were obtained from The Culture Collection of Algae and Protozoa (SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland). *P. subcapitata* was compared with *A. formosa* and *M. contortum* to ensure that *P. subcapitata* was representative of Irish aquatic algae (Table 2). Algae were sub-cultured every three days to ensure the growth rate remained in the exponential phase. Analysis was conducted as per the Water quality – Fresh water algal growth inhibition test with unicellular green algae ISO (8692:2012) guidelines. The *P. subcapitata* was exposed to the intake and output samples for 72 h at 23 °C ± 2 °C exposed to continuous illumination under static conditions. The percent of algal growth rate inhibition (E_rC_{50}) was calculated by comparing samples to a negative control containing just the Jarworski's culture medium. The E_rC_{50} is the concentration at which the has been a 50% reduction in the growth rate relative to the control within 72 h (ISO, 2012). Eqs. (1), (2) and (3) were taken directly from the ISO (8692:2012) guidelines (ISO, 2012) and calculations were conducted as follows;

$$\text{Algae cells mL}^{-1} = \frac{n}{0.02} \times 10^3 \quad (1)$$

where

n = the number of cells counted using a haemocytometer.

$$\text{Average specific growth rate } (\mu) = \frac{\ln X_n - \ln X_0}{T_n - T_0} \quad (2)$$

where

ln = natural log of
X_n = Algae cells mL^{-1} at 72 h
X_0 = Algae cells mL^{-1} at 0 h
T_n = Duration of test
T_0 = Time zero.

$$\text{Percent growth rate inhibition} = \frac{C\mu - T\mu}{C\mu} \times 100 \quad (3)$$

where

Cμ = Average specific growth rate for control
Tμ = Average specific growth rate for treatment.

Table 2
Mean concentrations calculated for each parameter investigated in this study conducted in 2019 (intake and output water 2019) and the previous study conducted in 2018 on the same fish farm (intake and output water 2018) by O'Neill et al. (2019). All data is based on the average across six months. S.D. has been indicated.

Parameter	Intake water		Output water	
	2018	2019	2018	2019
NH_4^+ (mg L^{-1})	0.16 ± 0.18	0.06 ± 0.09	1.16 ± 0.64	0.53 ± 0.53
NO_2^- (mg L^{-1})	0.02 ± 0.01	0.01 ± 0.01	0.32 ± 0.38	0.10 ± 0.07
NO_3^- (mg L^{-1})	3.62 ± 1.60	1.81 ± 1.27	5.29 ± 5.56	1.74 ± 1.10
PO_4^{3-} (mg L^{-1})	1.76 ± 0.84	0.63 ± 1.14	3.78 ± 2.00	0.77 ± 0.51
DO (mg O_2 L^{-1})	10.31 ± 0.87	10.76 ± 2.75	5.10 ± 2.85	7.66 ± 3.06
BOD (mg O_2 L^{-1})	2.27 ± 1.47	2.68 ± 0.70	3.24 ± 1.95	2.80 ± 0.96
COD (mg O_2 L^{-1})	45.91 ± 40.81	25.97 ± 9.98	76.44 ± 59.06	19.24 ± 11.68
Temperature (°C)	14.76 ± 2.53	13.85 ± 1.35	15.53 ± 2.66	14.23 ± 1.48
pH	7.76 ± 0.19	7.70 ± 0.14	7.11 ± 0.18	7.14 ± 0.06
Suspended solids (mg L^{-1})	40.17 ± 79.08	20.50 ± 8.00	83.67 ± 144.33	19.22 ± 9.23
Hardness (mg $CaCO_3$ L^{-1})	100.49 ± 9.22	106.24 ± 12.18	116.03 ± 16.80	111.58 ± 22.45
Alkalinity (mg $CaCO_3$ L^{-1})	122.55 ± 17.71	135.03 ± 20.49	128.91 ± 18.19	129.47 ± 17.98
P. subcapitata (% growth rate inhibition)	43.14 ± 18.47	13.66 ± 1.44	-2.70 ± 20.41	9.73 ± 2.03

NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand.

2.4. Statistical analysis

Statistical analyses were conducted using MINITAB 18 and GRAPHPAD PRISM 8. The generated data were grouped and subjected to normality testing (Anderson-Darling) to ensure all samples were normally distributed. Unpaired *t*-tests and ANOVA were used to identify any significant differences in the variables. $P < 0.05$ indicated a statistically significant difference. Pearson's correlation (r) was used to assess if any correlations between the algae and/or the physicochemical parameters existed (Fig. 3).

3. Results

3.1. Physicochemical analysis

Results determined for the physicochemical parameters investigated in this study on Irish freshwater fish farm intake, output and settlement pond water samples are displayed in Fig. 4. Increases in NH_4^+, NO_2, PO_4^{3-}, BOD and temperature, along with decreases in DO, COD, pH and alkalinity occurred when comparing the intake and output water from the fish farm. Fluctuations from month-to-month in NO_3^- and

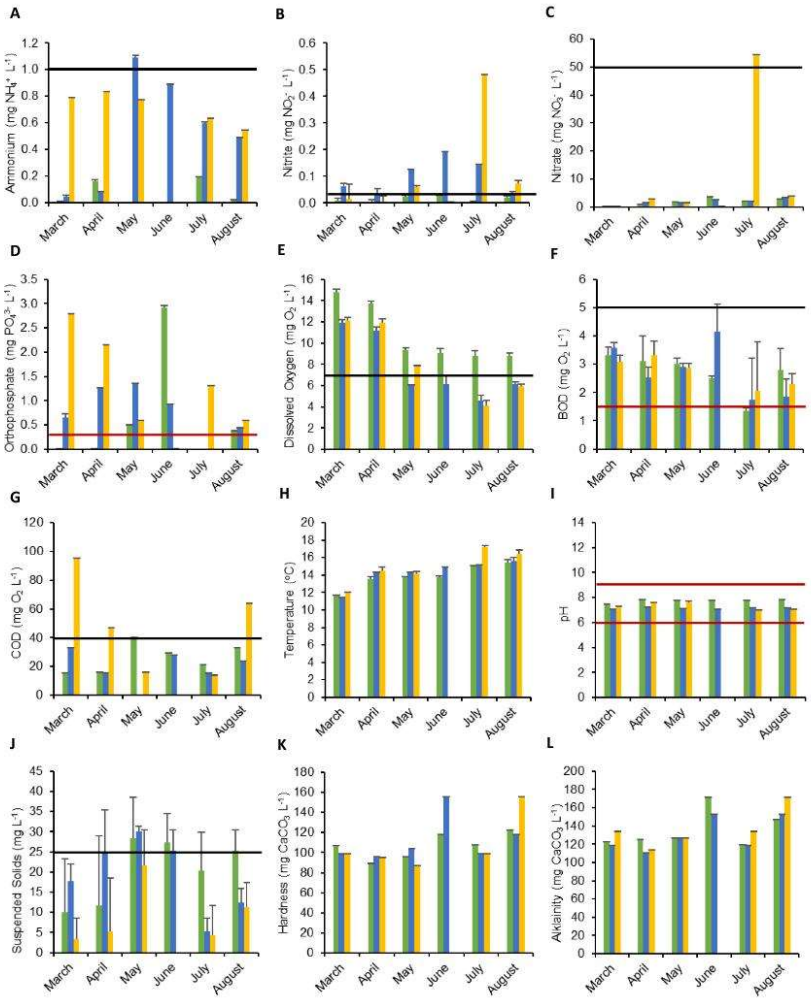

Fig. 3. Breakdown for the physicochemical parameters investigated on Irish freshwater aquaculture intake (green), output (blue) and settlement pond (yellow) water samples from March 2019 to August 2019. Parameters investigated were A) NH_4^+, B) NO_2, C) NO_3, D) PO_4^{3-}, E) DO, F) BOD, G) COD, H) temperature, I) pH, J) suspended solids, K) hardness and L) alkalinity. Red lines indicate levels set out by S.I. 272 of 2009 and 77 of 2019. Black lines indicate levels set out by the Irish EPAs parameters for water quality. NOTE: Dilution factor of the receiving water body has not been included. Lines do not appear on temperature and CaCO3 as no limits were indicated. S.D. indicated, $n = 9$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

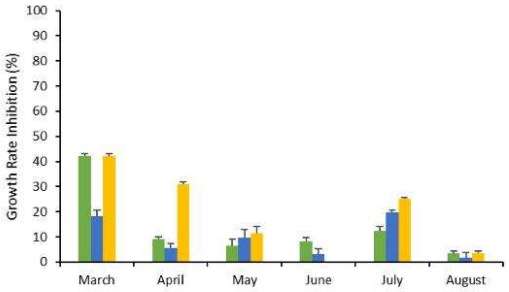

Fig. 4. Percentage growth rate inhibition observed in *P. subcapitata* proceeding exposure to Irish freshwater aquaculture intake (green), output (blue) and settlement pond (yellow) water samples for 72 h at 23 °C ± 2 °C under continuous illumination from March 2019 to august 2019. S.D. indicated, $n = 9$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hardness were also observed. With the exception of NO_2^- ($P = 0.011$) and pH ($P = 0.025$), no statistically significant (one-way ANOVA) differences were indicated. With the exception of NH_4^+ and NO_2^- levels in May and June, decreases were observed in the physicochemical parameters between the settlement pond and output water. With the exception of suspended solids ($P = 0.044$), no statistically significant (one-way ANOVA) differences were observed; one-way ANOVA was conducted across all three sampling points and no statistically significant differences were observed. Two-way ANOVA was conducted in order to take the sampling month into consideration; with the exception of BOD ($P = 0.083$) and suspended solids ($P = 0.150$), a statistically significant difference was observed in all parameters.

3.2. Algal bioassay analysis

The percentage growth rate inhibition observed in the intake, output and settlement pond water are displayed in Fig. 5. With the exception of samples for May and July, a decrease in growth rate inhibition between the intake and output samples was demonstrated. A decrease was also observed in all samples between the settlement pond and output water. No statistically significant (one-way ANOVA) differences were observed for either set of samples. One-way ANOVA was conducted across the three sampling points and no statistically significant differences were indicated. Two-way ANOVA was conducted to determine whether any statistically significant differences were observed when the sampling month was taken into consideration. A statistically significant difference ($P = 0.001$) was indicated when the sampling month was included.

3.3. Comparative study

Table 3 summarises an average of all results obtained during a previous study conducted at a similar time of year on the same fish farm during times of extreme weather conditions (heat wave and drought) by O'Neill et al. (2019) and those determined in this study which were conducted under normal weather conditions for the Republic of Ireland. With the exception of the dissolved oxygen, pH, alkalinity and hardness, all physicochemical concentrations decreased during the similar time periods of 2018 and 2019 in both the intake and output water samples. The pH, alkalinity and hardness remained similar whilst dissolved oxygen levels increased. For the *P. subcapitata*, considerable decreases in inhibition toxicity were observed in the intake water and no stimulation was observed in the output water from this study compared to 2018. Both of which demonstrated a statistically significant (*t*-tests) difference ($P = 0.030$ for the intake water and $P = 0.039$ for the output water).

3.4. Correlation studies for monitoring periods of freshwater aquaculture farm

Correlation studies were conducted between all parameters investigated at the three sampling points. A positive correlation between two parameters indicates that as one parameter increases or decreases, so too does the other parameter. A negative correlation between two parameters indicates that as one parameter increases or decreases, the opposite occurs with the other parameter i.e., an inverse relationship. All results for the intake, output and settlement pond water samples are displayed in Tables 3, 4, 5, and 6 respectively. In the intake samples a negative correlation was observed between *P. subcapitata* and temperature as well as pH. A negative correlation was indicated between NH_4^+ and NO_2^-. A positive correlation was identified between NO_2^- and alkalinity. The NO_3^- demonstrated a negative correlation with DO and a positive correlation with suspended solids. A positive correlation was observed between PO_4^{3-} and alkalinity. A negative correlation was identified between DO and suspended solids. In the output samples a positive correlation between temperature and NO_3^- was indicated. A negative correlation was identified between DO and NH_4^+ as well as NO_2^-. Hardness displayed a positive correlation between PO_4^{3-} and alkalinity. In the settlement pond a positive correlation between *P. subcapitata* and PO_4^{3-} was observed. Temperature demonstrated a positive correlation with pH, NH_4^+, hardness and alkalinity. The pH indicated a positive correlation with NH_4^+, BOD hardness and alkalinity.

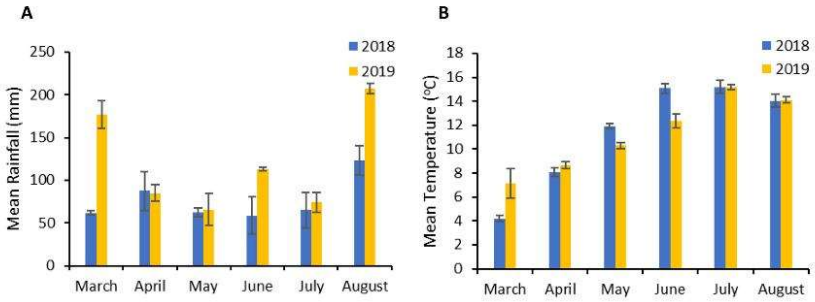

Fig. 5. Average A) rainfall and B) temperature recorded for 2018 (blue) and 2019 (yellow) at three Met Eireann weather stations surrounding the freshwater fish farm during the sampling period of March 2019 to August 2019. Stations were located at 1) Markree, Co. Sligo, 2) Knock, Co. Mayo and 3) Mount Dillon, Co. Roscommon. Stations were located north-west, south-west and south-east of the fish farm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture intake water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	**-0.830**	1.000							>0-0.3 = Weak relationship				
pH	**-0.951**	0.794	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	-0.183	0.360	0.451	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	-0.260	0.037	-0.020	**-0.845**	1.000				0.7-<1 = Strong relationship				
NO_3^-	-0.727	0.687	0.557	-0.173	0.656	1.000			1 = Perfect linear relationship				
PO_4^{3-}	-0.286	0.024	0.133	-0.444	0.785	0.728	1.000						
DO	0.726	-0.809	-0.553	0.057	-0.468	**-0.876**	-0.400	1.000					
BOD	0.307	-0.627	-0.351	-0.582	0.232	-0.465	-0.073	0.625	1.000				
COD	-0.623	0.448	0.372	-0.523	0.712	0.623	0.353	-0.749	0.007	1.000			
SS	-0.702	0.594	0.466	-0.380	0.730	**0.855**	0.559	**-0.915**	-0.295	0.924	1.000		
H	-0.061	0.326	-0.138	-0.426	0.588	0.638	0.471	-0.502	-0.264	0.254	0.438	1.000	
A	-0.394	0.201	0.218	-0.497	**0.831**	0.800	**0.916**	-0.440	0.017	0.400	0.577	0.659	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

A positive correlation was identified between NH_4^+ and DO as well as BOD. A positive correlation was observed between NO_2^- and NO_3^-. DO demonstrated positive correlations with PO_4^{3-} and BOD. Finally, a positive correlation was identified between hardness and alkalinity.

3.5. Weather conditions influencing water quality on monitored aquaculture farm

Due to observations determined in the previous study, conducted by same authors (O'Neill et al., 2019), as a result of dramatic weather conditions experienced during 2018 in the Republic of Ireland that coincided with occurrence of the hottest summer recorded to date by Irish Meteorological Office (Met Eireann) resulting in a nationwide hosepipe ban due to limit negative impacts of drought; weather conditions during the sampling period have been included. For continuity, mean temperature and rainfall data collected by Met Eireann at the same three weather stations surrounding the fish farm as the previous study have been included for this sample period (Met Éireann, 2019). Stations were situated at Markree Co. Sligo, Knock Co. Mayo and Mount Dillon Co. Roscommon, as shown in Fig. 1. Increases in the mean rainfall (Fig. 5A) and decreases in the mean temperature (Fig. 5B) were observed for 2019 versus the same time period in 2018 across the three stations. Maxima temperatures had also decreased (Fig. 6 – Supplementary). Statistical analysis found that the relationships between the algae and the rainfall and temperature switched. A moderately strong inverse relationship (r = -0.559) between the algae and temperature, and a weak inverse relationship (r = -0.209) between the algae and rainfall now existed.

4. Discussion

4.1. Physicochemical evaluation

In order to ascertain whether the processes conducted in the fish farm altered the quality of the water, the physicochemical results determined in the intake and output water were compared firstly to one another and then to the previous study conducted. It should be appreciated that the dilution factor of the receiving river on potential impact of aquaculture effluent has not been considered in this research. The presence of NH_4^+, NO_2^- and NO_3^- in the output water suggested that the nitrification process (enzymatic oxidation of NH_4^+ to NO_3^- by way of NO_2^-) was occurring. Increases in these parameters between the intake and output samples suggested that, for the most part, their production was due to practices within the farm. These were most likely due to the presence of fish waste and uneaten artificial pelleted feed used in the cultural process. Increases above guidance levels (1 mg NH_4^+ L^{-1} and 0.03 mg NO_2^- L^{-1}) in the parameters were only observed between May and June, which suggested a potential cause for concern. However, this was most likely attributed to the constructed wetland, which was not functioning to its optimal capacity due to undergoing maintenance works: no discharge of aquaculture effluent was released during these times. Levels of monitored physicochemical parameters dropped below

Table 4
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture output water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	-0.537	1.000							>0-0.3 = Weak relationship				
pH	-0.332	0.419	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	-0.210	0.496	-0.262	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	0.131	0.213	-0.645	0.733	1.000				0.7-<1 = Strong relationship				
NO_3^-	-0.627	**0.929**	0.234	0.439	0.161	1.000			1 = Perfect linear relationship				
PO_4^{3-}	-0.488	0.139	-0.500	0.433	0.656	0.213	1.000						
DO	0.296	-0.670	0.370	**-0.816**	**-0.811**	-0.700	-0.612	1.000					
BOD	-0.102	-0.518	-0.702	0.128	0.431	-0.405	0.766	-0.112	1.000				
COD	0.007	-0.362	-0.505	-0.529	-0.092	-0.070	0.252	0.095	0.385	1.000			
SS	-0.442	-0.194	-0.004	0.304	0.124	-0.267	0.525	0.033	0.634	-0.324	1.000		
H	-0.574	0.344	-0.514	0.494	0.603	0.522	**0.902**	-0.769	0.558	0.364	0.246	1.000	
A	-0.639	0.479	-0.327	0.494	0.299	0.736	0.544	-0.701	0.197	0.296	0.019	**0.839**	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

Table 5
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture settlement pond water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	0.386	1.000							>0-0.3 = Weak relationship				
pH	0.566	**0.938**	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	0.714	**0.816**	**0.958**	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	0.133	0.464	0.229	0.107	1.000				0.7-<1 = Strong relationship				
NO_3^-	0.168	0.417	0.182	0.079	**0.992**	1.000			1 = Perfect linear relationship				
PO_4^{3-}	**0.976**	0.393	0.580	0.715	-0.004	0.032	1.000						
DO	0.784	0.525	0.765	**0.883**	-0.288	-0.287	**0.847**	1.000					
BOD	0.679	0.786	**0.945**	**0.986**	-0.034	-0.060	0.714	**0.927**	1.000				
COD	0.593	0.362	0.523	0.532	-0.322	-0.336	0.730	0.744	0.616	1.000			
SS	-0.255	0.468	0.514	0.438	-0.085	-0.183	-0.271	0.201	0.441	-0.077	1.000		
H	0.251	**0.898**	**0.843**	0.677	0.205	0.147	0.341	0.512	0.709	0.609	0.412	1.000	
A	0.356	**0.942**	**0.920**	0.781	0.279	0.212	0.407	0.571	0.785	0.582	0.488	**0.974**	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

guidance values, once the wetland was fully functional where low levels were also observed in the intake water. It is likely that agricultural processes (cattle and sheep farming) and forestry processes (tree felling) occurring upstream of the fish farm contributed to these measured physicochemical parameters.

The PO_4^{3-} levels in the output water was greater than that of the input as a result of the processes within the aquaculture farm. However, levels observed in the intake suggested that agricultural and forestry processes upstream of the farm could have also contributed to levels. Concentrations of PO_4^{3-} were greater than guidance levels (0.35 mg PO_4^{3-} L^{-1}) suggesting a potential cause for concern as excess levels can result in the promotion of algal blooms leading to potential hypoxic conditions in the water body (O'Neill et al., 2019). However, once maintenance was completed on the constructed wetland, levels detected in the output water were reduced to guidance levels indicating no foreseen issues.

A decrease in oxygen levels was observed between the intake and output water due to the aquaculture process. This decrease may also have been due to changes in seasonality. According to Alam et al. (2007), da Silva et al. (2017) and O'Neill et al. (2019) oxygen levels ≥4 mg O_2 L^{-1} are sufficient to maintain aquatic life. Although levels in the output water dropped just below the guidance value (7 mg O_2 L^{-1} for cyprinid waters), levels remained above the critical 4 mg O_2 L^{-1} level and as a result had indicated no cause for concern. BOD levels between the intake and output water fluctuated i.e., BOD increased between the intake and output water during March, June and July, whilst decreases were observed in April, May and August. Despite fluctuations, BOD levels remained below the guidance value of 5 mg O_2 L^{-1} for cyprinid waters suggested by the Irish EPA. Additionally, the dilution factor of the receiving water system has not been included therefore BOD levels would further decrease upon release. With the exception of March, COD levels decreased between the intake and output water. This suggested that the COD levels were not due to processes within the farm and were more likely due to works being conducted upstream. Despite this, COD levels were well below the guidance value of 40 mg O_2 L^{-1}.

Suspended solids levels were greater between the intake and output water during the months of March, April and May which were most likely due to increases in production processes in the farm. However, this trend reversed for the months of June, July and August. This was most likely due to high levels of tree felling being conducted in the forestry upstream of the farm during this time. Levels were greater than the guidance level of 25 mg L^{-1} during May and June but this was most likely due to the constructed wetland maintenance work as once the wetland became fully functional again after the June maintenance, concentrations dropped well below this level. Once again, it should be noted that water did not leave the farm during this time.

Temperature between the intake and output water samples remained consistent with increases observed during the summer months, as would be expected. Fish farms must not release water that is greater than 20 °C. At no point during the study did temperatures rise to this level. Aquaculture waters are recommended to have a pH of between 6 and 9 (EPA, 2001). All samples remained within this range. The intake samples were slightly more alkaline than the output samples. Output samples had greater $CaCO_3$ levels and therefore a greater buffering capacity which may account for pH levels of just about neutral (pH 7) in the output water. $CaCO_3$ levels were measured for hardness. Results suggested that the water was slightly to moderately hard. This correlates with water hardness demonstrated around Boyle, Co. Roscommon (O'Neill et al., 2019).

All parameters were then compared to the previous year's study. In 2018, Ireland experienced its hottest summer on record whereby the country experienced long periods of drought. The physicochemical parameters were greater in 2018 than that of this study (2019) for the similar time period (O'Neill et al., 2019). This was most likely due to increased flow rates as a result of increased rainfall resulting in no drought conditions being observed in 2019. As this research only focused on one type of fish farm culturing one specific species of fish (European Perch) results from this study were also compared to previous aquaculture studies. These studies were located worldwide and encompassed a range of different aquaculture systems culturing several different species of fish, as shown in Table 6. The studies reviewed demonstrated similar or higher levels than the concentrations observed in this study.

4.2. Algal bioassay evaluation

Inhibition of the growth rate of the *P. subcapitata* was observed in both the intake and output water samples. The presence of growth rate inhibition suggested that algal blooms downstream of the fish farm would be unlikely. However, growth rate inhibition is still demonstration of a toxic effect. This inhibition may result in loss of biodiversity in the receiving water body (Rabalais, 2002; O'Neill et al., 2019). Exclusive of the months of May and July, the percentage of growth rate inhibition was found to decrease between the intake and output water samples. The inhibition toxicity detected throughout the study was at sub-lethal levels. Additionally, toxicity was reduced once the water had passed through the fish farm's constructed wetland. This suggested that the farm itself was successfully improving the water quality.

When results were compared to the previous study of 2018, a statistically significant difference was observed in both the intake

($P = 0.030$) and the output ($P = 0.039$) water samples. Unlike the previous study, no growth rate stimulation was observed in the output water. Equally, considerably lower levels (sub-lethal) of toxicity were observed in the intake water, e.g. levels of up to 75% growth rate inhibition were observed during the drought conditions of 2018 (O'Neill et al., 2019). This reduction was most likely due to the reduced temperatures and resulting increased flow rates.

Results were than compared to previous studies that utilised *P. subcapitata* to assess fish farm output water. Miashiro et al. (2012) demonstrated similar results in a Brazilian study (with a traditionally much warmer climate than Ireland) to the previous study conducted on the fish farm by O'Neill et al. (2019) during the heat wave and drought conditions, where by similar levels of growth rate stimulation were observed. The current study however, conformed to most of the available research on the effects of fish farm output water on *P. subcapitata*. Guéguen et al. (2004), Ivanova and Groudeva (2006) and Ma et al. (2006) all observed similar growth inhibition levels to those demonstrated. These studies were also conducted in countries (Poland and Bulgaria) with similar temperate weather conditions to those normally experienced in Ireland.

4.3. Constructed wetland evaluation

The previous study conducted by O'Neill et al. (2019) indicated that there may have been issues with the constructed wetland due to increased concentration of nitrogenous and phosphorus waste in the output water samples. However, it was unclear whether this issue may have been due to the extreme weather conditions experienced during 2018 in Ireland. As a result, samples were included at the exit point of the settlement pond to ascertain the efficacy of the wetland. This was the point at which the wastewater entered the constructed wetland. Evaluation of the settlement pond demonstrated that, when fully functional after the June maintenance, the constructed wetland was effective in the removal of waste products from the water. This efficacy may also be due in part to the re-introduction of duckweed (*Lemna minor*). The previous study found spikes in nitrogenous waste concentrations when the duckweed was removed from the farm. Duckweed has the ability to use NO_3^- as a nutrient source (O'Neill et al., 2019) and research is ongoing in this area.

4.4. Climate change

According to the Intergovernmental Panel on Climate Change (2020), the momentum of climate change had greatly increased in 2019. Climate change is the most troubling scientific issues of our time (Bulkeley and Newell, 2015). The Bulletin of the Atomic Scientists (2020) has now moved the hypothetical Doomsday Clock to 100 s to midnight which is the closest it's ever been to the "point of no return" represented by midnight. Originally introduced in 1947 due to the threat of nuclear weapons, climate change is now considered an equal threat to that (Weisberger, 2020). This research has further indicated that climate change has a direct impact on fish farming, as suggested by the lack of algal growth stimulation or high levels of growth inhibition due normal weather conditions reported in this study. Algal growth and temperature still demonstrate a strong correlationship ($r = -0.830$) in the intake samples. This research has further demonstrated the ability of *P. subcapitata* to be utilised as an early warning indicator for climate change ambiguity in freshwater aquaculture.

5. Conclusion

- The findings of this timely study responds to the main tenets of the recent intergovernmental report on global climate change (IPCC, 2021) that seeks urgent viable and resilient technological solutions to help future proof for a climate-smart environmentally friendly agri-food sector, including fisheries. Moreover, this 'code red for humanity' IPCC report on climate change clearly highlights that human or anthropogenic activity has contributed greatly to greenhouse gas levels in the atmosphere where there is a pressing need to reduce carbon dioxide and methane emissions, and to stall rising global temperatures that leads to extreme weather events.
- Regarding the latter, there is pressing need for countries to use innovative approaches to support and to develop sustainable food systems delivering benefits for the sector, for society, and for the environment. The findings of this present study will support and enable viable and resilient primary producers to provide food that are safe, nutritious and appealing; thus, using eco-technologies and talent to inform innovative, competitive and resilient agri-food sector regionally, and internationally (Rowan and Pogue, 2021).
- Specifically, this study revealed that the freshwater microalga *P. subcapitata* can be used for the real-time prediction of potential adverse environmental issues associated with freshwater aquaculture wastewater, which can be seen as complementary to relying upon using traditional physicochemical parametric measurements.
- As this research focused exclusively on one type of fish farm in the Republic of Ireland, use of this algal bioindicator technique should be also applied to evaluate different types of aquaculture farms including pond-based, flow-through, and recirculation in order to ensure harmonised results across a range of culture systems and fish species.
- Inclusion of additional ecotoxicological bioassays *such as* a full test battery, encompassing different trophic levels (e.g., *Daphnia magna* – primary consumer, *Vibrio fischeri* – decomposer) (Garvey et al., 2013) within the aquatic ecosystems should also be considered for future studies in order to develop a better understanding of the potential environmental effects' aquaculture processes could have on water bodies.
- There is merit in conducting molecular profiling of naturally occurring microalgae in order to incorporate these as a cocktail of native species representative of local natural aquatic ecosystems, which will support and inform biodiversity, conservation management along with enhanced bioindicator performance. For example, Kadam et al. (2020) identified 33 Taxa belonging to 27 genera of microalgae when they considered development of a putative 'Algal Genus Pollution Index' for potentially assessing water bodies in the Doon valley, India.
- The constructed wetland servicing this aquaculture farm needs to be increased in size in order to be effective in treating volume of the wastewater effluent where efficacy of treatment can be also influenced by extreme weather events that influence flow rates.
- The lack of growth rate stimulation and decrease in growth rate inhibition when compared to the previous study (O'Neill et al., 2019) supports future use of *P. subcapitata* as an early warning indicator to potential issues in fish farms associated with climate change where unpredictable and more erratic weather conditions may become more frequent. It is appreciated that there a dearth in evidence-based literature on the use of microalgae as a bioindicator for monitoring impact of climate change and its potential effects in aquaculture.
- Whilst this present research has demonstrated interested findings, there is a need to pursue catchment based-studies that incorporates an extended number of locations and inter-laboratory evaluations for to improve technological rigor and stakeholder acceptance including policy-makers.
- Increasing NH_3 levels in the monitored fish ponds can be potentially toxic to fish that require further investigation.
- There are emerging opportunities for use of natural microalgae in the development of predictive environmental risk models that will help inform the quality status of water catchments, along with evaluating commensurate efficacy of intervention strategies, such as municipal wastewater treatment plants (Tahar et al., 2017).

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CRediT authorship contribution statement

Emer A. O'Neill: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Neil J. Rowan:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no competing interests or conflict of interest with respect to the publication of this article.

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AIT Research

Development of ecotoxicological toolbox for assessing freshwater finfish aquaculture effluent.

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INTRODUCTION

The EcoAqua project focuses on improving production efficiency in Irish freshwater aquaculture. It aims to address critically important needs identified by the industry and aquaculture stakeholders[1]. Aquaculture is one of the fastest growing food producing industries in the world[2]. The Irish aquaculture sector was worth €163M, producing 44,000 tonnes of fish in 2016[3]. Food Wise 2025 predicts that the Irish agri-food industry has the potential to increase exports by €19B per annum by 2025. It proposes that aquaculture production should be increased to 81,700 tonnes by 2023 in order to assist in meeting this goal[4]. However issues with the aquaculture licensing process and the adoption of EU environmental protection directives, e.g. 92/43/EEC, 2009/147/EC and 2000/60/EC, have hampered the growth and development of the industry[5]. Freshwater aquaculture waste-water discharge is currently monitored by Irish Water however current regulations may not be specifically applicable to aquaculture[6]. Hence there is an urgent need to develop an ecotoxicological toolbox consisting of tests representative of the receiving freshwater aquatic ecosystem downstream of fish farms. This aims to assist in improving both the aquaculture licensing process and monitoring of concomitant waste-water discharge.

METHODOLOGY

A standard acute multi-trophic freshwater ecotoxicological test-battery consisting of a producer, consumer and decomposer. *Pseudokirchneriella subcapitata* (algae) and *Daphnia magna* (crustacean) have been validated and *Vibrio fischeri* (luminescent bacteria) is currently being validated. These bioassays will be employed to analyse freshwater aquaculture effluent.

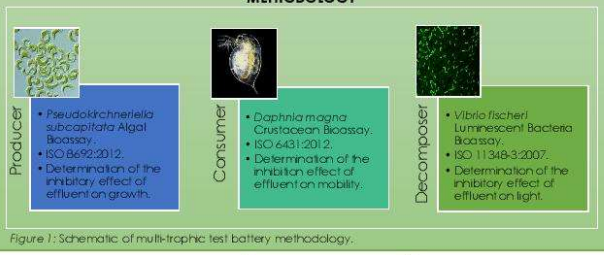

Figure 1: Schematic of multi-trophic test battery methodology.

Physico-chemical analysis to be performed will be in line with requirements set out by the EPAs Irish aquaculture licensing guidelines and will include; biochemical oxygen demand, chemical oxygen demand, dissolved oxygen, total nitrogen, total phosphate, total suspended solids, pH, and conductivity. Physico-chemical tests are currently being validated.

RESULTS

Algal growth inhibition assay

The toxicological response (E_rC_{50}) of the reference substance 3,5-Dichlorophenol after 72 hours was determined to be 3.09 mg/L (Figure 2). As per ISO 8692:2012, an E_rC_{50} value of 3.38 mg/L +/- 1.30 mg/L is required for the assay to be deemed valid. As the determined concentration fell within this range, this assay was deemed valid and ready for the analysis of the freshwater aquaculture effluent.

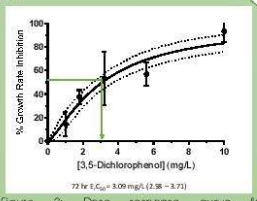

Figure 2: Dose response curve for *Pseudokirchneriella subcapitata* exposed to 3,5-dichlorophenol for 72 hours under static conditions. S.D. & 95% C.I. indicated.

Crustacean mobility inhibition assay

The toxicological response (EC_{50}) of the reference substances potassium dichromate and zinc sulphate after 24 hours were determined to be 1.06 mg/L and 2.53 mg/L, respectively (Figure 3). An EC_{50} value between the range 0.6 - 2.1 mg/L for potassium dichromate and 0.93 – 3.48 mg/L for zinc sulphate is required for the assay to be deemed valid. As the determined concentrations fell within this parameters, this assay was deemed valid and ready for the analysis of the freshwater aquaculture effluent.

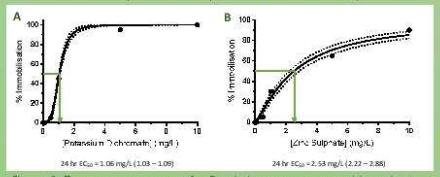

Figure 3: Dose response curves for *Daphnia magna* exposed to potassium dichromate & zinc sulphate for 24 hours. S.D. & 95% C.I. indicated.

DISCUSSION & FUTURE WORK

Upon validation of all bioassays and physicochemical tests, influent and effluent from a selected aquaculture site will be assessed every two weeks for approximately six months. Validation of all tests is expected to be completed by the end of March and testing will commence at the start of April.

Pseudokirchneriella subcapitata and *Daphnia magna* will only be used for validation purposes. An algae and micro-crustacean species representative of Irish freshwater aquatic ecosystems are currently being investigated and will be used for the monitoring process.

Correlations between the ecotoxicological bioassays and physicochemical data will then be established with the hope of providing an early warning system for any issues arising from aquaculture effluent discharge.

The main aim of this toolbox is to develop ecologically relevant test species for use in bioassays for EIAs (required for aquaculture licensing process) **and for monitoring of aquaculture waste water discharge.**

REFERENCES

EcoAqua

OÉ Gaillimh NUI Galway

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AIT Research

Sustainable Intensification of Freshwater Aquaculture using Irish Peatlands – Role of Algae!

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INTRODUCTION

Figure 1: Aerial view of the **AquaMona trial fish farm** located in the middle of Mount Lucas Wind Farm, Co. Offlay. The wind turbine used to provide all electrical requirements is displayed in the bottom left hand side of the image.

Aquaculture is one of the **fastest growing food** producing industries in the world[1]. Aquaculture will contribute to **meeting global need for sustainable food production**. The Irish aquaculture sector was worth €208.4M in 2017[2]. However, several factors have hampered the growth of the industry, including disease and environmental impacts[1,3]. **Bord Iascaigh Mhara (BIM)**, undertook a feasibility study to assess the potential use of **peatlands (bogs)**, for sustainable aquaculture diversification[4]. In April 2019 BIM, in conjunction with **Bord na Mona**, begun the **AquaMona project** and opened a trial fish farm at the Mount Lucas Wind Farm[4,5] (*Figure 1*). AquaMona is a **new concept** in **integrated multitrophic aquaculture (IMTA)**, which uses cutaway bogs to farm rainbow trout (*Oncorhynchus mykiss*) and Eurasian perch (*Perca fluviatilis*) with associated organic status that is powered by wind energy and utilises algae and duckweed to treat rearing water[5].

Algal communities have been previously found to contribute to wastewater process efficacy through assimilation of nitrate and ammonium, along with providing health benefits to fish both in terms of **nutrition** and bioactives[6] (*Figure 2*). However, there is a **lack of knowledge** in terms of the specific contributory role of algae in fish health and IMTA process enhancement.

The Aim of this AquaAlgal+ research is to develop a comprehensive understanding of the role of algal communities and emergence/predominance of specific speciation in freshwater aquaculture so as to specifically inform and guide the development of this novel innovative peatland cut-away IMTA process. This would provide unique new knowledge for aquaculture industry as to critical role of algal communities in maintaining optimal IMTA conditions.

Figure 2: The main benefits of utilising algae in aquaculture processes.

PROJECT OVERVIEW

WORKPACKAGE 1: Algal & Microbial Profiling

- Real time algal and microbial profiling at Mount Lucas over a 12-month duration.
- Elucidate algal communities and operating parameters influencing occurrence and predominance.
- Analysis of algal populations in freshwater samples.

WORKPACKAGE 2: Analysis of Bioactive Compounds

- Perform screening of different algal species in order to obtain bioactive compounds.
- Determine anti-microbial potential of extracts against fish pathogens.
- Determine immunological properties in extracts.
- Isolation and culturing of algae in bioreactor.

METHODOLOGY

Algal & Microbial Profiling

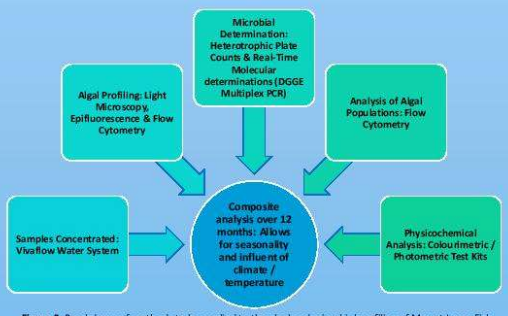

Figure 3: Breakdown of methods to be applied to the algal and microbial profiling of Mount Lucas Fish Farm.

Analysis of Bioactive Compounds

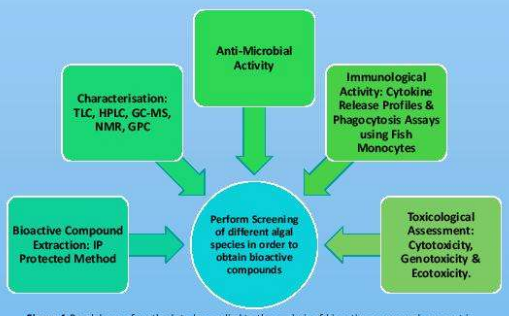

Figure 4: Breakdown of methods to be applied to the analysis of bioactive compounds present in algae.

DISCUSSION

This research will provide critical new information on the role of algae for augmenting Ireland's first peatland cutaway freshwater IMTA system where algae as a natural actor for waste removal and performance.

The algal and microbial profiling will result in;

- Providing new knowledge of the role of algae in optimising new aquaculture enterprise.
- Providing a new toolbox for profiling algae for aquaculture and result in a unique repository of species.
- Allowing for the adoption of new technologies for the detection and profiling of bacterial pathogens in aquaculture before the manifestation of diseases.
- Providing added-value to other adjacent projects under the AquaMona project to inform process performance and optimisation.

The analysis of bioactive compounds in algae will result in;

- Providing a unique knowledge of new bioactives isolated from algae that will help inform key species of benefit to the aquaculture process.
- Providing new products that may inform organic feed for fish health and wellbeing.
- Providing new technologies that will lead to IP and competitiveness of this new cutaway peatland process. A process that is currently unique to Ireland

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AQUAMONA

Ireland's European Structural and Investment Funds Programmes 2014-2020 Co-funded by the Irish Government and the European Union

EUROPEAN UNION Investing in your Future European Social Fund

BIM Ireland's Seafood Development Agency

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AIT Research

Developing Sustainable Freshwater Aquaculture using Irish Peatlands during COVID-19 Crisis

Ms. Emer O'Neill[1], Mr. Gustavo Fehrenbach, Dr. Emma Murphy, Dr. Robert Pogue, Dr. Mark Lynch, Prof. Neil Rowan.
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Introduction

Figure 1: Aerial view of the **AquaMona trial fish farm** located in the middle of Mount Lucas Wind Farm, Co. Offaly.

Aquaculture is one of the **fastest growing food** producing industries in the world[1]. Aquaculture will contribute to **meeting global need for sustainable food production**. The Irish aquaculture sector was worth €208.4M in 2017[2]. However, several factors have hampered the growth of the industry, including disease and environmental impacts[1,3]. **Bord Iascaigh Mhara (BIM),** undertook a feasibility study to assess the potential use of **peatlands (bogs)**, for sustainable aquaculture diversification[4]. In April 2019 BIM, in conjunction with **Bord na Mona**, begun the **AquaMona project** and opened a trial fish farm at the Mount Lucas Wind Farm[4,5] (Figure 1). AquaMona is a **new concept** in **integrated multitrophic aquaculture (IMTA)**, which uses cutaway bogs to farm rainbow trout (*Oncorhynchus mykiss*) and Eurasian perch (*Perca fluviatilis*) with associated organic status that is powered by wind energy and utilises algae and duckweed to treat rearing water[6]. There is total reliance upon **natural processes** for **water quality** and waste management negating the ability to use antibiotics or other chemical biocides that would provides **risks to process security and ecosystem.**

Algal communities have been exploiting during this a previous studies to address latter threat. This includes wastewater process efficacy through assimilation of nutrients, along with providing health benefits to fish both in terms of nutrition and bioactives[6]. However, there is a **lack of knowledge** on the specific contributory **role of algae** in the **aquaculture process for this purpose**. Equally missing is the **relationship** between algae, **microbial communities and the role of harmful cyanobacteria in this process.**

The Aim of this research is to develop a comprehensive understanding of the relationship between algal and microbial communities in freshwater aquaculture so as to specifically inform and guide the development of this novel innovative peatland cut-away IMTA process. This would provide unique new knowledge for aquaculture industry as to critical role of algal and microbial communities in maintaining optimal IMTA conditions.

Methodology

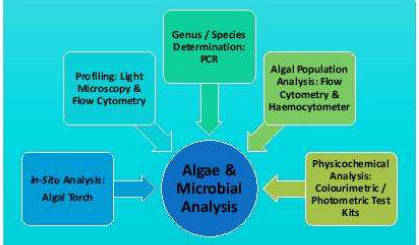

Figure 2: Breakdown of methods applied to assist in analysing the relationship between the algal and microbial communities of Mount Lucas Fish Farm.

Results to Date

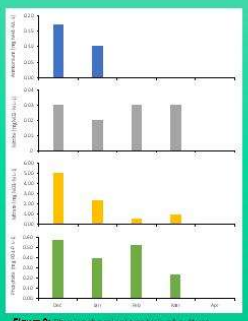

Figure 3: Physicochemical analysis of culture ponds from December 2019 to April 2020. n = 8

Physicochemical Analysis
Analysis included ammonium, nitrite, nitrate and phosphate analysis (Figure 3). Decreases were observed in ammonium and nitrate from February onwards.

Algae & Microbial Analysis
Analysis included algal counts, bacterial counts, cyanobacteria levels and chlorophyll concentrations (Figure 4). Increases in cyanobacteria coincided with decreases in algae from February. Fish mortalities have also been included.

Due to the COVID-19 pandemic, results for April have not yet been fully established.

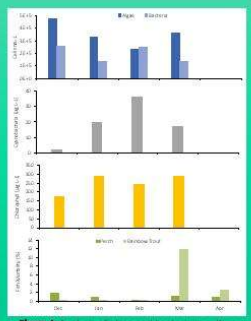

Figure 4: Analysis of algae and bacteria in culture ponds from December 2019 to April 2020. n = 8

Discussion

Unpredictable changes in our weather has had major repercussions on the fish farm.
- February 2020 displayed the highest level of rainfall for a single month in Ireland since records began.
- Traditional average = 70.3 mm of rainfall. February 2020 - 197.5 mm of rain fell.
- Algal and nitrate levels diluted. Algae's preferred nutrient source is nitrate.
- The nitrogen phosphorous ratio (N:P) was reduced = Cyanobacteria growth favoured.
- Cyanobacteria's preferred nutrient source is ammonium ∴ Nitrification process could not occur preventing nitrate from forming further limiting algae growth and recovery.

Cyanobacteria growth
- Cyanobacteria is always present at low levels within the farm. Approximate 90:10 Algae:Cyanobacteria.
- Higher ratios of algae keeps cyanobacterial levels in check preventing toxic conditions.
- Reduced algal levels and nitrate concentration increased favourable conditions for cyanobacteria.
- Subsequent burst in cyanobacterial growth = catastrophic implications for the fish.
- High mortality rates within the rainbow trout population of the farm were observed. Up to
- >18% mortality was recorded at times during the month of March.

Mitigation measures suggested and applied
- Introduction of straw to help absorb the cyanobacteria.
- Use of small amounts of activated charcoal to neutralise and eliminate the Cyanobacteria toxins.
- Cyanobacteria levels have began to decrease and algae / chlorophyll levels are on the rise.

More research needs to be conducted to establish the efficacy of the measures, and to also determine which cyanobacteria species are causing issues so as to ensure that the most appropriate mitigation processes are being applied.

Challenges & Solutions

- COVID-19 has had dramatic effects on the way research is now conducted.
- Limited data sharing ability in real-time.
- Although limited lab work was conducted, it was very effective where analysis has been conducted during the lockdown period supplemented with *in-situ* monitoring of Trout and Perch on commercial farm at Mount Lucas (Bord na Mona).
- Samples are also preserved for future analysis post COVID-19 restrictions.
- Clear effectives solutions have been furnished to BIM to help over come cyanobacteria problem in real time to alleviate fish mortality

Future Work

- Analysis of backlogged samples.
- Continued assessment of beneficial uses of algae. Mainly water quality and immunology.
- Molecular analysis in harmful cyanobacterial species present and their toxic compounds.
- Development of tools for monitoring and decision making

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Supporting the sustainable development of the Irish freshwater aquaculture industry

Final Report

A. Kennedy, A. Tahar, R. Cooney, S. Naughton, E. O'Neill,
A. Fogarty, S. Kavanagh, N. Rowan, E. Clifford

EXECUTIVE SUMMARY

Project Overview

Ireland currently ranks as 5th in value and 7th in volume for aquaculture products in Europe (EMFF, 2015). In the Department of Agriculture Food and the Marine's National Strategic Plan for Sustainable Aquaculture Development (DAFM, 2015), the aim is to sustainably grow production of the Irish aquaculture sector to 45,000 tonnes per annum across all species by 2020 (DAFM, 2015) compared to 40,140 tonnes in 2015. In the context of significant potential for growth the industry will need to innovate in order to sustainably intensify production for both domestic and export markets. Furthermore, the use of a detailed evidence base demonstrating the potential of the industry to be both high value and low impact will be of great aid to the industry and policy makers.

The EcoAqua project, project was developed to address critically important challenges identified by the Irish aquaculture industry and various stakeholders to analyse environmental sustainability and production efficiency of the sector.
The project team analysed 4 representative freshwater aquaculture sites spread across Ireland with respect to process water and discharge water quality, alignment with the water framework directive (WFD), life cycle impacts, operational parameters and environmental sustainability. *This study characterised 67% of salmon smolt, 47% of trout and 100% of perch production nationally based on 2016 figures (BIM 2017).*Table 1 gives an overview of the 4 farms studied.

Table 1: Stakeholder fish farms main characteristics

Farm	Species produced	Facility type	Notes
MOWI Altan (MA)	Salmon smolt	Tank based flow through	Water treatment – drum filter & belt filter
MOWI Pettigo (MP)	Salmon smolt	Tank based flow through	Water treatment – drum filter & belt filter
IDAS	Rainbow Trout	Traditional earth pond flow through	No water treatment
Keywater Fisheries (KF)	Perch	RAS & Split-pond	In-situ water recirculation, algae treatment for nutrient remediation

The work on each farm was tailored towards the operational characteristics of that farm and some results may be relevant to farms of that type only. This results can be used to inform decision making relating to operational and process interventions on each site (and the sector as a whole) and thereby support the sustainable development of the industry. An overview of the project structure is given in Figure 1.

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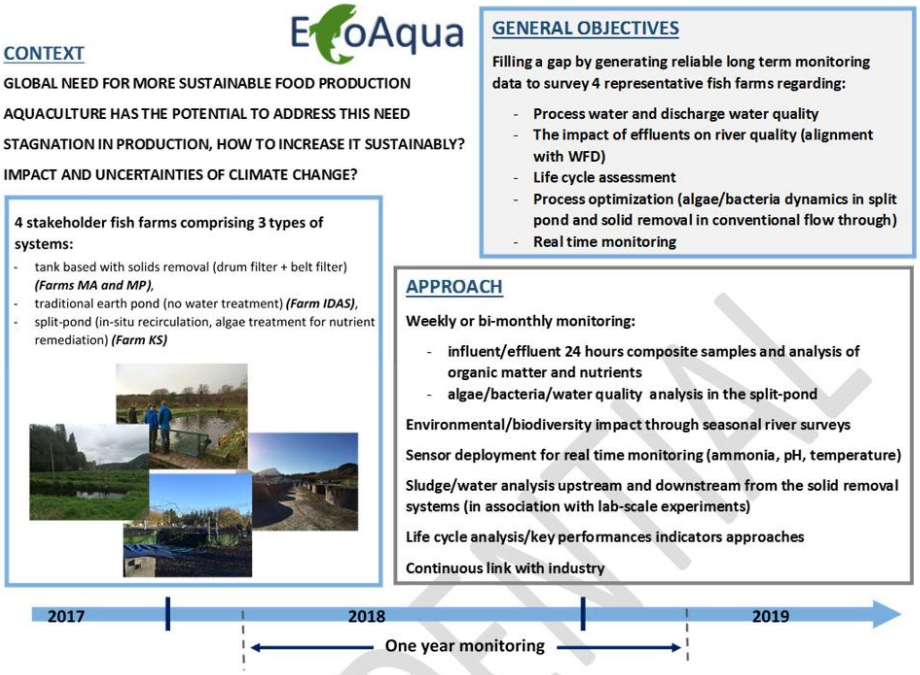

Figure 1: EcoAqua Project Schematic. EcoAqua was funded by the European Maritime Fisheries Fund (EMFF), administered by Bord Iascaigh Mhara, through the Knowledge Gateway Scheme, on behalf of the Department of Agriculture, Food and the Marine (Grant Number 17/KGS/004)

To meet the objectives of the project the key areas of research included (i) assessment water treatment technologies and their application to aquaculture (facilitating water reuse opportunities), (ii) life cycle assessment (LCA) studies, (iii) intensive continual monitoring of on-site environmental performance with a focus on treated water leaving the sites, and (iv) assessment for energy reduction potential within the industry. The project also developed a key collaboration with the Danish Technical University (DTU) and Chelsea Technologies to investigate the potential of new sensors within the sector to enable more efficient process control.

Link to online document:
https://qrty.io/KPAGpo

Figure A1.1: QR Code for access to the full EcoAqua Report. QR Code has been included due to the size and scale of the full report.

AquaAlgaePlus Project

Investigation to elucidate role and relationship between algal and microbial communities in freshwater aquaculture

Final Report

Project Reference BIM 19-KGS-008

The main aim of this research was to develop a comprehensive understanding of the role of algal communities and emergence/predominance of specific speciation in freshwater aquaculture so as to specifically inform and guide the development of the innovative peatland cut-away IMTA process at Mount Lucas. This would thereby provide the aquaculture industry with a holistic understanding of the putatively critical role of algal communities in maintaining optimal IMTA conditions and enable augmentation of this trial process at Mount Lucas in an environmentally sustainable manner that positively influences competitiveness. The main objectives of this research were;

1. Conduct physicochemical analysis to determine operating parameters that influence the occurrence and predominance of different algal communities.
2. Analyse algal and microbial populations in freshwater samples using flow cytometry parallel to physicochemical analysis in order to establish the different species present.
3. Identification of individual algal species present using next generation sequencing techniques and MinION technology in order to develop an algal repository for BIM.
4. Determination of immunological properties produced by bioactive compounds in algae using fish cell lines.
5. Isolation and cultivation of individual algae present in Mount Lucas using an algal bioreactor in order to provide a means to analyse the immunological potential of algae.

Given the size and scale of the final report, a copy of it has been attached to the following QR code.

Link to online document:
https://qrty.io/3cECVe

Figure A1.2: QR Code for access to the full AquaAlgaePlus Report. QR Code has been included due to the size and scale of the full report.

APPENDIX 2

SUPPLEMENTARY INFORMATION

Table A2.1: **The main issues concerned with the Irish Aquaculture industry** that were highlighted after SWOT analysis was performed as part of the National Strategic Plan for Sustainable Aquaculture Development. The SWOT data was based on information obtained from stakeholders concerned with the aquaculture industry. Bold points indicate those weaknesses and threats this research is aiming to address.

Strengths	**Opportunities**
i. Nutrient rich waters. ii. Environmentally sustainable production techniques. iii. Established production capabilities. iv. Technically advanced systems. v. Sheltered bays suitable for aquaculture production. vi. Global recognition as a leading producer of organic species. vii. Experienced operators with proven track record.	i. Employment potential in coastal communities. ii. Global demand for high quality seafood. iii. Cost / efficiency benefits from consolidation. iv. Land and sea-based nursery sites. v. Development of shellfish hatcheries. vi. Use of financial instruments to leverage resources. vii. Significant export potential. viii. Off-shore aquaculture sites. ix. Underutilised aquaculture sites. x. Market gaps such as oysters. xi. Novel species and niche products.
Weaknesses	**Threats**
i. Complex environmental requirements leading to delays in licensing process. ii. Insufficient product availability to meet market demand. iii. Lack of a co-ordinated route to market approach. iv. Lack of private investment. v. Lack of Irish packaging/distribution presence on mainland Europe. vi. Lack of support services and ancillary industries. **vii. Insufficient investment in research and development.** viii. Limited business planning for smaller operations. ix. Fragmentation within certain sectors. x. Overdependence on intermediaries to assess markets. xi. Overdependence on foreign seed suppliers for oysters.	i. Fish diseases and parasites. ii. Public opposition to industry. **iii. Spatial restrictions on aquaculture activities to protect Natura 2000 designated species and habitats.** iv. Competition in the organic Salmon sector v. Lack of access to finance. **vi. Climate change.** **vii. Impact on aquaculture due to eutrophication of marine waters.** viii. Co-existence with other marine activities. **ix. Natural occurring events such as algal blooms** and diseases such as AGD. x. Increased competition from companies outside the EU. xi. Further revisions of regulatory limits for bio-toxins. xii. Constrained exchequer funding. xiii. Impact on biodiversity from alien species.

Table A2.2: **The over-reaching needs** that have been identified **for the Irish aquaculture industry** after consultation with stakeholders and SWOT analysis. Bold points indicate the needs this research is aiming to address.

1.	**There is a need to grow the value, production and employment within the aquaculture sector.**
2.	**There is a need to develop the aquaculture sector in harmony with nature, in compliance with environmental law and with the confidence of the stakeholders.**
3.	**There is a need to foster knowledge, innovation and technology transfer to take advantage of opportunities growth and to better manage environmental impacts, fish diseases bio-toxins, *etc.***
4.	**There is a need for a streamlines and efficient licensing system that provides greater business certainty to applicants and more transparency to the general public.**
5.	**There is a need to develop marine spatial planning and justifiably incorporate aquaculture into the framework.**

Table A2.3: **Food Harvest 2020'**s overall **Smart, Green, Growth vision** for the sustainable development of the Irish Agri-Food Industry. Bold points indicate those with which this research is aiming to address.

Act Smart
i. Prioritise research and development.
ii. Maximise adoption of best practice.
iii. Rationalise and collaborate at industry level.
iv. Review institutional support and regulatory burden.
v. Improve skill levels.
vi. Foster creativity and entrepreneurship.
vii. Improve focus on customer preferences.
Think Green
i. Prioritise environmental protection.
ii. Build environmental credibility through research and actions.
iii. Satisfy consumer requirements and preferences.
iv. Capitalise on natural advantages and resources.
v. Develop an umbrella 'Brand Ireland'.
vi. Align sustainability across the supply chain.
vii. Conserve biodiversity.
Achieve Growth
i. Increase the value of primary output in the agriculture and fisheries by €1.5B by 2020.
ii. Increase valued-added output by €3B by 2020.
iii. Achieve an export target of €12B by 2020.

Table A2.4: **Main issues concerned with the Irish Agri-Food industry** that were highlighted after SWOT analysis was revised as part of the Food Wise 2025 strategy. The SWOT data was based on information obtained from stakeholders concerned with the aquaculture industry. Bold points indicate the threats this research is aiming to address.

Strengths i. Proximity to key fishing grounds. ii. Strong marine science capability. iii. Good market diversification by involvement in Origin Green. iv. Clean, green image of Atlantic waters.	**Opportunities** i. Increasing global demand with supply deficit. ii. Attract increased landings into Ireland. iii. Upscale and diversity production. iv. Stock recovery through Common Fisheries Policy (CFP), programs.
Weaknesses i. Small scale, fragmented industry with lack of large processing facilities. ii. Lack of continuous raw material supply. iii. Over-emphasis on commodity production. iv. Poor industry competitiveness and leadership.	**Threats** i. Stock depletion in wild fisheries. **ii. Slowness / uncertainty of aquaculture licence determination.** iii. Seafood safety issues and farmed fish disease. iv. Failure to scale, diversify, innovate and invest. **v. Failure to protect and measure the impact on the natural environment.**

Table A2.5: **List of criteria to be considered by the licensing authority** as per Section 61 of the Fisheries (Amendment), Act 1997, when assessing aquaculture licence applications.

i.	The suitability of the place or waters for the proposed.
ii.	The possibility of other beneficial uses of the place, be they existing or potential.
iii.	The particular statutory status of the proposed location, if any.
iv.	The likely effects of the proposed on the economy of the area.
v.	The likely ecological effects of the proposal on wild fisheries, natural habitats, flora and fauna.
vi.	The effects or likely effects of the proposal on the environment.
vii.	The effects or likely effects of the proposal on any man-made environment of heritage value.
viii.	Where the proposal is likely to have significant effects on Natura 2000 sites.
-	Screening for and / or appropriate assessment must be under taken before consideration and determination

(DAFM, 2018a)

Table A2.6: **Summary breakdown** of some of the **algal classification systems** set out between 1753 and 1969. The researcher responsible, the year, the classification and their main factors have been included.

Researcher	Year	Classification Number	Classification	Classification Factors	Reference
Linnaeus	1753	14 Classifications	Conferva, Ulva, Fucus, Chara (Only 4 now considered Algae)	R	(Baweja and Sahoo, 2015)
Eichler	1833	5 Classifications	Cyanophyceae, Diatomeae, Chlorophyceae, Phaeophyceae, Rhodophyceae	P	(Baweja and Sahoo, 2015)
Harvey	1836	4 Classifications	Chlorospermae, Melanospermae, Rhodospermae, Diatomacea	P	(Sambamurty, 2017)
Engler & Prantle	1912	6 Classifications	Schizophyta, Flagellatae, Dinoflagellata, Bacillariales, Euphyceae, Eumycetes	MS	(Baweja and Sahoo, 2015; Sambamurty, 2017)
West	1916	4 Classifications	Isokontae, Akontae, Stephanokontae, Heterokontae	R, F	(Baweja and Sahoo, 2015)
Pascher	1931	8 Classifications	Chrysophyta, Phaeophyta, Pyrrophyta, Euglenophyta, Chlorophyta, Charophyta, Rhodophyta, Cyanophyta	CC, MS, DS	(Baweja and Sahoo, 2015; Whittaker, 1959)
Tilden	1933	5 Classifications	Chlorophyceae, Myxophyceae, Rhodophyceae, Phaeophyceae, Chrysophyceae	RF, B, P, F	(Baweja and Sahoo, 2015; Sambamurty, 2017; Tilden, 1933)
Smith	1938	7 Classifications	Chlorophyta, Euglenophyta, Pyrrophyta, Chrysophyta, Phaeophyta, Cyanophyta, Rhodophyta	MS, DS, PR	(Sambamurty, 2017; Whittaker, 1959)
Papenfuss	1955	8 Classifications	Chlorophycophyta, Charophycophyta, Euglenophycophyta, Chrysophycophyta, Pyrrophycophyta, Phaeophycophyta, Schizophycophyta, Rhodophycophyta	MS, DS, PR	(Baweja and Sahoo, 2015; Sambamurty, 2017)
Chapman	1962	4 Classifications	Euphycophyta, myxophycophyta, Chrysophycophyta, Pyrrophycophyta	P, MS, B, PR	(Baweja and Sahoo, 2015; Chapman and Chapman, 1973)
Round	1965	8 Classifications	Cyanophyta, Chrysophyta, Chlorophyta, Euglenophyta, Pyrrophyta, Cryptophyta, Phaeophyta, Rhodophyta	N, CO, PR	(Baweja and Sahoo, 2015; Sambamurty, 2017)
Prescott	1969	9 Classifications	Chlorophyta, Euglenophyta, Chrysophyta, Pyrrophyta, Phaeophyta, Rhodophyta, Cyanophyta, Cryptophyta, Chloromonadophyta	N, P, B, R	(Baweja and Sahoo, 2015; Sambamurty, 2017)

B = Biochemistry, CC = Cell Characterisation, CO = Cell Organelles, DS = Developmental Similarities, F = Flagellation, MS = Morphological Similarities, N = Nucleus, P = Pigmentation, PR = Phylogenetic Relationships, R = Reproduction, RF = Reserve Food

Table A2.7: Summary of previous studies conducted on aquaculture discharge. Location, culture species and physicochemical parameters used have been listed. Many studies included additional parameters however, for the purpose of this paper, only the same physicochemical parameters investigated in this study have been included.

Culture Species	Location	Physicochemical Parameters	Reference
Rainbow Trout (*Oncorhynchus mykiss*)	Portugal	DO, BOD, A, NH_4-N, PO_4-P, SS, pH, H, NO_3-N, NO_2-N, T	Boaventura *et al.* (1997)
Rainbow Trout (*Oncorhynchus mykiss*) & Brown Trout (*Salmo trutta*)	Spain	H, pH, DO, T, NO_3^-	Camargo (1994)
Range of Marine & Freshwater Aquaculture	China	NH_4-H, COD, BOD, SS	Cao *et al.* (2007)
Rainbow Trout (*Oncorhynchus mykiss*)	Brazil	PO_4-P, NH_4-N, NO_2-N, NO_3-N, pH, SS, DO, T	Caramel *et al*. (2014)
Banana Shrimp (*Penaeus merguiensis*)	Australia	T, pH, SS, NH_4^+, NO_3^-	Costanzo *et al.* (2004)
Shrimp (*Litopenaeus vannamei*)	Brazil	pH, DO, SS, NO_2^-, NO_3^-, PO_4^{3-}, COD	da Silva *et al.* (2017)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	DO, BOD, PO_4-P, NO_3^-, NO_2^-, H, pH, SS	Fadaeifard *et al.* (2011)
Rainbow Trout (*Oncorhynchus mykiss*)	France	NH_4^+, NO_3^-, PO_4^{3-}, SS	Guilpart *et al.* (2012)
Atlantic Salmon (*Salmo salar*)	Canada	SS, NO_3^-, pH, A, H, PO_4^{3-}, BOD	Lalonde *et al.* (2014)
Nile Tilapia (*Oreochromis niloticus*)	Brazil	NH_4-N, PO_4^{3-}, pH, DO, T	Miashiro *et al.* (2012)
Freshwater Prawn (*Macrobrachium rosenbergii*)	Brazil	T, pH, NH_4^+, NO_2^-, NO_3^-, DO, BOD, H	Moreira *et al.* (2010)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	DO, pH, SS, T	Namin *et al.* (2013)
Rainbow Trout (*Oncorhynchus mykiss*)	Iran	T, pH, DO, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}	Noroozrajabi *et al.* (2013)
Rainbow Trout (*Oncorhynchus mykiss*)	Turkey	T, DO, pH, BOD, SS, NO_2^-, NO_3^-	Pulatsü *et al.* (2004)
Channel Catfish (*Ictalurus punctatus*)	United States of America	pH, DO, T, A, H, SS, PO_4^{3-}, NO_2^-, NO_3^-, NH_4-N	Stephens and Farris (2004a)
Channel Catfish (*Ictalurus punctatus*)	United States of America	NO_3^-, NO_2^-, SS, PO_4^{3-},	Stephens and Farris (2004b)
Rainbow Trout (*Oncorhynchus mykiss*)	Ireland	BOD, NO_2-N, PO_4-P, SS, NH_4-N, T, DO, pH	Tahar *et al.* (2018)
Range of Marine & Freshwater Fish & Shrimp	Hawaii	PO_4^{3-}, SS, NH_4-N	Ziemann *et al.* (1992)
Rainbow Trout (*Oncorhynchus mykiss*)	Serbia	PO_4^{3-}, NO_2^-, NO_3^-, DO, T, pH, NH_4^+	Živić *et al.* (2009)

T = temperature, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, NH_4-N / NH_4^+ = ammonium, NO_2-N / NO_2^- = nitrite, NO_3-N / NO_3^- = nitrate, PO_4-P / PO_4^{3-} = orthophosphate, A = alkalinity, H = hardness, SS = suspended solids.

Table A2.8: **EPA water quality parameters.** G = guidance values, I/PV = indicative value, S = salmonids, C = cyprinids. See following page for notes.

EU Directive or National [Ministerial] Regulations		Units of Analysis	G Value	I / PV Value	Note(s)
AMMONIA					
Freshwater Fish Directive [78/659/EEC]	(S)	mg/L NH_3	< 0.005	< 0.025	1
	(C)	mg/L NH_3	< 0.005	< 0.025	1
	(S)	mg/L NH_4	< 0.04	< 1	2
	(C)	mg/L NH_4	< 0.2	< 1	2
BIOCHEMICAL OXYGEN DEMAND					
Freshwater Fish Directive [78/659/EEC]	(S)	mg/L O_2	< 3	-	3
	(C)	mg/L O_2	< 6	-	3
CHEMICAL OXYGEN DEMAND					
Surface Water Regulations [1989]	A1 waters	mg/L O_2	n/a	-	4
	A2 waters	mg/L O_2	n/a	-	4
	A3 waters	mg/L O_2	n/a	40	4
CONDUCTIVITY					
Surface Water Regulations [1989]	A1 waters	µS/cm	n/a	1000	5
	A2 waters	µS/cm	n/a	1000	5
	A3 waters	µS/cm	n/a	1000	5
DISSOLVED OXYGEN					
Freshwater Fish Directive [78/659/EEC]	(S)	mg/L O_2	50% > 9	50% > 9	6
		mg/L O_2	100% > 7		
	(C)	mg/L O_2	50% > 8	50% > 7	7
		mg/L O_2	100% > 5		
HARDNESS					
			-	-	8
NITRATE					
Surface Water Regulations [1989]	A1 waters	mg/L NO_3	n/a	50	9
	A2 waters	mg/L NO_3	n/a	50	9
	A3 waters	mg/L NO_3	n/a	50	9
NITRITE					
Freshwater Fish Directive [78/650/EEC]	(S)	mg/L NO_2	< 0.01	-	
	(C)	mg/L NO_2	< 0.03	-	
pH					
Freshwater Fish Directive [78/650/EEC]	(S)	pH units	n/a	> 6 - < 9	10, 11
	(C)	pH units	n/a	> 6 - < 9	10, 11
PHOSPHATE					
Surface Water Regulations [1989]	A1 waters	mg/L P_2O_5	n/a	0.5	12, 13
	A2 waters	mg/L P_2O_5	n/a	0.7	12, 13
	A3 waters	mg/L P_2O_5	n/a	0.7	12, 13
Molybdate Reactive Phosphorus	Unpolluted		0.03		
SUSPENDED SOLIDS					
Freshwater Fish Directive [78/659/EEC]	(S)	mg/L	< 25	-	14
	(C)	mg/L	< 25	-	14
TEMPERATURE					
Surface Water Regulations [1989]	A1 waters	°C	n/a	25	15
	A2 waters	°C	n/a	25	15
	A3 waters	°C	n/a	25	15

(EPA, 2001)

Notes

1. Limits are for "Non-ionised Ammonia".
2. Limits are for "Total Ammonia".
3. Nitrification should not be inhibited.
4. Value specified in Regulations for A3 water only.
5. Measured at 20°C.
6. The I value for salmonid water contains the provision: "When the oxygen concentration falls below 6".
7. The I value for cyprinid water contains the provision: "When the oxygen concentration falls below 4".
8. There are no specified values for Hardness.
9. Departure from standard may be granted by Minister "in the case of surface water in shallow lakes or virtually stagnant water" or "where exceptional meteorological or geographical conditions have arisen".
10. "Artificial pH variations with respect to the unaffected values shall not exceed ± 0.5 of a pH unit within the limits 6 and 9 provided that these variations do not increase the harmfulness of other substances present in the water".
11. Derogations possible "because of exceptional weather or special geographical conditions".
12. This precisely defined unit is broadly equivalent to "orthophosphate".
13. Departure may be granted by the Minister "in the case of surface water in shallow lakes or virtually stagnant surface water".
14. "The values shown are average concentrations and do not apply with suspended solids with harmful chemical properties". "Floods are liable to cause particularly high concentrations". Departures are possible "of exceptional weather or geographical conditions".
15. Departure may be granted by the Minister "where exceptional meteorological or geographical conditions have arisen".

Table A2.9: **Water quality parameters** applicable to this research set out the Statutory Instrument (**SI**) **272 of 2009** – European Communities Environmental Objectives (Surface Waters), Regulations 2009 and the Statutory Instrument (**SI**) **77 of 2019** – European Communities Environmental Objectives (Surface Waters), Regulations Amendments 2019. Listed are the general **physicochemical** conditions supporting the biological elements for **river water bodies** set out by the SI.

OXYGENATION CONDITIONS	
Biochemical Oxygen Demand	Mean ≤ 1.30 mg O_2 L^{-1} – High Status Mean ≤ 1.50 mg O_2 L^{-1} – Good Status
ACIDIFICATION STAUTS	
pH	4.5 < pH < 9.0 – Soft Water 6.0 < pH 9.0 – Hard Water
NUTRIENT CONDITIONS	
Total Ammonia	Mean ≤ 0.040 mg TAN L^{-1} – High Status Mean ≤ 0.065 mg TAN L^{-1} – Good Status
Molybdate Reactive Phosphorus	Mean ≤ 0.025 mg MRP L^{-1} – High Status Mean ≤ 0.035 mg MRP L^{-1} – Good Status

(Irish Statutory Office, 2019, 2009)

APPENDIX 3

METHODOLOGY BREAKDOWN

BIOASSAY – CULTURING, VALIDATION & ANALYSIS

ALGAE CULTURE MEDIUM PREPARATION

PSEUDOKIRCHNERIELLA SUBCAPITATA – JARWORSKI'S MEDIUM

Each component for was first prepared as per Table A3.1. To prepare the Jarworski's medium (JM), vitamin free JM was first prepared by adding one mL of each stock solution, except for solution number seven, to one L dH_20. The vitamin free JM was autoclaved at 121°C for 15 min at 15 psi and then cooled. To finish preparing the JM, one mL of stock solution number seven (vitamin solution) was sterilised using membrane filtration and then added to the cooled vitamin free JM. The JM was stored at 4°C until required. NOTE: Vitamin solution will become denatured if autoclaving.

Table A3.1: Summary of the components required for the preparation of **Jarworski's Medium**.

Stock Solution Number	Component	Concentration ($200mL^{-1}$)
1	$Ca(NO_3)_2$	4g
2	KH_2PO_4	2.48g
3	$MgSO_4$	10g
4	$NaHCO_3$	3.18g
5	EDTAFeNa	0.45g
	$EDTANa_2$	0.45g
6	H_3BO_3	0.496g
	$MnCl_2$	0.278g
	$(NH_4)6Mo_7O_{24}$	0.2g
7	Cyanocobalamin	0.008g
	Thiamine	0.008g
	Biotin	0.008g
8	$NaNO_3$	16g
9	Na_2HPO_4	7.2g

ASTERIONELLA FORMOSA – DIATOM MEDIUM

Each component for was prepared as per Table A3.2. To prepare the diatom medium (DM), vitamin free JM was first prepared by adding one mL of each stock solution, except for solution number seven, to one L dH_20. The vitamin free DM was autoclaved at 121°C for 15 min at 15 psi and then cooled. To finish preparing the DM, one mL of stock solution number seven (vitamin solution) was sterilised using membrane filtration and then added to the cooled vitamin free DM. Finally, the pH was adjusted to

pH 6.9 using HCl and NaOH. The DM was stored at 4°C until required. NOTE: Vitamin solution will become denatured if autoclaving.

Table A3.2: Summary of the components required for the preparation of **Diatom Medium**.

Stock Solution Number	Component	Concentration ($200mL^{-1}$)
1	$Ca(NO_3)_2$	4g
2	KH_2PO_4	2.48g
3	$MgSO_4$	5g
4	$NaHCO_3$	3.18g
5	EDTAFeNa	0.45g
	$EDTANa_2$	0.45g
6	H_3BO_3	0.496g
	$MnCl_2$	0.278g
	$(NH_4)6Mo_7O_{24}$	0.2g
7	Cyanocobalamin	0.008g
	Thiamine	0.008g
	Biotin	0.008g
8	Na_2SiO_3	11.4g

MONORAPHIDIUM CONTORTUM – TRIPLE NITRATE BOLD'S BASAL MEDIUM WITH VITAMINS

Firstly, each component for the Bristol's solution, vitamin solution and PIV metal solution were prepared as per Table A3.3, Table A3.4, and Table A3.5, respectively. The triple nitrate Bold's Basal Medium with vitamins (3N-BBM+V) was then prepared as follows; The PIV metal solution was firstly prepared by adding each component listed in Table A3.5 to 500mL dH_2O. Each component was added in the exact order listed. The PIV solution was the adjusted to pH 6.4 using HCl and NaOH. One L Bristol's solution was then prepared by adding 30mL of component one and 10mL of the remaining components listed in Table A3.3 to 920mL dH_2O. The solution was then autoclaved at 121°C for 15 min at 15 psi and then cooled. Finally, the vitamin solution (Table A3.4) was added as follows; one mL of components one, two and three, and six mL of the PIV metal solution were sterilised using membrane filtration and then added to the one L Bristol's solution. The 3N-BBM+V was then stored at 4°C until required.

Table A3.3: Summary of the components required for the preparation of the **Bristol's Solution** required for the preparation of **Triple Nitrate Bold's Basal Medium with Vitamins**.

Stock Solution Number	Component	Concentration ($200mL^{-1}$)
1	$NaNO_3$	5g
2	$CaCl_2$	0.5g
3	$MgSO_4$	1.5g
4	K_2HPO_4	1.5g
5	KH_2PO_4	3.5g
6	$NaCl_2$	0.5g

Table A3.4: Summary of the components required for the preparation of the **Vitamin Solution** required for the preparation of **Triple Nitrate Bold's Basal Medium with Vitamins**.

Stock Solution Number	Component	Concentration
1	Thiamine	0.1g $100mL^{-1}$
2	Biotin	25µg $100mL^{-1}$
3	Vitamin B_{12}	15µg $100mL^{-1}$
4	PIV Metal Solution	See Table A3.5

Table A3.5: Summary of the components required for the preparation of the **PIV Metal Solution** required for the preparation of **Triple Nitrate Bold's Basal Medium with Vitamins**.

Stock Solution Number	Component	Concentration $500mL^{-1}$
1	Na_2EDTA	0.75g
2	$FeCl_2$	48.5mg
3	$MnCl_2$	20.5mg
4	$ZnCl_2$	2.5mg
5	$CoCl_2$	1mg
6	Na_2MoO_4	2mg

ALGAE CULTURING

A starter culture of *P. subcapitata* (CCAP 278/4), *A. formosa* (CCAP 1005/9) and *M. contortum* (CCAP 245/2) were obtained from The Culture Collection of Algae and Protozoa (SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.), and grown in their respective culture mediums at 23°C ± 2°C exposed to continuous illumination (lux 6,000 – 10,000). *P. subcapitata* and *M. contortum* were sub-cultured every Monday, Wednesday and Friday, and *A. formosa* was sub-cultured every Wednesday to ensure the growth rates remained in the exponential phase. For the sub-culturing; 15mL of the *P. subcapitata* was added to 85mL of fresh JM, 10mL of the *A. formosa* was added to 90mL of fresh DM and 20mL of the *M. contortum* was added to 80mL of fresh 3N-BBM+V. (NOTE: JM was also used for the growth of *A. formosa*). Culture flasks were autoclaved at 121°C for 15 min at 15 psi between each sub-culture to prevent contamination. Cultures should have ideally been incubated in a shaking phyto-incubator, as per ISO (8692:2012) guidelines however, due to limitations in availability of equipment, a static incubator was used and flasks were manually shaken by hand every 2h – 3h or whenever possible.

ALGAL BIOASSAY VALIDATION

Validation of the algal bioassays were carried out in general accordance with the ISO (8692:2012) guidelines. However, as previously mentioned, the procedures were modified for incubation under static conditions due to limitations in availability of a shaking phyto-incubator. Validation was performed using the ISO specified reference chemicals (3,5-DCP and $K_2Cr_2O_7$). Dilutions of the reference chemicals were prepared (1, 1.8, 3.2, 5.6 and 10 mg L^{-1}) using the respective mediums as

diluent. A 50mL working stock solution of algae ($2x10^5$ algae cells mL^{-1}) was prepared. Nineteen millilitres of each chemical treatment were placed into their respective 25mL Erlenmeyer flasks. Negative controls were also prepared using just medium. One mL of the working stock solution of algae was added to each flask resulting in a concentration of $1x10^4$ algal cells mL^{-1} per control and treatment. All flasks were then plugged with cotton wool to prevent evaporation. Each control and treatment were set up in triplicate and two independent tests were performed. Controls and treatments were then incubated at 23°C ± 2°C for 72h and 96h, respectively, under continuous illumination (lux 6000 – 10000). The percent growth rate inhibition for the control and each treatment was then calculated followed by the E_rC_{50} value.

DAPHNIA – MAINTENANCE & CULTURING

In-house cultures of *Daphnia magna* and *Daphnia pulex* were cultured in spring-water that had been aerated for a minimum of 24h. Using two litre beakers half filled with the aerated spring-water, the Daphnids were separated out into adults, juveniles and neonates using sieves of different pore sizes. The beakers were then topped up with the old culture water the Daphnids had just been removed from. The beakers were then placed in a water bath at 20°C ± 2°C and exposed to 16h of light and 8h of darkness. The Daphnids were then fed with *Daphnia* food pellets (one per beaker). This process was repeated every Tuesday and Friday as well as 24h prior to any planned tests. This was to ensure the neonates required for testing were less than 24h old, as per the ISO (6341:2012), guidelines. Ideally, *Daphnia magna* should be fed algae, which is more indicative of *in-situ* conditions; however, setbacks in obtaining algae for this purpose were experienced.

DAPHNIA BIOASSAY VALIDATION

Validation of the crustacean bioassays were carried out in general accordance with the ISO (6341:2012), guidelines. However, the procedure was modified slightly. The aerated spring-water was used as diluent as opposed to the suggested dilution water set out in the guidelines. This modification was conducted due to high levels of mortality observed when the *Daphnia* were transferred from the aerated spring-water to the suggested dilution water. The guidelines also stated that natural water may be used as an alternative if the *Daphnia* did not display any signs of stress in it. Validation was performed using the two ISO suggested reference chemicals; $K_2Cr_2O_7$ and $ZnSO_4$. Dilutions of each reference chemical were prepared (0.1, 0.5, 1, 5 and 10 mg L^{-1}), using the aerated spring-water as diluent. A negative control was also prepared. Ten mL of each control and chemical dilution treatment was placed into their respective 25mL beakers. The control and treatments were all set up in quadruplicate and two independent tests per reference chemical were performed. Using a dissecting

microscope, five neonates less than 24h old were placed into each beaker using a glass Pasteur pipette, ensuring the neonates were released just below the surface of the liquid to minimise stress conditions. The beakers were then incubated at 20°C ± 2°C, exposed to 16h of light and 8h of darkness, for 24h and 48h, respectively. The percent immobilisation for each control and treatment were calculated, followed by the EC_{50} value. Neonates are considered immobilised if no movement is observed for more than 15s under gentle agitation.

PHYSICOCHEMICAL – KIT VALIDATION & STANDARD CURVES

AMMONIUM

KIT VALIDATION

Validation was carried out in general accordance with the APHA 4500-NH_3-F guidelines. All reagents required for the procedure were first prepared. Due to restrictions, dH_2O was used as diluent instead of the suggested ammonium free water. For the boric acid solution; 20g boric acid (H_3BO_3) was dissolved in 500mL dH_2O and then topped up to 1L. For the 1N sodium hydroxide solution (NaOH); 40g NaOH was dissolved in 500mL dH_2O and dissolved to 1L. For the borate buffer solution; a sodium tetraborate solution ($Na_2B_4O_7$) was first prepared by adding 9.5g $Na_2B_4O_7$ to 1L dH_2O. Eighty-eight millilitres 0.1N NaOH was added to 500mL of the $Na_2B_4O_7$ solution and diluted to 1L. For the sodium thiosulphate solution ($Na_2S_2O_3$); 3.5g $Na_2S_2O_3$ was dissolved in 500mL dH_2O and diluted to 1L. For the 5N sulphuric acid (H_2SO_4); 139mL concentrated H_2SO_4 was carefully added to 500mL dH_2O, cooled to room temperature and diluted to 1L. For the phenol solution (C_6H_6OH); 11.1mL 95% (v/v) C_6H_5OH was added to 50mL dH_2O and diluted to 100mL. For the sodium hypochloride solution (NaOCl); 250mL Domestos bleach was added to 500mL dH_2O and mixed. Domestos was used as it contained the required 5% NaOCl. For the alkaline citrate solution; 200g trisodium citrate ($Na_3C_6H_5O_7$) and 10g NaOH was dissolved in 500mL dH_2O and diluted to 1L. For the oxidising solution; 100mL of the alkaline citrate solution was added to 25mL of the NaOCl solution. Finally, for the sodium nitroprusside solution ($Na_2[Fe(CN)_5NO]$); 0.5 g $Na_2[Fe(CN)_5NO]$ was dissolved in 1L dH_2O. The preliminary distillation step was then performed. Five hundred millilitres dH_2O and 20mL borate buffer solution were added together. The pH was adjusted to 9.5. The solution was placed in a distillation flask and attached to the distillation apparatus. The solution was then used to steam out the apparatus. The flask was left attached to the apparatus until it was ready to be used to prevent contamination. Dilutions of ammonium were prepared as per the standard curve method below. *To remove any chloride from the standard solutions, 5mL $Na_2S_2O_3$ solution was added to 500mL of the first standard. Then the standard was immediately neutralised to pH 7. Twenty-five mL borate buffer solution was added to

the standard and the pH was adjusted to 9.5. The standard was then placed into a new distillation flask and immediately swopped out with the steaming out flask. The standard was distilled at a rate of approximately 10mL min^{-1} into 0.04N H_2SO_4. The tip of the delivery tube was placed just below the surface of the receiving acid. Approximately 200 mL distillate was collected and the tip of the delivery tube was moved so it was no longer in contact with the liquid. Distillation was then continued for another two minutes to cleanse the condenser and delivery tube*. This process (* to *) was repeated for each control and standard, ensuring each was immediately neutralised. Each control and standard were set up in triplicate. Twenty-five mL of each control / standard was added to their respective 50mL Erlenmeyer flask. One mL C_6H_5OH solution, 1 mL $Na_2[Fe(CN)_5NO]$ solution and 2.5mL oxidising solution was added to each flask, ensuring that the flasks were mixed thoroughly after the addition of each solution. The flasks were then covered with parafilm and left to stand at room temperature, away from bright lights, for one hour to allow the colour to develop. The absorbance for each control / standard was read at 640nm and a standard curve was constructed.

STANDARD CURVE

Dilutions of ammonium were prepared (2.5, 5, 7.5, 10, 25, 50 and 75 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). As the standard solution was at a concentration of 1000 mg L^{-1}, serial dilutions were prepared (100, and 10 mg L^{-1}), to ensure the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. Five mL of reagent NH_4-1 was added to test tubes. Two hundred microlitres of each standard concentration was added to their respective test tubes and mixed. A negative control / colour blank was also prepared using dH_2O. Each control and standard concentration were set up in triplicate. One level micro-spoon of reagent NH_4-2 was added to each test tube and then vortexed to completely dissolve the reagent. The test tubes were left to stand at room temperature for 15 min to allow the colour to develop. The spectrometer was set at 640nm and auto-zeroed using dH_2O. The absorbance for each colour blank and standard concentration was then read and the ammonium standard curve was constructed. Statistical analysis was then conducted between the kit and the wet chemistry method to determine whether any significant difference was observed between the two methods.

NITRITE

KIT VALIDATION

Validation was carried out in general accordance with the EPA 345-1 guidelines. The buffer-colour reagent was first prepared. One hundred five millilitres concentrated HCl, 5g sulphanilamide

($H_2NSO_2NH_2$) and 0.5g N-(1-Naphthyl) ethylenediamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$) was added to 250mL dH_2O and stirred to dissolve. One hundred thirty-six grams' sodium acetate ($C_2H_3NaO_2$) was then added to the solution and stirred again to dissolve. Finally, the solution was diluted to 500mL and stored in the dark. The nitrite standards and colour blank were prepared as per the standard curve method below. Fifty mL of each standard concentration were added to their respective beakers. Each standard and colour blank were set up in triplicate. Two mL of the buffer-colour reagent were added to each beaker and mixed. The beakers were left to stand at room temperature to allow the colour to develop and absorbance was read at 540nm. Statistical analysis was then conducted between the kit and the wet chemistry method to determine whether any significant difference was observed between the two methods.

STANDARD CURVE

Dilutions of nitrite were prepared (0.2, 0.4, 0.6, 0.8 and 1 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). As the standard solution was at a concentration of 1000 mg L^{-1}, serial dilutions were prepared (100, 10 and 1 mg L^{-1}) to ensure the 1:10 dilution rule was adhered to *i.e.*, dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. Five mL of each standard concentration were added to their respective test tubes. A negative control / colour blank was also prepared using dH_2O. Each control and standard concentration were set up in triplicate. One level micro-spoon of reagent NO_2-1 was added to each test tube and then vortexed to completely dissolve the reagent. The test tubes were left to stand at room temperature for 10 min. The spectrometer was set at 540 nm and auto-zeroed using dH_2O. The absorbance for each colour blank and standard concentration was then read and the nitrite standard curve was constructed.

NITRATE

KIT VALIDATION

Validation was carried out in general accordance with the APHA 4500-NO_3 guidelines as the DIN 38405-9 guidelines could not be accessed. Dilutions of nitrate were prepared (0.5, 1, 5, 10, 50 and 100 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). During preparation, it was ensured that the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. One mL 0.1N HCl was added to 50mL of each standard dilution. Absorbance was read at 220nm and then at 275nm. Two times the absorbance observed at 275nm was then subtracted from the absorbance observed at

220nm for each solution. The standard curve was constructed. Each concentration was prepared in triplicate and two independent tests were performed.

STANDARD CURVE

Dilutions of nitrate were prepared (0.5, 1, 5, 10, 50 and 100 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). During preparation, it was ensured that the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. Four mL of reagent NO_3-1 was added to each test tube. Five hundred microlitres of each standard concentration were added to their respective test tubes. N.B. Samples WERE NOT mixed at this point. Five hundred microlitres of reagent NO_3-2 was added to each test tube. Then samples were then carefully mixed, only holding the top of the tubes as the solution gets very hot. A negative control was also prepared using dH_2O. The standard concentrations and negative control were prepared in triplicate. The tests tubes were left to sit at room temperature for 10 min. The spectrometer was set at 332nm and auto-zeroed using dH_2O. The absorbance of read was read and standard curve was constructed. Statistical analysis was conducted between the kit and the wet chemistry method to ensure no significant difference observed between the two methods.

ISE STANDARD CURVE

Dilutions of nitrate were prepared (0.5, 1, 5, 10, 50 and 100 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). During preparation, it was ensured that the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. The nitrate probe was attached to the pH meter and set to mV. The probe was left to soak in the 100 mg L^{-1} nitrate solution for at least five minutes. The probe was rinsed with dH_2O, placed into 100mL of the lowest concentration, left to stabilise and the reading was taken. This was repeated for each concentration, working from the lowest to the highest. Each concertation was set up in triplicate. The ISE standard curve was then constructed. Ionic strength adjustment buffer was not required for concentrations <50 mg L^{-1}. A difference of between 55mV and 59mV between each log concentration needed to be observed for validation.

ISE WATER SAMPLE ANALYSIS

Turbid samples were filtered prior to testing to prevent interferences. Samples were tested within 24h of collection to remove the need for preservation. One hundred mL of the individual water samples were placed into their respective beakers. All samples were prepared in triplicate. The nitrate probe

was attached to the pH meter and set to mV. The probe was left to soak in the 100 mg L^{-1} nitrate solution for at least five min. The probe was rinsed with dH_2O, placed into the first sample, left to stabilise and the reading was taken. This was repeated for each sample, ensuring the probe was well rinsed between readings.

ORTHOPHOSPHATE

KIT VALIDATION

Validation was carried out in general accordance with the APHA 4500-P-C guidelines. The vanadate molybdate reagent was first prepared. Twenty-five grams' ammonium molybdate ($[NH_4]_6Mo_7O_{24}$), was added to 300mL dH_2O and dissolved. This solution was labelled A. Twelve hundred fifty mg ammonium metavanadate (NH_4VO_3), was added to another 300mL dH_2O and boiled to dissolve. Three hundred thirty millilitres concentrated HCl was added and mixed. This solution was labelled B. Solution A was then poured into solution B and diluted to one litre with dH_2O. The orthophosphate standards and colour blank were prepared as per the standard curve method below. Thirty-five mL of each standard and colour blank were added to their respective beakers. Each standard and colour blank were set up in triplicate. Ten mL of the vanadate molybdate reagent was added to each beaker and mixed. Samples were then diluted to 50mL with dH_2O and left to stand at room temperature for ten minutes to allow the colour to develop. Absorbance was read at 400nm and the standard curve was constructed.

STANDARD CURVE

Dilutions of orthophosphate were prepared (2, 4, 6, 8 and 10 mg L^{-1}) using a manufacturers standard solution (Sigma-Aldrich). As the standard solution was at a concentration of 1000 mg L^{-1}, serial dilutions were prepared (100 and 10 mg L^{-1}) to ensure the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. Five mL of each standard concentration were added to their respective test tubes. A negative control / colour blank was also prepared using dH_2O. Each control and standard concentration were set up in triplicate. Twelve hundred µL of reagent PO_4-1 was added to each test tube and then vortexed to mix the reagent. The spectrometer was set at 400nm and auto-zeroed using dH_2O. The absorbance for each colour blank and standard concentration was then read and the orthophosphate standard curve was constructed. Statistical analysis was then conducted between the kit and the wet chemistry method to determine whether any significant difference was observed between the two methods.

CHEMICAL OXYGEN DEMAND

KIT VALIDATION

Validation was carried out in general accordance with the APHA 5220-D guidelines. The reagents were first prepared. For the digestion solution, 1.5g $K_2Cr_2O_7$, 84mL concentrated HCl and 16.7g mercuric sulphate ($HgSO_4$), were added to 250mL dH_2O and dissolved. The solution was then diluted to 500mL. For the catalyst solution, 22g silver sulphate (Ag_2SO_4), was added to 4.09Kg concentrated HCl. Finally, for the sampler wash solution, 250mL concentrated H_2SO_4 was added to 250mL dH_2O. The COD standards and colour blank were prepared as per the standard curve method below. A colour blank was also prepared using dH_2O. Two thousand five hundred µL of each standard and colour blank was pipetted into their respective test tubes. Each standard and colour blank were set up in triplicate. Fifteen hundred µL of the digestion solution was added to each test tube and mixed. Three thousand five hundred µL of the catalyst solution was then added to each test tube. The test tubes were capped tightly and shaken to mix. The test tubes were placed into a pre-heated COD reactor at 150^{o}C for 120 min. After incubation, the cells were moved to a rack, mixed and left to cool. Absorbance was read at 600nm and the standard curve was constructed. Statistical analysis was then conducted between the kit and the wet chemistry method to determine whether any significant difference was observed between the two methods.

STANDARD CURVE

Dilutions for the standard curve were prepared (50, 100, 150, 200 and 250 mg L^{-1}) using a manufacturers COD standard solution (Sigma-Aldrich). As the standard solution was at a concentration of 1000 mg L^{-1}, serial dilutions were prepared (500 and 50 mg L^{-1}) to ensure the 1:10 dilution rule was adhered to *i.e.,* dilutions were not prepared from solutions that were greater than 10X the desired concentration. Distilled water was used as diluent. Two mL of each standard concentration were slowly pipetted down the side of their respective COD reaction cells. A negative control was also prepared using dH_2O. Each control and standard concentration were set up in triplicate. The cells were capped tightly and shaken vigorously, ensuring to only hold the cap as the cell gets very hot. The caps were loosened slightly and the cells were placed into a pre-heated COD reactor at 148^{o}C for 120 min. After incubation, the cells were moved to a rack to cool for at least 30 min. Cells were shaken ten min into the cooling process. The spectrometer was set at 600nm and auto-zeroed using dH_2O. The absorbance for each colour blank and standard concentration was then read and the COD standard curve was constructed.

APPENDIX 4

RESULTS & STATISTICS BREAKDOWN

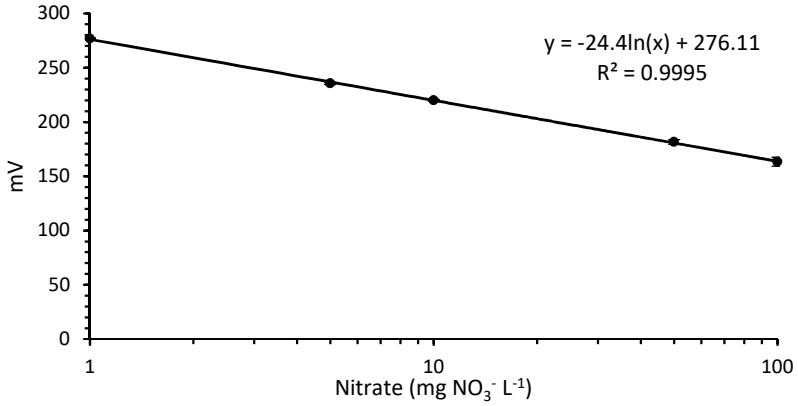

Figure A4.1: **Nitrate standard curve prepared via the ion selective electrode method.** The concentration of nitrate in mg NO_3^- L^{-1} has been plotted against the mV. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9999).

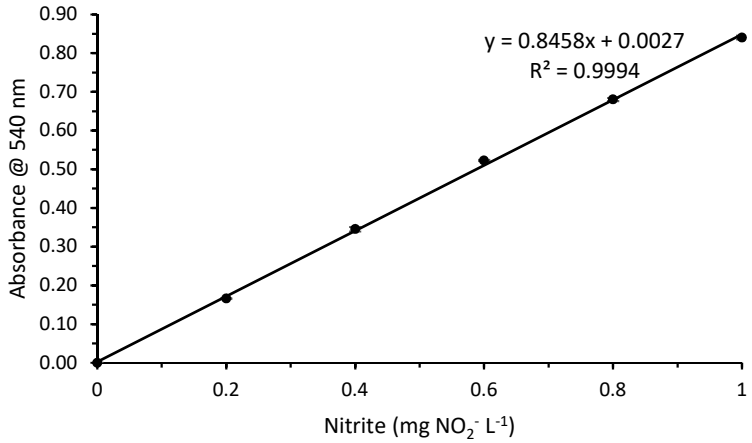

Figure A4.2: **Nitrite standard curve prepared via the Spectroquant Colourimetric Nitrite Test Kit.** The concentration of nitrite in mg NO_2^- L^{-1} has been plotted against the absorbance. Absorbance has been measured at 540nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9994).

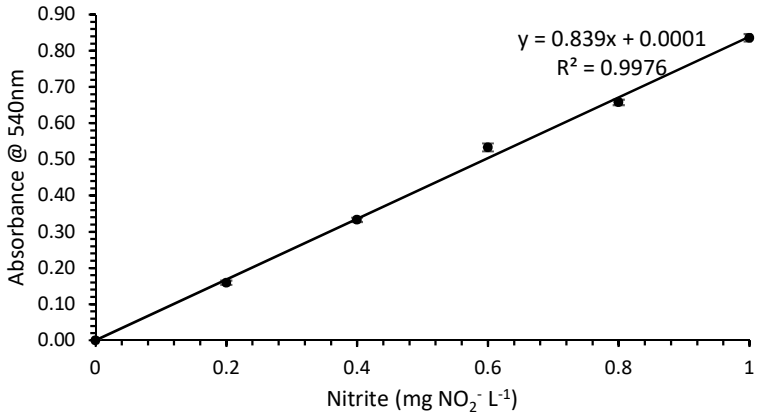

Figure A4.3: **Nitrite standard curve prepared via the Nitrite wet chemistry method as per the EPA 345-1 guidelines.** The concentration of nitrite in mg NO_2^- L^{-1} has been plotted against the absorbance. Absorbance has been measured at 540nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9996).

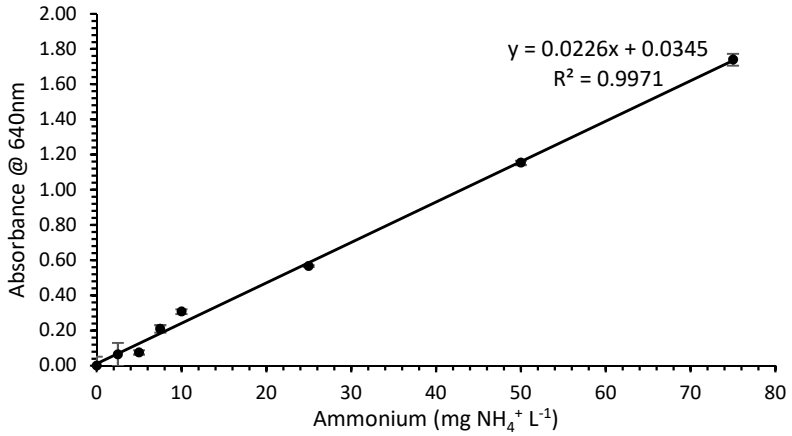

Figure A4.4: **Ammonium standard curve prepared via the Spectroquant Colourimetric Ammonium Test Kit.** The concentration of ammonium in mg NH_4^+ L^{-1} has been plotted against the absorbance. Absorbance has been measured at 640nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9958).

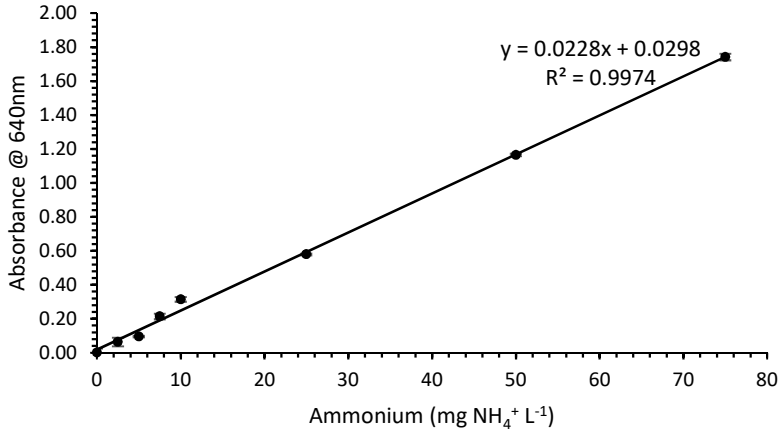

Figure A4.5: **Ammonium standard curve prepared via the wet chemistry method as per the APHA 4500-NH_3-F guidelines.** The concentration of ammonium in mg NH_4^+ L^{-1} has been plotted against the absorbance. Absorbance has been measured at 640nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9962).

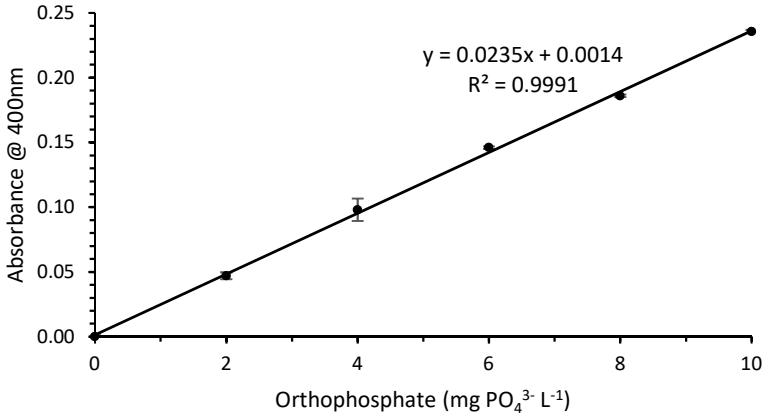

Figure A4.6: **Orthophosphate standard curve prepared via the Spectroquant Colourimetric Phosphate Test Kit.** The concentration of orthophosphate in mg PO_4^{3-} L^{-1} has been plotted against the absorbance. Absorbance has been measured at 400nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9982).

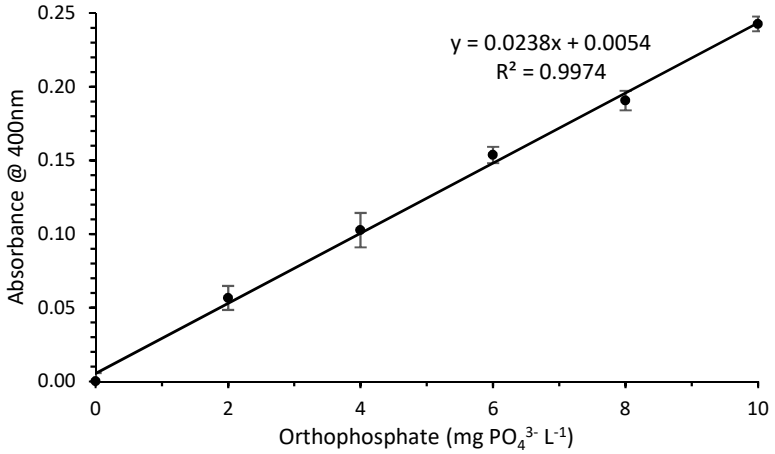

Figure A4.7: **Orthophosphate standard curve prepared via the wet chemistry method as per the APHA 4500-P-C guidelines.** The concentration of ammonium in mg PO_4^{3-} L^{-1} has been plotted against the absorbance. Absorbance has been measured at 400nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9977).

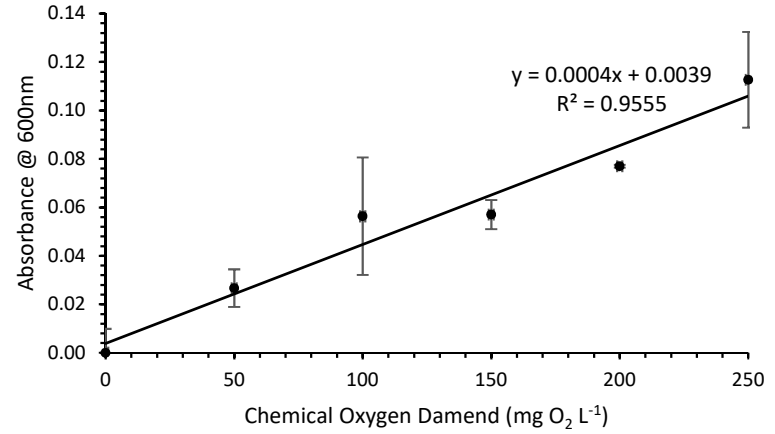

Figure A4.8: **Chemical Oxygen Demand (COD), standard curve prepared via the Spectroquant COD Test Kit.** The concentration of COD in mg O_2 L^{-1} has been plotted against the absorbance. Absorbance has been measured at 600nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.9300).

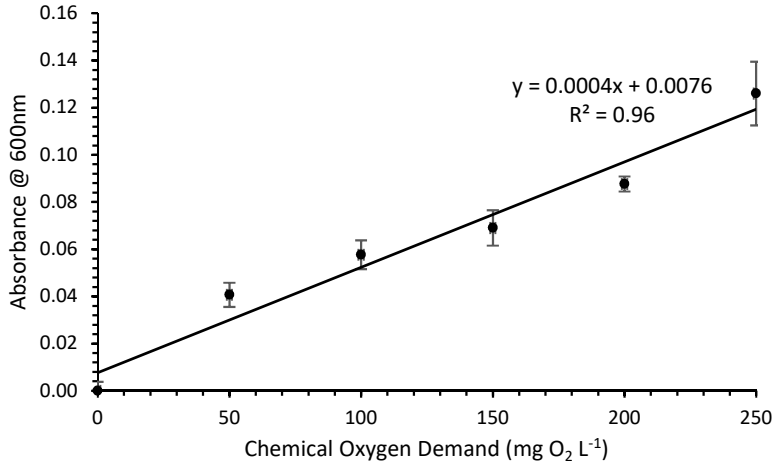

Figure A4.9: **Chemical Oxygen Demand (COD), standard curve prepared via the wet chemistry method as per the APHA 5220-D guidelines.** The concentration of COD in mg L^{-1} has been plotted against the absorbance. Absorbance has been measured at 600nm. Results display two independent tests with triplicates per test. N = 6, S.D., R^2 and equation of the line indicated. (p = 0.8678).

***Table A4.1:* Summary of results for all bioassays investigated on freshwater aquaculture intake water from April 2018 to October 2018 at Keywater Fisheries.** Grey sections indicate samples that could not be tested. All samples were run with a minimum of triplicates per test. All figures represent percentage.

Bioassay	Sample Date													
	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	02-Sep	04-Oct
***P. subcapitata* 72h Inhibition**	19.67	53.20	11.32	55.36	21.15			75.00	42.59	62.96	42.59	44.64	40.35	48.39
***P. subcapitata* 96h Inhibition**	7.27	38.03	0	30.61	17.78			46.67	53.06	31.25	25.00	23.91	22.92	9.26
***D. magna* 24h Immobility**	10	10	5	5	5			5	5	5				
***D. magna* 48h Immobility**	10	25	15	10	5			10	5.26	10				
***D. pulex* 24h Immobility**	10	5	16.67	10	10			5						
***D. pulex* 48h Immobility**	15	20	22.22	10.53	15			15						

***Table A4.2:* Summary of results for all bioassays investigated on freshwater aquaculture output water from April 2018 to October 2018 at Keywater Fisheries.** Grey sections indicate samples that could not be tested. All samples were run with a minimum of triplicates per test. All figures represent percentage. Red figures indicate stimulation.

Bioassay	Sample Date													
	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	02-Sep	04-Oct
***P. subcapitata* 72h Inhibition**	13.11	**50.47**	24.53	8.93	15.38			**7.69**	**14.81**	**25.93**	**5.56**	0	5.26	4.84
***P. subcapitata* 96h Inhibition**	5.45	**6.58**	0	10.20	13.30			**2.22**	**6.12**	**8.33**	**4.17**	0	6.25	1.85
***D. magna* 24h Immobility**	0	0	5	0	0			5	15	5				
***D. magna* 48h Immobility**	10	5	5	10	5			10	15.79	15				
***D. pulex* 24h Immobility**	5	10	16.67	5	5			10						
***D. pulex* 48h Immobility**	15	15	22.22	15.79	15			20						

Table A4.3: **Summary of results for all physicochemical analyses performed on freshwater aquaculture intake samples from April 2018 to October 2018 at Keywater Fisheries.** Grey sections indicate samples that could not be tested. All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested with 24 hours of collection to prevent the need for preserving samples.

Parameter	Sample Date													
	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	20-Sep	04-Oct
pH	7.81	7.67	7.66	7.99	7.96			7.86	7.92	7.92	7.33	7.70	7.65	7.70
Temperature (°C)	10.6	17.3	12.1	12.6	16.3			16.2	19.8	15.9	15.0	14.5	13.8	13.0
Ammonium (mg NH_4^+ L^{-1})		0.49	0.09	0.31	0.47			0.10	0	0.09	0	0.13	0	0.05
Nitrite (mg NO_2^- L^{-1})	0.02	0.03	0.03	0.01	0			0	0	0.01	0.03	0.02	0.02	0.02
Nitrate (mg NO_3^- L^{-1})					3.20			5.19	6.35	2.32	3.12	1.80	4.62	2.38
Orthophosphate (mg PO_4^{3-} L^{-1})	1.79	2.51	2.65	1.30	0.55			0.14	1.01	2.71	2.10	2.23	2.25	1.90
DO (mg O_2 L^{-1})	10.62	9.64	9.21	11.40	9.95			9.54	9.85	9.82	9.52	11.60	11.12	11.41
BOD (mg O_2 L^{-1})	1.14	3.11	0.59	5.03	2.77			2.61	3.83	1.65	2.67	3.05	1.25	0.55
COD (mg O_2 L^{-1})		0	53.34	151.67	20.84			30.00	45.00	0	39.17	55.00	51.67	58.34
Suspended Solids (mg L^{-1})	30	290	18	4	25			19	19	19	11	5	27	15
Hardness (mg $CaCO_3$ L^{-1})		98.1	108.0	101.5	106.8			117.2	82.8	94.0	93.2	96.0	106.8	101.0
Alkalinity (mg $CaCO_3$ L^{-1})		105.5	112.8	108.7	131.5			171.5	118.5	116.5	123.5	120.0	121.5	118.0
Conductivity (µS cm^{-1})	191.0	167.2	165.0	232.0	308.0			318.0	324.0	306.0	206.0	251.0	264.0	234.0

DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand

Table A4.4: **Summary of results for all physicochemical analyses performed on freshwater aquaculture output samples from April 2018 to October 2018 at Keywater Fisheries.** Grey sections indicate samples that could not be tested. All samples were run with a minimum of triplicates per test**.** Samples were analysed every two weeks. Samples were tested with 24 hours of collection to prevent the need for preserving samples.

Parameter	Sample Date													
	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	20-Sep	04-Oct
pH	7.50	7.18	7.21	7.29	7.11			7.14	7.06	7.14	6.96	6.89	6.91	6.95
Temperature (°C)	10.8	17.5	12.6	12.1	17.8			17.6	19.2	17.6	16.9	15.1	15.0	14.2
Ammonium (mg NH_4^+ L^{-1})		0.98	0.64	0.49	0.76			1.57	1.60	1.39	1.41	2.63	0.63	0.70
Nitrite (mg NO_2^- L^{-1})	0.06	0.17	0.09	0.13	0.05			0.05	0.05	1.15	0.76	0.88	0.22	0.20
Nitrate (mg NO_3^- L^{-1})					1.34			2.79	3.96	4.73	1.36	4.70	18.58	4.89
Orthophosphate (mg PO_4^{3-} L^{-1})	2.84	3.79	2.75	1.91	1.18			0.57	5.56	7.36	3.91	5.28	5.03	5.12
DO (mg O_2 L^{-1})	9.64	6.31	7.64	7.00	3.63			1.25	1.72	3.15	1.04	5.51	6.67	7.58
BOD (mg O_2 L^{-1})	1.91	6.31	4.71	0.62	3.63			1.25	1.72	3.15	1.04	4.88	3.62	5.97
COD (mg O_2 L^{-1})		14.17	105.00	169.17	27.50			80.84	197.50	32.50	35.84	53.34	59.17	65.84
Suspended Solids (mg L^{-1})	70	520	56	18	24			170	21	55	11	11	29	19
Hardness (mg $CaCO_3$ L^{-1})		110.5	128.6	118.0	133.2			154.8	106.8	96.0	105.2	109.2	104.0	110.0
Alkalinity (mg $CaCO_3$ L^{-1})		101.1	137.6	129.8	163.5			151.5	125.0	120.0	126.5	103.5	128.5	131.0
Conductivity (µS cm^{-1})	202.0	258.0	206.0	259.0	358.0			368.0	357.0	349.0	320.0	299.0	302.0	300.0

DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand

Table A4.5: **Correlation table** of studies carried out on result obtained from analysis of **intake** from **April 2018 to October 2018** *i.e.,* during the **pilot study, at Keywater Fisheries**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***D magna***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
D magna	-0.167														
D pulex	***-0.795***	-0.350													
pH	0.157	-0.531	-0.166												
Temp	0.401	-0.232	-0.667	0.173											
NH_4^+	-0.083	0.592	-0.372	0.357	0.129										
NO_2^-	-0.310	0.602	0.337	**-0.803**	-0.459	-0.079									
NO_3^-	0.112	0.000	0.000	0.253	*0.666*	-0.263	-0.533								
PO_4^{3-}	-0.194	0.354	0.427	*-0.509*	-0.310	-0.169	**0.807**	-0.583							
DO	0.080	0.112	-0.056	0.137	-0.421	-0.062	0.003	-0.429	0.060						
BOD	0.376	-0.198	-0.479	0.342	0.466	0.406	-0.400	0.410	-0.425	0.082					
COD	-0.028	-0.364	0.330	0.184	*-0.553*	-0.064	-0.043	0.091	-0.158	*0.590*	0.407				
SS	0.133	*0.688*	-0.507	-0.145	0.312	**0.601**	0.366	0.499	0.269	-0.271	0.120	-0.422			
Hard.	0.004	-0.124	-0.015	0.080	-0.469	0.197	-0.066	0.010	-0.304	-0.045	-0.320	0.057	-0.061		
Alk.	0.379	-0.351	-0.405	0.132	0.197	-0.171	-0.529	0.383	**-0.707**	-0.243	-0.043	-0.217	-0.294	*0.572*	
Cond.	0.417	*-0.636*	0.379	*0.563*	**0.575**	-0.184	**-0.896**	0.541	**-0.608**	0.011	0.257	-0.158	-0.421	-0.044	*0.559*

Table A4.6: **Correlation table** of studies carried out on result obtained from analysis of **output** from **April 2018 to October 2018** *i.e.,* during the **pilot study, at Keywater Fisheries**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***D magna***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
D magna	-0.142														
D pulex	-0.029	*0.787*													
pH	0.112	-0.552	-0.337												
Temp	**-0.619**	0.489	0.044	-0.480											
NH_4^+	-0.285	*0.692*	0.121	-0.437	0.414										
NO_2^-	-0.307	0.046	0.144	-0.389	0.213	*0.554*									
NO_3^-	0.164	0.605	0.000	-0.468	-0.475	-0.346	-0.100								
PO_4^{3-}	-0.393	0.478	0.247	-0.435	0.209	0.308	**0.651**	0.320							
DO	0.356	-0.594	-0.016	0.408	**-0.846**	-0.492	-0.298	0.578	-0.028						
BOD	-0.197	-0.230	0.513	-0.307	0.008	-0.065	0.048	0.251	0.310	0.422					
COD	0.270	0.593	-0.016	0.310	-0.216	-0.083	-0.430	-0.003	-0.088	0.004	-0.495				
SS	**-0.727**	-0.289	0.225	0.228	0.244	-0.064	-0.191	-0.121	-0.139	0.069	0.399	-0.345			
Hard.	0.343	-0.215	0.097	0.393	-0.021	-0.095	*-0.580*	-0.335	**-0.885**	-0.156	-0.199	0.085	0.115		
Alk.	**0.607**	-0.089	-0.205	0.224	0.034	-0.408	-0.493	-0.228	**-0.657**	-0.240	-0.359	0.098	-0.368	**0.701**	
Cond.	-0.292	0.440	-0.255	*-0.547*	**0.841**	0.394	0.251	-0.485	0.138	**-0.851**	-0.227	-0.083	-0.174	0.101	0.344

P sub = Pseudokirchneriella subcapitata, D magna = Daphnia magna, *D pulex = Daphnia pulex*, Temp = Temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = Dissolved oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity.

Table A4.7: **Summary of all p-values** determined during **normality testing** using Anderson-Darling tests after Grubb's test for outliers was conducted on all bioassays and parameters for the pilot study conducted between April 2018 and October 2018 at Keywater Fisheries. Normal distribution = $p > 0.050$.

	Intake	Output
Pseudokirchneriella subcapitata	0.519	0.359
Daphnia magna	0.625	0.120
Daphnia pulex	0.128	0.079
pH	0.201	0.536
Temperature	0.962	0.253
NH_4^+	0.090	0.106
NO_2^-	0.084	0.171
NO_3^-	0.404	0.116
PO_4^{3-}	0.265	0.837
Dissolved Oxygen	0.054	0.806
Biochemical Oxygen Demand	0.549	0.528
Chemical Oxygen Demand	0.134	0.065
Suspended Solids	0.523	0.370
Alkalinity	0.845	0.066
Hardness	0.854	0.411
Conductivity	0.445	0.312

Table A4.8: **Summary of results for all bioassays investigated on freshwater aquaculture intake from March 2019 to August 2019 at Keywater Fisheries.** All samples were run with a minimum of triplicates per test. All data represent percentage.

Bioassay	Sample Month					
	March	**April**	**May**	**June**	**July**	**August**
P. subcapitata **72h Inhibition**	42.11	9.09	6.58	8.20	12.50	3.45
P. subcapitata **96h Inhibition**	17.65	8.51	3.92	7.84	2.27	2.22
A. formosa **72h Inhibition**	18.18	32.14	55.17	55.56	17.39	11.54
A. formosa **96h Inhibition**	14.29	14.81	19.35	68.00	7.50	0.00
D. pulex **24h Immobility**	5.00	5.00	10.00	5.00	5.00	10.00
D. pulex **48h Immobility**	10.00	5.00	10.53	15.79	11.11	10.00

Table A4.9**: Summary of results for all bioassays investigated on freshwater aquaculture output from March 2019 to August 2019 at Keywater Fisheries.** All samples were run with a minimum of triplicates per test. All data represent percentage.

Bioassay	Sample Month					
	March	**April**	**May**	**June**	**July**	**August**
P. subcapitata **72h Inhibition**	18.42	5.45	9.84	3.28	19.64	1.72
P. subcapitata **96h Inhibition**	8.82	4.26	1.96	1.96	9.09	2.22
A. formosa **72h Inhibition**	4.55	10.71	3.45	3.70	17.39	3.85
A. formosa **96h Inhibition**	4.76	25.93	28.81	0.00	7.50	4.35
D. pulex **24h Immobility**	5.00	5.00	10.00	5.00	5.00	10.00
D. pulex **48h Immobility**	15.00	20.00	15.79	10.53	16.67	10.00

Table A4.10: **Summary of results for all bioassays investigated on freshwater aquaculture settlement pond samples from March 2019 to August 2019 at Keywater Fisheries.** All samples were run with a minimum of triplicates per test. All figures represent percentage. Grey sections indicate where samples could not be obtained.

Bioassay	Sample Month					
	March	April	May	June	July	August
P. subcapitata **72h Inhibition**	42.11	30.91	11.48		25.00	3.45
P. subcapitata **96h Inhibition**	17.65	12.77	5.88		18.18	4.44
A. formosa **72h Inhibition**	4.55	17.86	31.03		41.30	0.00
A. formosa **96h Inhibition**	9.52	18.52	38.71		25.00	8.70
D. pulex **24h Immobility**	5	10	5		20.00	20.00
D. pulex **48h Immobility**	15	10	10.53		27.78	25.00

Table A4.11: **Summary of results for all physicochemical analyses performed on freshwater aquaculture intake samples from March 2019 to August 2019 at Keywater Fisheries.** All samples were run with a minimum of triplicates per test. Samples were analysed once a month. Samples were tested with 24 hours of collection to prevent the need for preserving samples. Grey sections indicated where samples could not be obtained.

Parameter	Sample Month					
	March	April	May	June	July	August
pH	7.43	7.81	7.73	7.72	7.74	7.76
Temperature (°C)	11.57	13.60	13.77	13.73	15.03	15.40
Ammonium (mg NH_4^+ L^{-1})	0.00	0.16	0.00	0.00	0.19	0.02
Nitrite (mg NO_2^- L^{-1})	0.01	0.00	0.02	0.03	0.00	0.02
Nitrate (mg NO_3^- L^{-1})	0.00	0.83	1.76	3.51	2.03	2.74
Orthophosphate (mg PO_4^{3-} L^{-1})	0.00	0.01	0.49	2.92	0.00	0.37
DO (mg O_2 L^{-1})	14.78	13.77	9.34	9.09	8.80	8.78
BOD (mg O_2 L^{-1})	3.32	3.10	3.01	2.51	1.36	2.78
COD (mg O_2 L^{-1})	15.42	16.25	40.42	29.58	21.25	32.92
Suspended Solids (mg L^{-1})	10	11.67	28.33	27.33	20.33	25.33
Hardness (mg $CaCO_3$ L^{-1})	106.67	89.33	96.00	117.33	106.80	121.33
Alkalinity (mg $CaCO_3$ L^{-1})	121.67	125.00	126.67	171.67	118.50	146.67
Conductivity (µS cm^{-1})	113.10	132.40	144.70	91.50	150.20	158.00

Table A4.12: **Summary of results for all physicochemical analyses performed on freshwater aquaculture output samples from March 2019 to August 2019 at Keywater Fisheries.** All samples were run with a minimum of triplicates per test. Samples were analysed once a month. Samples were tested with 24 hours of collection to prevent the need for preserving samples. Grey sections indicated where samples could not be obtained.

Parameter	Sample Month					
	March	April	May	June	July	August
pH	7.08	7.23	7.15	7.07	7.14	7.18
Temperature (°C)	11.40	14.27	14.23	14.80	15.10	15.57
Ammonium (mg NH_4^+ L^{-1})	0.05	0.08	1.09	0.89	0.60	0.48
Nitrite (mg NO_2^- L^{-1})	0.06	0.03	0.12	0.19	0.14	0.03
Nitrate (mg NO_3^- L^{-1})	0.00	1.33	1.41	2.40	2.04	3.24
Orthophosphate (mg PO_4^{3-} L^{-1})	0.65	1.25	1.36	6.16	0.00	0.44
DO (mg O_2 L^{-1})	11.91	11.15	6.01	6.93	4.61	6.14
BOD (mg O_2 L^{-1})	3.59	2.54	2.90	4.16	1.74	1.86
COD (mg O_2 L^{-1})	32.92	15.42	0.00	27.92	15.42	23.75
Suspended Solids (mg L^{-1})	17.67	24.67	30.00	25.33	5.33	12.33
Hardness (mg $CaCO_3$ L^{-1})	98.67	96.00	104.00	154.67	98.80	117.33
Alkalinity (mg $CaCO_3$ L^{-1})	118.33	110.00	126.67	151.67	118.50	151.67
Conductivity (µS cm^{-1})	120.30	163.50	198.40	180.40	178.10	181.00

Table A4.13: Summary of results for all physicochemical analyses performed on freshwater aquaculture settlement pond samples from March 2019 to August 2019 at Keywater Fisheries. All samples were run with a minimum of triplicates per test. Samples were analysed once a month. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicated where samples could not be obtained.

Parameter	Sample Month					
	March	April	May	June	July	August
pH	7.29	7.56	7.62		6.99	7.04
Temperature (°C)	11.93	14.47	14.13		17.17	16.43
Ammonium (mg NH_4^+ L^{-1})	0.79	0.83	0.77		0.63	0.54
Nitrite (mg NO_2^- L^{-1})	0.02	0.01	0.06		0.48	0.07
Nitrate (mg NO_3^- L^{-1})	0.00	2.64	1.39		54.58	3.73
Orthophosphate (mg PO_4^{3-} L^{-1})	2.78	2.14	0.58		1.32	0.59
DO (mg O_2 L^{-1})	12.14	11.93	7.80		4.12	5.91
BOD (mg O_2 L^{-1})	3.08	3.32	2.88		2.06	2.30
COD (mg O_2 L^{-1})	95.42	47.08	16.25		13.75	63.75
Suspended Solids (mg L^{-1})	3.33	5.33	21.67		4.33	11.33
Hardness (mg $CaCO_3$ L^{-1})	98.67	94.67	86.67		98.80	154.67
Alkalinity (mg $CaCO_3$ L^{-1})	133.33	113.33	126.67		133.50	171.67
Conductivity (µS cm^{-1})	116.80	147.00	188.40		304.00	258.00

Table A4.14: **Summary of results for all bioassays investigated on freshwater aquaculture intake from May 2019 to August 2019 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. All figures represent percentage.

Bioassay	Sample Month			
	May	June	July	August
P. subcapitata **72h Inhibition**	7.14	22.95	1.69	5.17
P. subcapitata **96h Inhibition**	4.26	7.84	4.26	6.25
A. formosa **72h Inhibition**	0.00	3.70	10.00	6.90
A. formosa **96h Inhibition**	5.00	12.00	14.81	3.57
D. pulex **24h Immobility**	5.00	5.00	5.00	10.00
D. pulex **48h Immobility**	11.11	5.00	15.79	10.00

Table A4.15: **Summary of results for all bioassays investigated on freshwater aquaculture output from May 2019 to August 2019 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. All figures represent percentage. Red figures indicate where stimulation was observed instead of inhibition.

Bioassay	Sample Month			
	May	June	July	August
P. subcapitata **72h Inhibition**	1.79	3.28	10.17	3.45
P. subcapitata **96h Inhibition**	-2.13	3.92	6.38	8.33
A. formosa **72h Inhibition**	36.36	0	16.67	0.00
A. formosa **96h Inhibition**	15.00	4.00	18.52	3.57
D. pulex **24h Immobility**	15.00	5.00	10.00	5.00
D. pulex **48h Immobility**	22.22	10.00	21.05	15.00

Table A4.16: **Summary of results for all physicochemical analyses performed on freshwater aquaculture intake samples from May 2019 to August 2019 at Oasis Fish Farm.** Samples were analysed once a month. Samples were tested with 24 hours of collection to prevent the need for preserving samples.

Parameter	Sample Month			
	May	June	July	August
pH	7.62	7.43	7.71	7.70
Temperature (°C)	14.90	15.33	15.33	15.17
Ammonium (mg NH_4^+ L^{-1})	0.00	1.16	3.30	0.05
Nitrite (mg NO_2^- L^{-1})	0.04	0.11	0.15	0.03
Nitrate (mg NO_3^- L^{-1})	5.28	5.69	6.32	5.77
Orthophosphate (mg PO_4^{3-} L^{-1})	1.64	1.95	2.71	1.25
DO (mg O_2 L^{-1})	6.92	7.40	7.43	7.43
BOD (mg O_2 L^{-1})	0.79	1.15	1.44	1.09
COD (mg O_2 L^{-1})	37.08	23.75	28.75	39.58
Suspended Solids (mg L^{-1})	3.00	11.33	10.00	23.67
Hardness (mg $CaCO_3$ L^{-1})	106.67	94.00	106.67	106.67
Alkalinity (mg $CaCO_3$ L^{-1})	121.67	116.67	133.33	131.67
Conductivity (µS cm^{-1})	337.00	295.00	300.00	308.00

Table A4.17: **Summary of results for all physicochemical analyses performed on freshwater aquaculture output samples from May 2019 to August 2019 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed once a month. Samples were tested with 24 hours of collection to prevent the need for preserving samples.

Parameter	Sample Month			
	May	June	July	August
pH	7.54	7.77	7.63	7.74
Temperature (°C)	15.07	15.80	15.77	15.53
Ammonium (mg NH_4^+ L^{-1})	0.00	0.00	0.63	0.00
Nitrite (mg NO_2^- L^{-1})	0.46	0.41	0.40	0.37
Nitrate (mg NO_3^- L^{-1})	10.05	7.04	9.09	7.12
Orthophosphate (mg PO_4^{3-} L^{-1})	7.56	2.10	3.20	2.14
DO (mg O_2 L^{-1})	7.20	7.68	7.49	7.50
BOD (mg O_2 L^{-1})	1.32	3.80	1.87	1.95
COD (mg O_2 L^{-1})	49.58	47.08	46.25	42.92
Suspended Solids (mg L^{-1})	37.67	12.33	17.67	25.33
Hardness (mg $CaCO_3$ L^{-1})	104.00	96.00	105.33	98.67
Alkalinity (mg $CaCO_3$ L^{-1})	123.33	120.00	133.33	130.00
Conductivity (µS cm^{-1})	276.00	247.00	284.00	280.00

Table A4.18: **Correlation table** of studies carried out on result obtained from analysis of **intake** from **March 2019 to August 2019** *i.e.*, during the **monitoring program in Keywater Fisheries**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***A for***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
A for	-0.330														
D pulex	-0.470	0.067													
pH	**-0.830**	-0.168	0.423												
Temp	**-0.951**	0.234	0.267	0.794											
NH_4^+	-0.183	-0.350	-0.452	0.360	0.451										
NO_2^-	-0.260	0.548	0.426	0.037	-0.020	**-0.845**									
NO_3^-	-0.727	0.335	0.268	0.687	0.557	-0.173	0.656								
PO_4^{3-}	-0.286	0.662	-0.137	0.024	0.133	-0.444	0.785	0.728							
DO	0.726	-0.217	-0.479	-0.809	-0.553	0.057	-0.468	**-0.876**	-0.400						
BOD	0.307	0.176	0.237	-0.627	-0.351	-0.582	0.232	-0.465	-0.073	0.625					
COD	-0.623	0.503	**0.830**	0.448	0.372	-0.523	0.712	0.623	0.353	-0.749	0.007				
SS	-0.702	0.498	0.614	0.594	0.466	-0.380	0.730	**0.855**	0.559	**-0.915**	-0.295	**0.924**			
Hard.	-0.061	-0.253	0.154	0.326	-0.138	-0.426	0.588	0.638	0.471	-0.502	-0.264	0.254	0.438		
Alk.	-0.394	0.427	0.062	0.201	0.218	-0.497	**0.831**	0.800	**0.916**	-0.440	0.017	0.400	0.577	0.659	
Cond.	-0.388	-0.486	0.606	0.641	0.411	0.374	-0.395	-0.047	-0.704	-0.325	-0.252	0.261	0.139	-0.125	-0.520

Table A4.19: **Correlation table** of studies carried out on result obtained from analysis of **output** from **March 2019 to August 2019** *i.e.,* during the **monitoring program in Keywater Fisheries**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***A for***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
A for	0.512														
D pulex	-0.396	-0.495													
pH	-0.537	0.240	0.353												
Temp	-0.332	0.320	0.299	0.419											
NH_4^+	-0.210	-0.234	0.467	0.496	-0.262										
NO_2^-	0.131	0.062	-0.237	0.213	-0.645	0.733									
NO_3^-	-0.627	0.004	0.413	**0.929**	0.234	0.439	0.161								
PO_4^{3-}	-0.488	-0.407	-0.254	0.139	-0.500	0.433	0.656	0.213							
DO	0.296	0.044	-0.134	-0.670	0.370	**-0.816**	**-0.811**	-0.700	-0.612						
BOD	-0.102	-0.584	-0.339	-0.518	-0.702	0.128	0.431	-0.405	0.766	-0.112					
COD	0.007	-0.187	-0.488	-0.362	-0.505	-0.529	-0.092	-0.070	0.252	0.095	0.385				
SS	-0.442	-0.600	0.163	-0.194	-0.004	0.304	0.124	-0.267	0.525	0.033	0.634	-0.324			
Hard.	-0.574	-0.459	-0.032	0.344	-0.514	0.494	0.603	0.522	**0.902**	-0.769	0.558	0.364	0.246		
Alk.	-0.639	-0.573	0.418	0.479	-0.327	0.494	0.299	0.736	0.544	-0.701	0.197	0.296	0.019	**0.839**	
Cond.	-0.459	0.015	0.559	**0.848**	0.294	**0.822**	0.400	0.712	0.211	-0.704	-0.308	-0.696	0.180	0.310	0.408

P sub = Pseudokirchneriella subcapitata, A for = Asterionella formosa, *D pulex = Daphnia pulex*, Temp = Temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = Dissolved oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity

Table A4.20: **Correlation table** of studies carried out on result obtained from analysis of **settlement pond water** from **March 2018 to August 2018** *i.e.,* during the **monitoring program in Keywater Fisheries**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***A for***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
A for	0.242														
D pulex	0.024	0.323													
pH	0.386	0.527	0.777												
Temp	0.566	0.450	0.525	**0.938**											
NH_4^+	0.714	0.449	0.300	**0.816**	**0.958**										
NO_2^-	0.133	0.737	0.659	0.464	0.229	0.107									
NO_3^-	0.168	0.721	0.635	0.417	0.182	0.079	**0.992**								
PO_4^{3-}	**0.976**	0.069	0.052	0.393	0.580	0.715	-0.004	0.032							
DO	0.784	0.046	0.003	0.525	0.765	**0.883**	-0.288	-0.287	**0.847**						
BOD	0.679	0.308	0.272	0.786	**0.945**	**0.986**	-0.034	-0.060	0.714	**0.927**					
COD	0.593	-0.440	0.143	0.362	0.523	0.532	-0.322	-0.336	0.730	0.744	0.616				
SS	-0.255	0.347	0.097	0.468	0.514	0.438	-0.085	-0.183	-0.271	0.201	0.441	-0.077			
Hard.	0.251	0.112	0.770	**0.898**	**0.843**	0.677	0.205	0.147	0.341	0.512	0.709	0.609	0.412		
Alk.	0.356	0.267	0.713	**0.942**	**0.920**	0.781	0.279	0.212	0.407	0.571	0.785	0.582	0.488	**0.974**	
Cond.	0.114	0.590	**0.906**	**0.911**	0.724	0.527	0.710	0.655	0.087	0.133	0.466	0.093	0.409	0.799	**0.829**

Table A4.21: **Correlation table** of studies carried out on result obtained from analysis of **intake** from **May 2019 to August 2019** *i.e.,* during the **monitoring program in Oasis Fish Farm**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***A for***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
A for	-0.436														
D pulex	-0.288	0.272													
pH	0.271	0.741	-0.041												
Temp	**-0.985**	0.510	0.437	-0.199											
NH_4^+	-0.211	0.725	-0.465	0.699	0.172										
NO_2^-	0.105	0.561	-0.610	0.764	-0.150	0.948									
NO_3^-	-0.349	**0.964**	0.008	0.798	0.388	0.881	0.757								
PO_4^{3-}	-0.128	0.510	-0.687	0.554	0.046	**0.961**	**0.951**	0.716							
DO	0.093	0.829	0.359	0.916	0.031	0.495	0.484	0.777	0.268						
BOD	-0.158	0.920	-0.069	0.895	0.199	0.891	0.829	**0.980**	0.740	0.829					
COD	-0.628	-0.177	0.662	-0.722	0.661	-0.624	-0.839	-0.383	-0.681	-0.397	-0.543				
SS	-0.093	0.525	0.905	0.383	0.263	-0.171	-0.259	0.305	-0.432	0.714	0.285	0.289			
Hard.	**-0.971**	0.225	0.333	-0.485	**0.951**	-0.014	-0.320	0.117	-0.067	-0.280	-0.081	0.776	0.052		
Alk.	-0.874	0.791	0.487	0.176	0.927	0.389	0.091	0.673	0.196	0.400	0.520	0.422	0.469	0.765	
Cond.	-0.337	-0.700	-0.071	**-0.988**	0.245	-0.580	-0.659	-0.727	-0.418	-0.944	-0.833	0.673	-0.488	0.532	-0.137

P sub = Pseudokirchneriella subcapitata, A for = Asterionella formosa, *D pulex = Daphnia pulex*, Temp = Temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = Dissolved oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity

Table A4.22: **Correlation table** of studies carried out on result obtained from analysis of **output** from **May 2019 to August 2019** *i.e.*, during the **monitoring program in Oasis Fish Farm**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	P sub	***A for***	***D pulex***	**pH**	**Temp**	**NH_4^+**	**NO_2^-**	**NO_3^-**	**PO_4^{3-}**	**DO**	**BOD**	**COD**	**SS**	**Hard.**	**Alk.**
A for	-0.068														
D pulex	-0.025	**0.999**													
pH	0.599	-0.765	-0.740												
Temp	-0.059	**-0.985**	**-0.990**	0.723											
NH_4^+	**0.980**	0.132	0.174	0.450	-0.253										
NO_2^-	-0.358	0.851	0.837	-0.671	-0.751	-0.178									
NO_3^-	0.149	0.976	0.985	-0.634	**-0.991**	0.342	0.758								
PO_4^{3-}	-0.335	**0.963**	**0.950**	-0.889	-0.916	-0.142	0.895	0.882							
DO	0.251	**-0.908**	**-0.899**	0.925	0.913	0.076	-0.669	-0.852	-0.930						
BOD	-0.099	-0.702	-0.707	0.691	0.790	-0.225	-0.253	-0.730	-0.644	0.870					
COD	-0.217	0.772	0.764	-0.473	-0.670	-0.050	**0.971**	0.707	0.780	-0.502	-0.090				
SS	-0.483	0.756	0.737	**-0.981**	-0.746	-0.338	0.562	0.654	0.851	**-0.952**	-0.809	0.353			
Hard.	0.505	0.794	0.817	-0.379	-0.880	0.656	0.404	0.900	0.615	-0.703	-0.805	0.374	0.474		
Alk.	0.754	-0.032	0.000	0.217	-0.138	0.730	-0.537	0.143	-0.227	-0.073	-0.546	-0.553	-0.028	0.552	
Cond.	0.421	0.411	0.429	-0.335	-0.552	0.486	-0.127	0.513	0.283	-0.584	-0.907	-0.244	0.505	0.766	0.847

P sub = Pseudokirchneriella subcapitata, A for = Asterionella formosa, *D pulex = Daphnia pulex*, Temp = Temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = Dissolved oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity

Table A4.23: **Summary of all p-values** determined during **normality testing** conducted on **Keywater Fisheries** samples using Anderson-Darling tests. Normality testing was conducted after Grubb's test for outliers was conducted on all bioassays and parameters. Normal distribution = $p > 0.050$.

	Intake	Output	Settlement Pond
Pseudokirchneriella subcapitata	0.620	0.346	0.982
Asterionella formosa	0.124	0.115	0.934
Daphnia pulex	0.100	0.100	0.161
pH	0.334	0.745	0.344
Temperature	0.410	0.074	0.776
NH_4^+	0.114	0.534	0.393
NO_2^-	0.415	0.408	0.430
NO_3^-	0.979	0.944	0.090
PO_4^{3-}	0.150	0.075	0.401
Dissolved Oxygen	0.146	0.577	0.391
Biochemical Oxygen Demand	0.142	0.684	0.621
Chemical Oxygen Demand	0.556	0.712	0.559
Suspended Solids	0.206	0.749	0.130
Alkalinity	0.079	0.132	0.252
Hardness	0.754	0.191	0.145
Conductivity	0.580	0.150	0.755

Table A4.24: **Summary of all p-values** determined during **normality testing** on **Oasis** samples using Anderson-Darling tests. Normality testing was conducted after Grubb's test for outliers was conducted on all bioassays and parameters. Normal distribution = $p > 0.050$.

	Intake	Output
Pseudokirchneriella subcapitata	0.193	0.099
Asterionella formosa	0.973	0.258
Daphnia pulex	0.120	0.273
pH	0.190	0.602
Temperature	0.197	0.257
NH_4^+	0.198	0.120
NO_2^-	0.416	0.783
NO_3^-	0.815	0.266
PO_4^{3-}	0.838	0.055
Dissolved Oxygen	0.052	0.591
Biochemical Oxygen Demand	0.896	0.204
Chemical Oxygen Demand	0.601	0.900
Suspended Solids	0.649	0.799
Alkalinity	0.419	0.714
Hardness	0.120	0.513
Conductivity	0.267	0.106

Table A4.25: **Comparative investigation** between ***Pseudokirchneriella subcapitata*** and ***Asterionella formosa***. Results between the alga and diatom conducted on **Keywater Fisheries and Oasis Fish Farm influent and effluent samples** determined during the monitoring program. The p values for each set of comparative samples are listed. P values <0.05 indicate a significant difference.

Parameter	Intake	Output
Keywater Fisheries – *Pseudokirchneriella subcapitata* Vs *Asterionella formosa*	0.0984	0.5449
Oasis Fish Farm – *Pseudokirchneriella subcapitata* Vs *Asterionella formosa*	0.4595	0.3692

Table A4.26: **Comparative** investigation between the results determined in the bioassay and physicochemical parameters for the **pilot study and monitoring program** conducted on **Keywater Fisheries intake and output** samples. The p values for each set of comparative samples are listed. P values <0.05 indicate a significant difference. Red figures indicate where statistically significant differences have been observed. Samples for the pilot study were averaged for each month. Samples between **April 2018 to August 2018**, and **April 2019 to August 2019** were compared**.** As Asterionella formosa had not been included in the pilot study, no comparative investigation could be conducted.

Parameter	Intake	Output
Pseudokirchneriella subcapitata	**0.0302**	**0.0385**
Daphnia pulex	0.4569	0.5121
pH	0.3558	0.3528
Temperature	0.5280	0.5632
NH_4^+	0.3476	0.4062
NO_2^-	0.9999	0.4598
NO_3^-	0.8926	0.4084
PO_4^{3-}	0.4485	0.0698
Dissolved Oxygen	0.3938	0.1179
Biochemical Oxygen Demand	0.4644	0.5569
Chemical Oxygen Demand	0.9154	0.1680
Suspended Solids	0.5353	0.2165
Alkalinity	0.2358	0.4598
Hardness	0.1767	0.2793

Table A4.27: **Comparative** investigation between the results determined in the bioassay and physicochemical parameters conducted on **Keywater Fisheries and Oasis Fish Farm intake and output** samples. The p values for each set of comparative samples are listed. P values <0.05 indicate a significant difference. Red figures indicate where statistically significant differences have been observed. Samples between **May 2019 and August 2019** were compared**.**

Parameter	Intake	Output
Pseudokirchneriella subcapitata	0.7695	0.4123
Asterionella formosa	**0.0486**	0.5324
Daphnia pulex	0.5370	0.6704
pH	0.1103	**<0.0001**
Temperature	0.1639	0.1083
NH_4^+	0.2139	**0.0274**
NO_2^-	0.0688	**0.0003**
NO_3^-	**0.0003**	**0.0004**
PO_4^{3-}	0.2469	0.0603
Dissolved Oxygen	**<0.0001**	**0.0042**
Biochemical Oxygen Demand	**0.0158**	0.6012
Chemical Oxygen Demand	0.8253	**0.0033**
Suspended Solids	**0.0286**	0.5509
Alkalinity	0.3322	0.2160
Hardness	0.2742	0.2933

Table A4.28: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of culture Pond 1 from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)	T Acidity (mg/L)
03-Dec	0.52	0.03	6.74	1.33	6.00	167.68	7.43	1.10	11.70	7.71	97.68	136.74	
17-Dec													
07-Jan	0.00	0.03	2.97	0.35	14.00	165.12	9.89	1.18	11.10	7.71	95.12	132.96	
22-Jan	0.00	0.02	2.15	3.29	24.00	161.28	8.30	3.49	8.90	7.82	101.28	132.15	
05-Feb	0.00	0.03	1.28	1.88	7.67	165.76	9.80	8.04	5.20	8.01	105.76	131.27	
19-Feb	0.00	0.02	0.98	1.04	32.33	152.32	7.56	9.39	9.40	7.65	95.32	120.97	
27-Feb													
04-Mar	0.00	0.04	1.67	0.77	18.67	142.08	8.54	9.44	5.80	7.61	92.08	121.67	
22-Jun	0.14	0.04	3.09	1.98	68.00	188.80		6.06	17.25	7.82	128.80	133.09	20.00
29-Jun	0.11	0.23	0.16	0.53	78.00	187.52		6.70	15.55	8.03	127.52	120.15	52.00
08-Jul	0.03	0.05	2.53	1.60	68.00	176.00		6.22	17.00	7.86	116.00	122.53	36.00
15-Jul	0.00	0.03	1.55	0.37	82.00	170.24	8.10	22.10	16.30	7.86	110.24	121.54	20.00
22-Jul	0.00	0.02	1.41	0.63	122.00	172.80	5.50	23.50	20.95	7.94	112.80	121.41	22.00
27-Jul	0.00	0.07	1.52	1.23	78.00	168.32	4.99	21.10	18.05	8.09	108.32	111.50	16.00
05-Aug	0.04	0.06	1.69	0.94	116.00	168.32	8.14	17.50	19.00	7.46	108.32	111.68	33.60
12-Aug	0.04	0.11	3.00	1.32	74.00	172.16	6.40	15.30	18.05	7.78	102.16	122.99	31.20
19-Aug	0.11	0.16	2.94	1.26	76.00	172.80	9.53	27.70	18.40	7.03	102.80	122.94	19.60
25-Aug	0.10	0.11	2.81	1.87	42.00	167.68	7.03	27.30	17.30	7.04	107.68	112.80	26.80
02-Sep	0.17	0.17	4.04	1.84	54.00	166.40	6.08	16.60	16.50	7.01	106.40	104.03	30.40
09-Sep	0.21	0.19	3.43	2.22	38.00	168.96	4.94	19.9	16.40	7.21	108.96	103.43	18.40
16-Sep	0.22	0.24	5.54	2.07	46.00	171.52	6.98	20.70	19.30	7.00	111.52	115.53	18.40
23-Sep	0.19	0.18	4.94	0.00	34.00	172.16	7.01	29.70	13.90	7.04	112.16	114.93	31.20
30-Sep	0.10	0.11	4.55	1.74	26.00	170.24	5.84	22.70	13.30	7.41	110.24	124.55	27.60
07-Oct	0.55	0.11	3.82	1.90	40.00	170.24	6.96	33.20	12.90	7.76	110.24	133.81	22.40
14-Oct	0.77	0.11	3.00	1.69	22.00	169.60	6.37	23.20	12.10	7.65	109.60	122.99	21.20

Table A4.29: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of culture Pond 2 from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)
03-Dec	0.11	0.03	6.39	5.49	5.00	167.04	7.50	1.95	11.80	7.93	97.04	136.38
17-Dec	0.04	0.04	3.43	0.00	8.00	163.84	8.59	1.58	12.95	7.49	93.84	133.43
07-Jan	0.00	0.03	2.49	0.36	22.00	165.12	9.90	1.55	11.50	7.92	96.95	132.49
22-Jan	0.00	0.01	2.14	1.28	28.00	161.92	9.21	4.54	8.60	7.96	101.92	132.13
05-Feb	0.00	0.03	0.78	1.87	5.67	165.12	9.61	7.32	5.60	8.06	105.12	130.78
19-Feb	0.00	0.01	0.62	1.16	41.33	149.76	7.99	10.15	10.20	7.98	89.76	120.62
04-Mar	0.00	0.03	0.80	0.88	25.33	143.36	8.99	9.47	5.70	7.94	93.36	120.79
22-Jun	0.09	0.04	2.03	2.75	92.00	188.80		4.28	16.40	7.93	128.80	132.02
29-Jun	0.11	0.20	0.00	0.87	66.00	187.52		8.73	15.70	8.06	127.52	120.36
08-Jul	0.03	0.05	3.16	1.42	58.00	176.00		4.27	16.10	7.89	116.00	123.16
15-Jul	0.00	0.03	1.47	0.90	74.00	172.16	7.03	22.20	16.25	7.96	112.16	121.46
22-Jul	0.01	0.01	1.55	0.77	130.00	172.80	4.93	20.80	18.95	8.00	112.80	121.54
27-Jul	0.00	0.05	1.82	1.04	64.00	169.60	5.06	24.40	17.70	8.16	109.60	111.82
05-Aug	0.03	0.06	0.95	1.29	112.00	168.32	7.58	18.40	18.15	7.68	108.32	110.94
12-Aug	0.06	0.11	2.37	1.38	76.00	172.16	6.81	14.00	17.75	7.78	102.16	122.36
19-Aug	0.08	0.16	2.61	1.32	68.00	170.88	9.68	29.40	18.15	7.01	100.88	122.61
25-Aug	0.13	0.15	2.53	1.73	38.00	167.68	7.43	27.60	16.85	7.04	107.68	112.53
02-Sep	0.16	0.18	3.95	1.71	38.00	165.12	5.73	22.80	15.90	7.00	105.12	103.95
09-Sep	0.20	0.20	3.71	2.17	30.00	167.68	5.33	24.7	16.00	7.18	107.68	103.70
16-Sep	0.22	0.23	5.59	2.24	24.00	170.24	6.39	21.30	19.20	6.97	110.24	115.59
23-Sep	0.18	0.16	5.13	0.00	36.00	171.52	7.00	29.60	13.50	7.00	111.52	115.12
30-Sep	0.11	0.11	4.69	1.71	30.00	170.24	5.37	19.90	13.35	7.40	110.24	124.69
07-Oct	0.31	0.09	2.40	1.53	30.00	172.16	6.98	32.50	12.95	7.86	112.16	132.39
14-Oct	0.23	0.08	3.38	1.53	10.00	169.60	6.49	24.00	12.10	7.68	109.60	123.37

Table A4.30: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of culture Pond 3 from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)
03-Dec												
17-Dec												
07-Jan												
22-Jan	0.00	0.01	1.88	0.38	32.00	163.20	8.90	3.19	8.70	7.86	103.20	131.87
05-Feb	0.00	0.04	0.73	1.53	9.33	166.40	9.12	8.48	5.80	7.93	106.40	130.72
19-Feb	0.00	0.02	0.06	1.42	49.33	153.60	8.01	10.31	11.20	7.81	93.60	120.06
04-Mar	0.00	0.03	0.46	0.58	23.33	144.00	8.86	9.16	5.50	7.96	94.00	120.45
22-Jun	0.13	0.03	2.14	4.07	78.00	190.08		4.48	16.45	7.94	120.08	132.13
29-Jun	0.15	0.19	0.54	0.57	86.00	188.80		2.48	15.65	8.07	128.80	120.53
08-Jul	0.03	0.05	2.15	1.90	72.00	177.92		4.05	16.60	7.98	117.92	122.15
15-Jul	0.00	0.03	1.25	3.83	72.00	172.80	6.54	19.30	16.15	8.02	112.80	121.24
22-Jul	0.00	0.01	1.08	0.82	120.00	173.44	5.01	25.50	18.65	8.02	113.44	121.19
27-Jul	0.02	0.05	1.71	1.04	84.00	170.24	5.19	21.80	18.25	8.13	100.24	111.71
05-Aug	0.06	0.07	1.55	1.09	128.00	170.24	8.05	16.20	18.30	7.73	100.24	111.54
12-Aug	0.07	0.11	2.48	1.36	68.00	173.44	6.96	19.10	18.05	7.87	103.44	122.47
19-Aug	0.09	0.15	3.43	1.98	78.00	172.16	9.72	30.30	18.85	7.00	102.16	123.43
25-Aug	0.14	0.14	3.33	1.81	38.00	168.32	7.11	30.50	17.65	7.03	108.32	113.32
02-Sep	0.21	0.17	3.93	10.18	40.00	166.40	5.98	22.90	16.00	6.99	106.40	103.92
09-Sep	0.21	0.21	3.63	2.45	30.00	168.32	5.24	22.9	16.10	7.17	108.32	103.62
16-Sep	0.23	0.24	5.51	2.21	34.00	170.24	6.91	20.40	19.60	6.97	110.24	115.51
23-Sep	0.19	0.18	4.91	0.12	26.00	172.16	6.98	27.70	14.00	6.98	112.16	114.90
30-Sep	0.11	0.12	4.83	1.83	20.00	169.60	5.71	20.20	13.30	7.53	119.60	124.82
07-Oct	0.66	0.11	2.07	1.60	34.00	171.52	6.88	32.20	12.99	7.92	111.52	132.06
14-Oct	0.34	0.08	3.33	1.74	12.00	170.24	6.58	26.90	12.15	7.77	100.24	123.32

Table A4.31: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of culture Pond 4 from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)	T Acidity (mg/L)
03-Dec													
17-Dec	0.01	0.04	3.33	0.00	6.67	163.84	8.74	1.55	8.30	7.97	94.00	133.32	
07-Jan	0.00	0.03	2.00	2.56	20.33	167.04	9.57	1.44	11.10	7.83	97.04	132.00	
22-Jan	0.00	0.01	2.31	0.02	26.00	162.56	9.08	4.06	8.20	7.95	102.56	132.31	
05-Feb	0.00	0.03	0.31	1.88	7.33	165.76	9.88	8.70	6.00	7.97	105.76	130.30	
19-Feb	0.00	0.02	0.05	1.61	52.00	152.96	8.01	10.54	10.60	7.84	92.96	120.04	
04-Mar	0.00	0.03	0.55	0.52	22.00	143.36	8.79	9.83	5.30	7.99	93.36	120.55	
22-Jun	0.09	0.03	2.60	2.76	86.00	189.44		4.04	17.20	7.91	129.44	132.60	40.00
29-Jun	0.13	0.30	0.00	0.84	86.00	188.80		6.17	15.65	8.07	128.80	120.22	24.00
08-Jul	0.01	0.06	2.23	1.39	66.00	177.28		3.89	15.90	8.04	117.28	122.23	24.00
15-Jul	0.00	0.03	1.47	5.35	78.00	172.16	6.47	27.10	16.95	8.04	112.16	121.46	16.00
22-Jul	0.00	0.01	1.47	0.82	108.00	172.80	4.88	16.10	19.00	8.00	112.80	121.46	14.00
27-Jul	0.02	0.05	1.88	1.11	72.00	169.60	5.45	20.40	17.80	8.20	109.60	111.87	8.80
05-Aug	0.04	0.07	1.36	0.97	124.00	168.96	7.82	18.00	18.25	7.77	108.96	111.35	13.60
12-Aug	0.13	0.11	2.10	1.31	66.00	172.16	7.02	9.90	17.75	7.82	102.16	122.09	14.40
19-Aug	0.11	0.16	2.86	2.25	78.00	172.16	9.72	29.80	18.50	7.00	102.16	122.86	15.60
25-Aug	0.15	0.14	2.59	1.83	40.00	168.32	6.99	32.90	17.10	7.02	108.32	112.58	26.00
02-Sep	0.10	0.16	3.84	1.80	36.00	165.12	6.41	28.10	16.10	6.97	105.12	103.84	15.20
09-Sep	0.15	0.19	3.76	2.07	36.00	168.32	5.21	24.3	16.30	7.16	108.32	103.76	16.00
16-Sep	0.16	0.23	4.91	14.13	26.00	170.24	6.54	19.90	20.20	6.97	110.24	114.90	18.40
23-Sep	0.12	0.18	5.02	1.67	46.00	172.16	6.98	34.60	13.80	6.98	112.16	115.01	27.20
30-Sep	0.08	0.11	4.96	1.69	28.00	170.24	5.65	21.70	13.40	7.51	110.24	124.96	19.20
07-Oct	0.49	0.11	2.72	1.63	34.00	170.24	6.98	30.70	13.03	7.94	110.24	132.72	22.40
14-Oct	0.30	0.07	2.29	1.86	10.00	169.60	6.66	27.00	12.10	7.70	109.60	122.28	16.80

Table A4.32: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of the duckweed lagoon inflow point from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)	T Acidity (mg/L)
03-Dec													
17-Dec													
07-Jan													
22-Jan	0.00	0.01	2.18	0.00	19.00	161.92	8.05	3.75	8.40	8.02	101.92	132.17	
05-Feb	0.01	0.03	0.20	3.52	11.67	164.48	6.12	9.67	6.00	8.13	104.48	130.19	
19-Feb	0.00	0.01	0.10	1.16	44.00	151.68	7.13	10.85	10.40	7.94	91.68	120.10	
04-Mar	0.00	0.04	0.13	0.61	12.67	143.36	7.59	8.80	5.60	8.10	93.36	120.12	
22-Jun	0.11	0.02	3.64	2.66	74.00	190.08		2.31	16.80	7.77	120.08	133.63	60.00
29-Jun	0.15	0.21	0.95	0.50	86.00	188.80		5.94	15.55	8.01	118.80	120.94	28.00
08-Jul	0.01	0.07	1.93	1.49	64.00	177.28		3.22	15.85	8.00	117.28	121.93	28.00
15-Jul	0.00	0.03	1.71	0.68	68.00	172.80	6.03	25.20	17.40	8.01	112.80	121.71	16.00
22-Jul	0.00	0.01	1.33	0.99	126.00	172.80	4.38	12.80	19.50	7.95	112.80	121.33	20.00
27-Jul	0.00	0.04	1.63	1.08	66.00	168.96	4.95	23.10	17.70	8.09	108.96	111.63	11.20
05-Aug	0.01	0.06	2.37	1.06	90.00	168.96	7.86	23.10	18.60	7.73	108.96	112.36	13.20
12-Aug	0.04	0.11	2.61	1.29	56.00	172.16	6.91	10.20	17.75	7.85	102.16	122.61	24.40
19-Aug	0.10	0.15	3.68	2.07	68.00	171.52	9.66	29.80	18.30	7.00	101.52	123.68	16.80
25-Aug	0.08	0.13	2.31	1.74	44.00	168.32	6.90	27.50	17.95	7.01	108.32	112.31	19.20
02-Sep	0.12	0.16	3.98	1.91	42.00	165.12	6.32	29.80	16.20	6.96	105.12	103.98	14.00
09-Sep	0.16	0.20	3.82	1.94	32.00	167.68	5.00	22.10	16.40	7.06	107.68	103.81	17.60
16-Sep	0.14	0.23	7.64	2.12	28.00	170.24	6.88	21.90	19.80	6.96	110.24	117.64	16.40
23-Sep	0.14	0.17	5.10	5.01	30.00	171.52	6.97	31.10	13.90	6.97	111.52	115.10	18.00
30-Sep	0.11	0.11	4.83	1.60	22.00	171.52	5.51	24.60	13.65	7.50	111.52	124.82	24.80
07-Oct	0.37	0.10	2.45	1.55	34.00	172.16	6.97	30.40	13.10	7.91	112.16	132.45	19.60
14-Oct	0.19	0.07	1.41	1.63	20.00	172.16	6.41	27.50	12.35	7.70	102.16	121.41	14.40

Table A4.33: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of the duckweed lagoon outflow point from December 2019 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)	T Acidity (mg/L)
03-Dec	0.00	0.03	8.35	1.11	6.00	170.88	6.14	1.86	11.60	7.94	100.88	138.35	
17-Dec	0.00	0.03	3.86	0.00	6.00	163.84	6.32	0.63	8.30	8.05	94.94	133.85	
07-Jan	0.00	0.03	1.70	0.00	20.67	165.12	6.52	0.62	11.10	8.01	96.12	131.69	
22-Jan	0.00	0.01	5.37	0.00	32.00	161.92	8.70	3.69	8.10	8.04	101.92	135.37	
05-Feb	0.00	0.03	0.40	1.50	10.33	164.48	6.61	9.29	6.20	8.14	104.48	130.40	
19-Feb	0.00	0.01	0.14	1.16	49.33	152.32	7.94	10.46	10.40	7.97	92.32	120.14	
04-Mar	0.00	0.04	0.20	0.44	17.33	142.72	7.98	9.51	5.70	8.20	92.72	120.19	
22-Jun	0.09	0.02	3.94	1.38	56.00	190.08		1.00	17.35	7.69	120.08	133.93	28.00
29-Jun	0.12	0.21	1.22	0.27	76.00	188.80		6.22	15.50	8.00	118.80	121.22	20.00
08-Jul	0.01	0.04	3.00	2.12	64.00	177.28		2.81	17.55	7.92	117.28	122.99	20.00
15-Jul	0.00	0.03	1.90	0.50	66.00	172.80	4.42	19.10	16.10	7.89	112.80	121.90	8.00
22-Jul	0.00	0.00	1.41	1.35	124.00	172.80	4.20	22.20	19.20	7.92	112.80	121.41	12.80
27-Jul	0.00	0.03	1.69	1.15	70.00	168.96	3.71	21.10	17.60	7.97	108.96	111.68	8.80
05-Aug	0.01	0.06	1.80	0.94	76.00	168.32	6.31	20.50	18.65	7.61	108.32	111.79	8.40
12-Aug	0.03	0.11	2.42	1.26	64.00	172.80	5.93	22.00	17.70	7.70	102.80	122.42	32.40
19-Aug	0.22	0.14	3.02	1.26	78.00	172.16	9.16	25.20	18.20	7.00	102.16	123.02	15.20
25-Aug	0.13	0.13	2.75	1.87	38.00	168.32	6.98	27.40	17.70	7.01	108.32	112.75	26.40
02-Sep	0.17	0.16	4.42	1.71	38.00	165.76	5.99	28.50	16.80	6.96	105.76	104.41	18.40
09-Sep	0.21	0.19	4.04	2.32	34.00	167.68	5.09	22.00	16.50	7.07	107.68	104.03	19.20
16-Sep	0.23	0.23	4.50	2.18	34.00	170.88	6.71	20.70	20.30	6.97	110.88	114.50	17.60
23-Sep	0.19	0.16	5.05	0.13	32.00	171.52	6.97	32.10	13.90	6.97	111.52	115.04	20.40
30-Sep	0.10	0.11	5.43	1.73	16.00	170.88	5.48	25.40	13.65	7.51	110.88	125.42	22.80
07-Oct	0.35	0.10	2.70	1.60	24.00	172.80	6.88	29.30	12.99	7.79	112.80	132.69	27.60
14-Oct	0.15	0.07	2.18	1.64	10.00	171.52	6.45	27.70	12.25	7.69	101.52	122.17	19.60

Table A4.34: **Summary of results for all physicochemical analyses performed on freshwater aquaculture samples taken out of the overflow tank at the discharge point from December 2019 to June 2020 and from the reservoir from June 2020 to October 2020 at Oasis Fish Farm.** All samples were run with a minimum of triplicates per test. Samples were analysed every two weeks. Samples were tested within 24 hours of collection to prevent the need for preserving samples. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)
03-Dec												
17-Dec												
07-Jan												
22-Jan												
05-Feb	0.00	0.03	0.98	1.73	15.00	166.40	5.99	7.96	6.40	8.01	106.40	130.97
19-Feb	0.00	0.01	0.35	0.94	48.00	152.32	4.83	9.33	13.60	7.12	92.32	120.34
04-Mar	0.00	0.02	0.95	0.45	39.33	143.36	6.65	9.16	7.00	8.07	93.36	120.94
22-Jun												
29-Jun	0.17	0.02	0.78	0.58	42.00	248.96		2.51	15.85	7.37	98.96	120.78
Date	NH_4^+ (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	S Solids (mg/L)	D Solids (mg/L)	DO	BOD (mg/L)	T (°C)	pH	Hardness (mg/L)	Alkalinity (mg/L)
22-Jun	0.00	0.04	5.28	0.00	48.00	176.64		6.83	18.25	8.12	116.64	135.27
29-Jun	0.04	0.09	1.14	0.00	44.00	183.04		5.60	17.00	8.07	113.04	121.13
08-Jul	0.00	0.05	5.48	1.60	24.00	171.52		2.12	17.25	7.87	111.52	125.48
15-Jul	0.00	0.09	5.35	0.62	34.00	171.52	5.58	14.90	16.65	8.01	101.52	125.34
22-Jul	0.02	0.05	6.03	0.46	80.00	174.08	4.88	19.10	20.75	7.99	114.08	126.03
27-Jul	0.01	0.08	4.99	1.09	40.00	174.08	4.83	21.60	18.90	8.10	104.08	114.99
05-Aug	0.07	0.16	5.02	1.02	46.00	177.28	8.04	12.10	18.45	7.75	107.28	115.01
12-Aug	0.01	0.23	9.28	1.63	22.00	179.20	7.02	17.90	18.60	7.76	109.20	129.28
19-Aug	0.02	0.23	7.75	1.32	44.00	178.56	9.02	23.20	19.55	7.00	108.56	127.75
25-Aug	0.40	0.21	7.20	2.01	66.00	176.00	7.01	27.70	17.70	7.00	116.00	117.20
02-Sep	0.40	0.20	7.89	1.98	56.00	176.00	5.43	27.60	16.70	6.97	116.00	107.88
09-Sep	0.22	0.18	7.51	1.81	46.00	174.08	5.98	22.20	16.90	6.97	114.08	107.50
16-Sep	0.06	0.07	4.75	1.60	52.00	178.56	6.99	20.00	19.70	6.98	118.56	114.74
23-Sep	0.05	0.05	3.83	0.00	30.00	179.84	6.97	32.70	15.30	6.97	119.84	113.83
30-Sep	0.07	0.07	6.00	1.59	20.00	178.56	6.00	21.70	13.30	7.70	118.56	123.13
07-Oct	0.93	0.08	3.05	1.45	28.00	179.84	6.95	30.80	13.00	7.91	119.84	133.05
14-Oct	0.24	0.06	3.24	1.60	22.00	174.08	6.78	26.40	12.10	7.89	104.08	123.24

Table A4.35: **Summary of algal counts established for at all eight individual sampling points from December 2019 to October 2020 at Oasis Fish Farm using Flow Cytometry.** All samples were run with a minimum of triplicates per count. Samples were analysed every two weeks. Samples were preserved immediately after collection as testing within 24 hours could not be conducted. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	Pond 1	Pond 2	Pond 3	Pond 4	Inflow	Outflow	Discharge	Reservoir
03-Dec	30167	41137				36885		
17-Dec		81211		87393		98907		
07-Jan	651756	674011		690230		694144		
22-Jan	112985	145570	120744	159052	159404	123026		
05-Feb	408756	468170	558659	537044	338285	229519	243026	
19-Feb	386078	311159	339830	409578	159922	194615	297907	
27-Feb	512389	486852	627041	527478	409670	352352	255844	
04-Mar	847322	698478	743215	810744	488715	406348	312463	
18-Mar	84270		66030	55860	47910	66310	70240	
18-May	339850	392790	282390	298840	357850	385460	290610	
25-May	733610	634580	952050	525630	946260	795790	663470	
03-Jun	412740	433550	418490	533640	580320	383380	246200	167430
10-Jun	573520	533470	300870	318390	318470	528460	391890	174450
15-Jun	284170	508570	352330	768220	691420	807830		490090
22-Jun	272650	323333	430817	410757	335297	253757		157010
29-Jun	121863	170187	158963	160177	105090	125127	60667	22447
08-Jul	190210	146730	248297	178017	173860	199437		90293
15-Jul	285203	262370	280010	275747	284247	226523		21533
22-Jul	450733	386113	455690	454257	476713	468473		21840
27-Jul	317557	316097	328250	375490	343213	301317		23320
05-Aug	417617	628597	445857	631860	553083	509133		44333
12-Aug	193347	205597	252067	247627	233957	215527		24620
19-Aug	270510	200427	242007	335250	207807	156623		88090
25-Aug	238663	179743	189803	264047	192903	185557		98297
02-Sep	203417	162900	169153	179153	221170	178360		60507
09-Sep	216950	130853	179593	208500	157600	221640		67337
16-Sep	109543	66627	89850	115037	138887	139540		44600
23-Sep	143197	195583	175153	187050	137400	185400		216500
30-Sep	100580	187127	171137	139067	97377	113507		72370
07-Oct	265467	229913	214443	170783	213250	200150		145770
14-Oct	115700	213717	248530	245197	196033	225283		105040

Table A4.36: **Summary of cyanobacterial counts established for at all eight individual sampling points from December 2019 to October 2020 at Oasis Fish Farm using Flow Cytometry.** All samples were run with a minimum of triplicates per count. Samples were analysed every two weeks. Samples were preserved immediately after collection as testing within 24 hours could not be conducted. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	Pond 1	Pond 2	Pond 3	Pond 4	Inflow	Outflow	Discharge	Reservoir
03-Dec	741	648				763		
17-Dec		3678		3952		3444		
07-Jan	11370	15552		12907		14070		
22-Jan	7215	7811	5896	8011	11696	6093		
05-Feb	88352	78622	88378	95244	77400	54033	65622	
19-Feb	61763	45915	5644	81304	29293	49811	79052	
27-Feb	11200	9148	10256	11285	5822	6133	5467	
04-Mar	26115	16807	15815	19207	7893	7304	23989	
18-Mar	8430		5470	5660	11130	9310	10780	
18-May	1339650	1063060	1313050	500820	1385840	1272530	719580	
25-May	439870	464280	672590	284730	671790	498370	499280	
03-Jun	553760	535860	557860	556260	32620	465650	100320	101580
10-Jun	664880	589680	400520	397800	383690	612140	157780	155580
15-Jun	247730	411030	306370	529280	518350	530110		130860
22-Jun	18293	20223	28880	27657	23410	18380		5360
29-Jun	14853	22680	25307	23257	14890	17517	2733	1370
08-Jul	57363	43483	56483	35663	45640	59817		1757
15-Jul	85323	84457	81600	91083	84017	71367		1373
22-Jul	17997	33273	14820	22003	20303	8743		1797
27-Jul	61580	57723	51727	76243	39123	39427		337
05-Aug	126633	198297	119523	154347	178700	138610		1207
12-Aug	14650	21967	21560	26447	19410	17707		1697
19-Aug	11137	8980	11433	11690	7080	3853		643
25-Aug	18540	11890	12520	16277	13080	10267		4560
02-Sep	23873	12647	9327	14507	19677	16770		33887
09-Sep	8027	7183	5790	7803	5607	8483		3840
16-Sep	8327	4157	5533	8510	8240	7797		4860
23-Sep	4727	8420	8053	8753	5343	8010		8500
30-Sep	16890	17070	11810	13463	11997	12307		14867
07-Oct	29473	17150	20217	20783	22127	19963		22513
14-Oct	2840	21680	26843	37270	27407	34303		23260

Table A4.37: **Summary of general bacteria counts established for at all eight individual sampling points from December 2019 to October 2020 at Oasis Fish Farm using Flow Cytometry.** All samples were run with a minimum of triplicates per count. Samples were analysed every two weeks. Samples were preserved immediately after collection as testing within 24 hours could not be conducted. Grey sections indicate when sampling and analysis could not be conducted. Red line indicates the COVID-19 lockdown period where restrictions prevented collection and analysis to be conducted.

Date	Pond 1	Pond 2	Pond 3	Pond 4	Inflow	Outflow	Discharge	Reservoir
03-Dec	3993	2793				2833		
17-Dec		6533		8374		4141		
07-Jan	120911	103956		160585		117889		
22-Jan	44237	35530	36381	38863	30752	26152		
05-Feb	209074	238485	268519	436741	431356	404796	425204	
19-Feb	165900	177796	325604	436148	460622	377963	414311	
04-Mar	188719	221600	141381	388230	456989	377556	49263	
22-Jun	254357	280647	284803	307380	343900	246833		16247
29-Jun	35890	22133	67117	93850	74450	41790	28117	6557
08-Jul	48943	46733	47763	42223	62223	46407		7977
15-Jul	45547	44513	48350	29270	84963	56217		3950
22-Jul	62313	122093	66153	95550	81667	20393		11030
27-Jul	41027	50787	35397	52050	61560	45390		3287
05-Aug	25270	50490	75143	68747	36783	37963		6030
12-Aug	31183	34697	38207	46397	49473	83143		16800
19-Aug	34917	26767	35117	47747	38180	49843		4433
25-Aug	50550	67190	42320	60650	65333	42527		17293
02-Sep	19987	22663	34290	40123	33607	42383		9253
09-Sep	12250	9783	15387	26067	9070	17510		7023
16-Sep	18310	17937	13347	20577	22877	20253		6037
23-Sep	16523	24680	28870	36307	37940	26560		12853
30-Sep	42850	40117	15113	29883	41867	43597		22583
07-Oct	53433	50257	18557	56480	20527	27120		34233
14-Oct	7123	51857	7227	21557	24470	26040		14667

Table A4.38: Summary of all p-values determined during **normality testing** on all **Oasis** sample sets from **December 2019 to October 2020** using **Anderson-Darling** tests. Normality testing was conducted after Grubb's test for outliers was conducted on all bioassays and parameters. Normal distribution = $p > 0.050$.

	Pond 1	Pond 2	Pond 3	Pond 4	Inflow	Outflow	Discharge	Reservoir
Algae	0.0509	0.0253	0.0640	0.0744	0.0640	0.3000	0.0925	0.0505
Cyano	0.0822	0.0503	0.0920	0.0980	0.0904	0.0515	0.0932	0.0564
Bac	0.0567	0.0543	0.0503	0.0885	0.0916	0.0694	0.0528	0.0987
NH_4^+	0.1000	0.1240	0.1500	0.2002	0.4600	0.1000	0.1200	0.1111
NO_2^-	0.0790	0.4000	0.1125	0.1460	0.1199	0.4800	0.6830	0.2100
NO_3^-	0.3902	0.5357	0.5174	0.4810	0.2844	0.5144	0.1191	0.7844
PO_4^{3-}	0.5179	0.2200	0.1000	0.1001	0.3270	0.1591	0.3791	0.2130
SS	0.2216	0.0550	0.0847	0.2191	0.2786	0.1711	0.2134	0.4351
DS	0.0800	0.1300	0.1600	0.1400	0.2500	0.2300	0.0995	0.4970
DO	0.7701	0.5018	0.3974	0.3001	0.5291	0.4476	0.6996	0.2793
BOD	0.2133	0.5090	0.1313	0.1203	0.1600	0.0950	0.0604	0.4655
T	0.1129	0.7060	0.1790	0.4980	0.2010	0.0539	0.2565	0.1838
pH	0.1110	0.2000	0.1001	0.1006	0.1011	0.3000	0.2861	0.3000
H	0.0921	0.2182	0.8631	0.0606	0.4891	0.6762	0.4308	0.3212
A	0.2055	0.1934	0.2410	0.1425	0.2342	0.3350	0.0590	0.6413
TA	0.4260	-	-	0.0541	0.1000	0.4767	-	-

Cyano = cyanobacteria, Bac = bacteria, H = hardness, A = alkalinity, TA = total acidity.

Table A4.39: **Correlation table** of studies carried out on result obtained from analysis of **Pond 1** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H	A
Cyano	0.288														
Bac	**0.803**	0.212													
NH_4^+	**-0.451**	-0.398	-0.418												
NO_2^-	**-0.458**	-0.402	**-0.485**	0.269											
NO_3^-	**-0.476**	**-0.514**	**-0.462**	**0.539**	0.312										
PO_4^{3-}	-0.400	-0.186	-0.164	0.200	0.043	0.212									
SS	0.028	0.370	-0.340	-0.335	0.053	-0.404	-0.290								
DS	**-0.555**	-0.142	**-0.701**	0.155	0.368	0.091	-0.001	**0.446**							
DO	0.423	0.248	**0.517**	-0.245	-0.364	-0.208	-0.163	-0.271	-0.310						
BOD	-0.178	-0.040	-0.368	0.269	**0.434**	0.162	-0.084	0.280	0.098	-0.431					
T	-0.317	-0.010	**-0.701**	-0.059	0.383	0.097	-0.101	**0.797**	**0.612**	**-0.500**	**0.433**				
pH	0.192	0.306	0.294	-0.150	**-0.595**	**-0.545**	-0.108	0.133	0.110	0.075	**-0.464**	-0.243			
H	**-0.433**	-0.009	**-0.510**	0.093	0.366	-0.097	0.037	**0.463**	**0.872**	**-0.531**	0.160	**0.510**	0.138		
A	0.050	-0.140	0.282	0.172	**-0.532**	0.083	0.079	-0.357	0.054	**0.536**	**-0.423**	**-0.474**	**0.523**	-0.107	
TA	-0.267	0.083	0.034	-0.192	0.252	-0.333	-0.368	0.150	0.404	0.152	-0.482	-0.133	0.156	0.374	-0.032

Table A4.40: **Correlation table** of studies carried out on result obtained from analysis of **Pond 2** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H
Cyano	**0.417**													
Bac	**0.617**	0.148												
NH_4^+	**-0.485**	-0.368	-0.334											
NO_2^-	**-0.457**	-0.292	**-0.466**	**0.667**										
NO_3^-	**-0.589**	**-0.468**	**-0.479**	**0.530**	0.368									
PO_4^{3-}	-0.331	-0.125	0.051	0.254	0.027	**0.447**								
SS	0.309	**0.489**	0.197	-0.301	-0.093	**-0.441**	-0.194							
DS	-0.232	0.024	0.075	0.287	0.315	0.075	0.127	**0.431**						
DO	0.334	0.014	0.306	-0.396	-0.352	-0.314	-0.135	-0.326	**-0.438**					
BOD	-0.151	0.033	-0.311	**0.569**	**0.532**	0.149	-0.161	0.172	0.098	**-0.541**				
T	-0.228	0.144	-0.309	0.216	**0.464**	0.165	0.001	**0.647**	**0.604**	**-0.577**	**0.446**			
pH	**0.409**	0.293	**0.421**	**-0.544**	**-0.759**	**-0.585**	0.017	0.192	0.002	0.171	**-0.542**	-0.357		
H	-0.118	0.111	0.174	0.304	0.308	-0.089	0.040	**0.460**	**0.876**	**-0.631**	0.199	**0.474**	0.059	
A	0.039	-0.213	0.299	-0.198	**-0.589**	-0.006	0.175	-0.260	0.060	**0.555**	**-0.587**	**-0.472**	**0.509**	-0.127

Table A4.41: **Correlation table** of studies carried out on result obtained from analysis of **Pond 3** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H
Cyano	0.282													
Bac	**0.592**	0.174												
NH_4^+	-0.411	-0.268	-0.340											
NO_2^-	**-0.653**	-0.374	**-0.483**	**0.473**										
NO_3^-	**-0.650**	-0.392	**-0.557**	0.392	**0.710**									
PO_4^{3-}	-0.166	-0.045	0.018	0.148	0.195	0.257								
SS	0.222	0.409	0.043	-0.331	-0.237	-0.373	-0.066							
DS	-0.303	0.178	-0.064	0.195	0.229	0.153	0.090	**0.434**						
DO	0.298	0.147	0.422	-0.226	-0.227	-0.304	-0.258	-0.145	-0.425					
BOD	-0.281	-0.226	**-0.526**	**0.512**	0.403	**0.530**	0.111	-0.098	-0.066	-0.443				
T	-0.392	0.030	-0.410	0.077	0.401	0.374	0.199	**0.631**	**0.578**	**-0.482**	0.394			
pH	**0.508**	0.424	0.320	-0.245	**-0.750**	**-0.801**	-0.329	0.328	0.098	-0.004	**-0.535**	-0.296		
H	-0.341	-0.017	-0.115	0.179	0.289	0.181	0.078	0.160	**0.794**	**-0.501**	-0.174	0.302	0.062	
A	0.214	0.047	0.345	0.049	**-0.512**	-0.322	-0.347	-0.117	0.162	**0.471**	-0.335	-0.431	**0.521**	0.183

Table A4.42: **Correlation table** of studies carried out on result obtained from analysis of **Pond 4** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H	A
Cyano	**0.398**														
Bac	**0.654**	0.300													
NH_4^+	-0.399	-0.258	-0.283												
NO_2^-	**-0.503**	-0.340	-0.371	**0.441**											
NO_3^-	**-0.565**	**-0.519**	**-0.561**	0.306	0.381										
PO_4^{3-}	-0.178	-0.056	-0.084	0.141	0.339	0.368									
SS	0.264	**0.443**	-0.021	-0.181	0.034	-0.286	-0.101								
DS	-0.242	-0.042	-0.137	0.230	0.386	0.151	0.122	**0.482**							
DO	0.377	0.062	0.429	-0.260	-0.312	-0.374	-0.164	-0.305	-0.406						
BOD	-0.260	-0.019	-0.393	**0.534**	0.397	**0.448**	0.189	0.033	-0.001	**-0.498**					
T	-0.155	0.069	-0.406	0.182	**0.431**	0.297	0.394	**0.676**	**0.605**	**-0.607**	0.403				
pH	0.299	0.370	0.280	-0.267	**-0.602**	**-0.689**	-0.389	0.184	0.022	0.141	**-0.639**	-0.344			
H	-0.223	0.036	-0.064	0.240	0.371	0.066	0.138	**0.487**	**0.882**	**-0.667**	0.078	**0.523**	0.070		
A	0.092	-0.134	0.300	-0.005	**-0.483**	-0.208	-0.161	-0.239	0.085	**0.566**	**-0.535**	**-0.497**	**0.542**	-0.060	
TA	-0.162	-0.333	**0.694**	0.162	0.079	0.126	0.010	-0.055	**0.670**	0.179	-0.247	-0.236	-0.043	**0.694**	0.462

Table A4.43: **Correlation table** of studies carried out on result obtained from analysis of the Duckweed Lagoon **Inflow** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H	A
Cyano	0.452														
Bac	0.339	0.052													
NH_4^+	-0.433	-0.341	-0.324												
NO_2^-	-0.562	-0.358	-0.498	0.546											
NO_3^-	-0.381	-0.261	-0.443	0.391	0.650										
PO_4^{3-}	-0.158	-0.108	0.223	0.273	0.300	0.426									
SS	0.463	0.311	-0.054	-0.248	-0.143	-0.152	-0.270								
DS	-0.158	0.025	-0.180	0.339	0.268	0.286	0.166	0.502							
DO	-0.098	0.057	-0.030	0.031	0.092	0.056	-0.013	-0.181	-0.242						
BOD	-0.125	0.020	-0.484	0.477	0.418	0.423	0.310	-0.153	-0.058	0.010					
T	0.062	0.101	-0.493	0.097	0.385	0.490	-0.017	0.670	0.578	-0.171	0.329				
pH	0.388	0.316	0.378	-0.374	-0.736	-0.766	-0.496	0.182	-0.060	-0.172	-0.632	-0.425			
H	-0.045	0.104	-0.235	0.273	0.225	0.320	0.167	0.468	0.871	-0.461	-0.039	0.529	-0.025		
A	-0.014	-0.059	0.364	0.071	-0.466	-0.210	-0.056	-0.075	0.204	0.283	-0.456	-0.389	0.534	0.104	
TA	-0.019	-0.184	0.897	0.038	-0.231	0.024	0.153	0.191	0.821	-0.074	-0.705	-0.063	0.228	0.621	0.593

Table A4.44: **Correlation table** of studies carried out on result obtained from analysis of the Duckweed Lagoon **Outflow** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed ($p < 0.05$).

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H	A
Cyano	0.504														
Bac	0.211	0.143													
NH_4^+	-0.302	-0.315	-0.292												
NO_2^-	-0.333	-0.273	-0.355	0.745											
NO_3^-	-0.523	-0.407	-0.487	0.234	0.219										
PO_4^{3-}	-0.204	0.043	0.043	0.424	0.325	0.097									
SS	0.294	0.284	-0.082	-0.126	-0.025	-0.349	0.059								
DS	-0.164	0.013	-0.222	0.284	0.278	0.243	0.187	0.379							
DO	-0.208	-0.193	0.223	0.196	0.085	0.022	-0.229	-0.302	-0.433						
BOD	-0.061	0.021	-0.314	0.617	0.502	-0.003	0.406	0.158	0.020	-0.194					
T	0.070	0.162	-0.396	0.326	0.434	0.102	0.489	0.680	0.598	-0.407	0.450				
pH	0.225	0.198	0.318	-0.696	-0.750	-0.382	-0.462	-0.035	-0.181	-0.095	-0.674	-0.577			
H	-0.116	0.152	-0.206	0.325	0.311	0.135	0.332	0.460	0.825	-0.514	0.204	0.638	-0.238		
A	-0.112	-0.211	0.200	-0.274	-0.547	0.195	-0.390	-0.309	0.112	0.285	-0.616	-0.524	0.609	-0.153	
TA	-0.482	-0.524	0.369	0.381	0.246	0.345	0.270	-0.436	0.300	0.364	-0.045	-0.286	-0.138	0.053	0.397

***Table A4.45**: **Correlation table** of studies carried out on result obtained from analysis of the overflow tank at the **Discharge Point** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed (p < 0.05).*

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H
Cyano	0.552													
Bac	0.449	**0.963**												
NH_4^+	**-0.975**	-0.595	**-1.000**											
NO_2^-	-0.213	-0.138	0.024	0.000										
NO_3^-	-0.253	-0.509	-0.183	0.034	0.886									
PO_4^{3-}	0.318	0.735	0.757	-0.400	0.561	0.147								
SS	-0.084	-0.235	-0.367	0.272	-0.928	-0.695	-0.813							
DS	**-0.971**	-0.476	-0.987	**0.981**	0.119	0.067	-0.212	0.117						
DO	-0.830	-0.873	-0.987	**0.981**	0.629	0.918	-0.230	-0.402	-0.236					
BOD	**0.989**	0.548	0.977	**-0.982**	-0.174	-0.153	0.240	-0.086	**-0.995**	-0.268				
T	-0.554	-0.108	-0.702	0.724	-0.621	-0.664	-0.421	0.698	0.683	-0.904	-0.625			
pH	0.178	-0.277	0.269	-0.386	0.772	0.904	0.220	-0.693	-0.366	0.952	0.283	-0.910		
H	-0.282	0.151	0.057	0.124	0.890	0.590	0.767	-0.904	0.296	0.222	-0.310	-0.333	0.418	
A	0.161	0.417	0.489	-0.321	0.843	0.536	0.914	**-0.979**	-0.147	0.206	0.136	-0.627	0.556	0.900

***Table A4.46:** **Correlation table** of studies carried out on result obtained from analysis of the **Reservoir** in **Oasis Fish Farm** from **December 2019 to October 2020**. Figures represent Pearson's Coefficient (r). Bold figures that are in red highlight where statistically significant differences were observed (p < 0.05).*

	Algae	Cyano	Bac	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SS	DS	DO	BOD	T	pH	H
Cyano	**0.694**													
Bac	**0.488**	**0.493**												
NH_4^+	0.325	**0.655**	**0.691**											
NO_2^-	-0.262	-0.020	-0.132	0.140										
NO_3^-	-0.219	-0.072	-0.106	-0.127	**0.707**									
PO_4^{3-}	-0.189	0.370	0.165	0.414	**0.527**	**0.532**								
SS	-0.248	-0.166	-0.284	-0.009	0.173	0.191	-0.100							
DS	0.199	0.074	0.328	0.163	0.133	-0.282	-0.242	-0.096						
DO	0.363	-0.154	0.067	0.049	0.426	0.034	0.115	-0.210	**0.598**					
BOD	0.363	**0.532**	0.378	**0.557**	0.232	0.150	0.348	0.026	0.148	0.038				
T	-0.456	**-0.663**	**-0.597**	**-0.515**	0.269	0.402	-0.163	**0.648**	-0.095	0.012	-0.364			
pH	-0.251	-0.174	0.138	-0.153	**-0.522**	-0.431	-0.435	-0.243	-0.156	-0.374	**-0.547**	-0.100		
H	0.507	0.303	**0.534**	0.379	-0.145	-0.055	0.002	0.187	**0.496**	0.037	0.264	-0.130	-0.406	
A	0.164	-0.132	0.468	0.022	-0.260	-0.117	-0.288	-0.261	0.087	0.231	-0.307	-0.071	**0.606**	-0.060

Table A4.47: **Summary of all p-values** observed during on **Oasis** samples from **December 2019 to October 2020** using **T-tests** and **ANOVA** to determine whether any statistically significant differences existed between the culture pond samples, between the duckweed lagoon entry and exit points and between all sampling points. Statistical analysis was conducted on all bioassays and parameters before and after Grubb's test for outliers was conducted. Statistically significant difference = $p > 0.050$.

	All Sampling Points	Ponds	Duckweed Lagoon
Algae	0.3400	0.9178	0.7725
Cyanobacteria	0.8098	0.9511	0.9817
Bacteria	0.1404	0.4404	0.5275
NH_4^+	0.5520	0.4638	0.9757
NO_2^-	0.4867	0.9711	0.5948
NO_3^-	<0.0001	0.7041	0.4775
PO_4^{3-}	0.2160	0.3885	0.0756
Suspended Solids	0.8604	0.8915	0.4999
Dissolved Solids	0.3172	0.9628	0.9102
Dissolved Oxygen	0.1421	0.9602	0.5718
BOD	0.5464	0.9148	0.5166
Temperature	0.1671	0.9716	0.6865
pH	0.9952	0.8759	0.9001
Hardness	0.5237	0.4560	0.9674
Alkalinity	0.4806	0.4507	0.9769
Total Acidity	0.0769	0.2140	0.5128