An Investigation into Critical Factors Influencing Ecosystem Dynamics in Fish Rearing Water and Disease Mitigation Eco-Innovations for the Enhancement of Irish Freshwater Aquaculture

A thesis being submitted for the degree of

Doctor of Philosophy

by

Sarah M. Naughton, B.Sc (Hons) Toxicology

Based on research carried out under the Supervision of

Dr. Siobhán Kavanagh, Dr. Alex Tahar, Dr. Andy Fogarty & Prof. Neil Rowan

Declaration

I hereby declare that this thesis, submitted to the Athlone Institute of Technology for the degree of Doctor of Philosophy, is a result of my own work and has not in the same or altered form, been presented to this institute or any other institute in support of any degree other than for which I am now a candidate.

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Sarah Naughton, B.Sc (Hons)

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Date

Confidentiality Statement

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

Dedication

This thesis is dedicated to the memory of my Grandad, Michael Hunt, who always believed in me. I know he was with me for this entire journey.

Acknowledgements

First off, I would like to thank my supervisors that were involved in this project. Prof. Neil Rowan, who has been with me on this journey from the beginning, was always available for advice and believed I could do it. Dr. Andy Fogarty and Dr. Alex Tahar, who were there for the first phase of this project, without their guidance I would not have made it this far. Dr. Siobhán Kavanagh who was at the end of an email, a phone call or a Whatsapp message morning, noon and night, without your constant availability, expertise and faith in me, I would not have got to this final stage.

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Abstract

Aims and Rationale: The aquaculture sector is becoming the fastest growing food sector in the world, with an increasing requirement for higher production rates. With the maximum sustainable yield almost exhausted in terms of the global capture fisheries, aquaculture has an important role to help meet the global demand for seafood. The Irish aquaculture industry is worth an estimated €208.4 million, yet stringent limits on water abstraction rates and concerns over pollution of receiving water with aquaculture effluent have hampered intensive sustainability with increasing trends towards recirculation of processed water. Insufficiencies in traditional freshwater aquaculture practices may heighten the potential for growth of harmful finfish pathogens, thus indicating a commensurate need for improved wastewater remediation and disinfection regimes. The aim of this project was to determine the interaction dynamic between biological and physiochemical parameters in freshwater rearing water, to inform for future use as a low cost, natural wastewater remediation process in land-based aquaculture. Studies were also conducted to investigate the development of pulsed ultraviolet (PUV) light as an energy-efficient, eco-friendly disease mitigation technology for land-based aquaculture.

Methods and Results: Phytoplankton are an essential component of the aquatic ecosystem as primary producers, providing the potential for improving culture water quality if monitored and managed correctly. They also act as a natural sustainable food source for fish and other organisms present in culture water. To determine the ecosystem dynamic of rearing water, a study was carried out in a freshwater pill-pond aquaculture farm in the Republic of Ireland, producing Eurasian Perch, *Perca fluviatilis*, over a 10-month monitoring period. The study involved an investigation of both phytoplankton and bacterial communities (species composition and numbers) in the water and their correlations with selected physiochemical parameters, such as nitrates, nitrites and phosphates. This encompassed a variety of techniques, including molecular methodologies, microscopy, real-time flow cytometry (FCM) and sequencing. Findings showed that both phytoplankton and bacteria numbers peaked in the summer months reaching levels of 1.57×10^5 cells/ml and 9.70×10^6 cells/ml, respectively. *Chlorophyta, Bacillariophyta* and *Cryptophyta* were the most dominant phytoplankton divisions observed in the rearing water. Proteobacteria, Cyanobacteria and unclassified bacteria were the most relevant bacterial communities observed through denaturating gradient gel electrophoresis (DGGE) community profiling and sequencing of bands excised from gels. Principle component analysis (PCA) established that temperature, nitrates and bacterial numbers were the parameters that had the strongest correlation with the phytoplankton numbers. PCA analysis also demonstrated a strong relationship between use of sophisticated flow cytometry and *in situ* AlgaeTorch technologies for determining phytoplankton and cyanobacteria numbers.

In order to advance disinfection processes, the fish pathogens *Aeromonas salmonicida* and *Flavobacterium psychrophilum* were subjected to PUV light treatment. Ultraviolet (UV) light prevents DNA replication of microbes due to

alteration of pyrimidine bases of nucleic acids resulting in thymine dimers. The PUV system produces an intense UV flash with high peak power and high current resulting in irreversible DNA alteration and rapid inactivation. This offers an advantage over conventional UV due to a more energy efficient system. The results indicated a decrease in the bacterial load to a non-detectable number. Disinfection *via* conventional UV light was also investigated as a comparative method for both bacteria and it proved sufficient as a disinfection treatment. The effects of utilizing PUV alongside chemical disinfectants, bronopol and chloramine-T, was also deemed safe, without the production of any toxic by-products.

Conclusions and Significance: Traditional aquaculture produces large amounts of wastewater, threatening its global sustainability. In-depth knowledge of an ecosystem allows for immediate identification of a problem should the dynamic change from the norm, providing a quick resolution time to avoid loss of fish stocks or financial resources. This novel baseline study was conducted as an initial step into the introduction of utilizing natural phytoplankton processes to aid aquaculture practices. Determining baseline biological communities in rearing water, followed by elucidating physicochemical parameters governing wastewater treatment performance, can inform future intensification and diversification of freshwater aquaculture by exploiting and replicating knowledge of favourable algal-microbial ecosystems. Based on preliminary results PUV penetrance is sufficient to kill the pathogens investigated, that are commonly present in processed water used in aquaculture. Overall, PUV light technology offers promising results as a disinfection technology, with the further optimisation required prior to implementation into an *in situ* setting.

Project Background

As consumption rates of fish *per capita* continue to increase worldwide, overfishing depletes the wild fish stocks to try to meet the demands of the human population. The aquaculture industry can provide a solution to this dilemma. However, many obstacles prevent this industry from reaching its full potential. The doctoral study was a product of the overarching MOREFISH project that was funded by the Department of Agriculture, Food and the Marine (DAFM), which aims to promote innovation, sustainability and improved efficiencies in Irish freshwater aquaculture. The legislative framework of freshwater aquaculture in Ireland stipulates stringent discharge limits and restricted freshwater abstraction rates. The MOREFISH project has brought and will continue to bring significant aquacultural progresses achieved worldwide and novel innovations to an Irish aquaculture setting. The overall MOREFISH project addresses critical pressure points identified by industry end-users in the form of advanced aeration, efficient production management and deployment of next-generation pulsed light disinfection technologies. The key impacts of these innovations will be: (i) Enhanced production efficiency and sustainability, (ii) Reduced environmental impact of aquaculture production and (iii) Improved fish health and reduced finfish diseases/mortalities in rearing systems. The aims of the project are fulfilled *via* long-term on-site studies to represent the Irish freshwater aquaculture setting; determination of the environmental effects of the industry *via* lifecycle analysis; and disinfection studies using pulsed ultraviolet light (PUV). A second project developed through the MOREFISH platform called ECOAQUA, which was funded by Bord Iascaigh Mhara (BIM). ECOAQUA aims to test and optimise innovative technologies and processes developed through the MOREFISH project. ECOAQUA focuses more specifically on (i) Environmental and energy performance of three aquaculture sites; (ii) Modelling and profiling the global dynamic of the rearing water ecosystem; (iii) Piloting novel innovations; and (iv) Continuing to act as a communication platform for industry stakeholders, policy-makers and commercial operators involved in the freshwater aquaculture industry in Ireland. The

combination of the two projects brings together a critical mass of engineering and scientific expertise, and industry stakeholders, who aim to enhance the Irish freshwater aquaculture industry so that it will remain a part of our future.

Within both projects, my work packages included the characterisation of the baseline rearing water dynamic as an insight into the complex ecosystem that exists and investigating the use of PUV light for the disinfection of harmful finfish pathogens, which are presented in this thesis.

List of Abbreviations

AGD:	Amoebic Gill Disease
ANOVA:	Analysis of Variance
AO:	Acridine Orange
APC:	Allophycocyanin
API:	Analytical Profile Index
ASEAN:	The Association of Southeast Asian Nations
ATCC:	American Type Culture Collection
AWS:	Automatic Weather Station
bp:	Base Pair
BCWD:	Bacterial Cold Water Disease
BIM:	Bord Iascaigh Mhara/Irish Sea Fisheries Board
BLAST:	Basic Local Alignment Search Tool
BNE:	2-Bromo-2-Nitroethanol
BNM:	Bromonitromethane
BOD:	Biochemical Oxygen Demand
BSA:	Bovine Serum Albumin
BVDV:	Bovine Viral Diarrhoea Virus
CCAP:	Culture Collection of Algae and Protozoa
CFP:	Common Fisheries Policy
CFU:	Colony Forming Units
CFV:	Crossflow Velocity
CSA:	Climate-Smart Aquaculture
CW:	Continuous Wave
CWD:	Cold Water Disease
DAFM:	Department of Food Agriculture and the Marine
DAPI:	4', 6-diamidino-2-phenylindole
DGGE:	Denaturing Gradient Gel Electrophoresis
DF:	Dilution Factor
DNA:	Deoxyribose Nucleic Acid

dNTP:	Deoxyribonucleotide triphosphate
DO:	Dissolved Oxygen
EDTA:	Ethylenediaminetetraacetic Acid
EFSA:	European Food Safety Authority
EMFF:	European Maritime and Fisheries Fund
EPA:	Environmental Protection Agency
ERD:	Enteric Redmouth Disease
EU:	European Union
FA:	Fatty Acid
FADH:	Flavin Adenine Dinucleotide
FAO:	Food and Agriculture Organization of the United Nations
FCM:	Flow Cytometry
FDA:	Food and Drug Association
FEAP:	Federation of European Aquaculture Producers
FISH:	Fluorescent *in situ* Hybridization
FITC:	Fluoresceinisothiocyanate
FM:	Fish Meal
FO:	Fish Oil
FP:	Fish Pond
FSC:	Forward Scatter
FT:	Flow through
HPLC:	High-Performance Liquid Chromatography
HTS:	High-Throughput Sequencing
HUFA:	Highly Unsaturated Fatty Acid
IPN:	Infectious Pancreatic Necrosis
IROMP:	Iron-regulated Outer Membrane Protein
ISO:	International Organization for Standardization
JC:	Jukes-Cantor
JM:	Jaworski's Media
kb:	Kilobase
KWF:	Keywater Fisheries

LCA:	Life Cycle Analysis
LED:	Light-Emitting Diode
LOEC:	Lowest Observed Effect Concentration
LP:	Low Pressure
LP-UV:	Low Pressure Ultraviolet Light
MB:	Microbubble
Mbp:	Megabase pair
MEGA:	Molecular Evolutionary Genetic Analysis
MHI:	Marine Harvest Ireland
ML:	Maximum Likelihood
MP:	Medium Pressure
MPN:	Most Probable Number
MPUV:	Medium Pressure Ultraviolet Light
MR:	Methyl Red
MSDS:	Material Safety Data Sheet
MWCO:	Molecular Weight Cut Off
NB:	Nanobubble
NCIB:	National Centre for Biotechnology Information
NCIMB:	National Collection of Industrial Food and Marine Bacteria
NJ:	Neighbor Joining
NOEC:	No Observed Effect Concentration
NTU:	Nephelometric Turbidity Units
OD:	Optical Density
OECD:	Organisation for Economic Co-operation and Development
OTU:	Operational Taxonomic Unit
PAA:	Peracetic Acid
PAS:	Partitioned Aquaculture System
PBS:	Phosphate Buffered Saline
PC:	Phycocyanin
PCA:	Principle Component Analysis
PCR:	Polymerase Chain Reaction

PE:	Phycoerythrin
PEPPA:	Perspectives of Plant Protein Usages in Aquaculture
PES:	Polyethersulfone
PI:	Propidium Iodide
PPE:	Personal Protective Equipment
pps:	Pulses per Second
PUFA:	Polyunsaturated Fatty Acid
PUV:	Pulsed Ultraviolet Light
RAFOA:	Research Alternatives to Fish Oils
RAS:	Recirculating Aquaculture System
rDNA:	Ribosomal Deoxyribose Nucleic Acid
RNA:	Ribose Nucleic Acid
RTFS:	Rainbow Trout Fry Syndrome
SD:	Serial Dilution
SDG:	Sustainable Development Goal
SEM:	Standard Error of the Mean
SOM:	Soil Organic Matter
SPC:	Sodium Percarbonate
SRP:	Sediment Reactive Phosphate
SS:	Suspended Solids
SSC:	Side Scatter
TAE:	Tris-Acetate EDTA
TAN:	Total Ammonia Nitrogen
TEM:	Transmission Electron Microscopy
TEMED:	Tetramethylethylenediamine
THM:	Trihalomethane
TOC:	Total Organic Carbon
TP:	Treatment Pond
T-RFLP:	Terminal-Restriction Fragment Length Polymorphism
UAE:	United Arab Emirates
UPGMA:	Unweighted Pair Group Method with Arithmetic Mean

UV:	Ultraviolet
VBNC:	Viable but Non-Culturable
VP:	Vogues Proskauer
WFD:	Water Framework Directive
WHO:	World Health Organisation
WWTP:	Waste Water Treatment Plant
Yo-Pro:	{4-[3-methly-2, 3-dihydro-(benzo-1, 3-oxazole)-2-methlymethlyedene]-1-(3'-trimethlylammoniumpropyl)-quinoliniumdiiodide}

Units of Measurement

CFU/ml:	Colony forming units per millilitre
cm:	Centimetres
CV:	Coefficient of variance
D-value:	Death value
E_rC_{50}:	Effective concentration that causes 50% reduction in growth
EC_{50}:	The concentration that causes adverse effects on 50% of organisms
J:	Joules
LC_{50}:	The concentration that causes lethality to 50% of the population
mJ:	Millijoules
mJ/cm^2:	Millijoules per centimetre squared
ml:	Millilitres
mm:	Millimetres
mW:	Milliwatts
mW/cm^2:	Milliwatts per centimetre squared
ng/µl:	Nanogram per microlitre
nm:	Nanometre
pg/µl:	Picogram per microlitre
ppm:	Parts per million
pps:	Pulses per second
ppt:	Parts per trillion
R^2:	Coefficient of determination
St. Dev.:	Standard deviation
µl:	Microlitres
V:	Volts

Chapter 1

Introduction

Context – The Importance of Aquaculture

There are many definitions to describe aquaculture, however, Harrell (2006) simply defines aquaculture as "the farming and husbandry of aquatic organisms under controlled or semi-controlled conditions". The difference between aquaculture and wild/capture fishing is the intervention of humans and technology in the rearing process. This is outlined in the more specific definition of aquaculture described by the Food and Agriculture Organization of the United Nations (FAO) which describes aquaculture as "the farming of aquatic organisms in both coastal and inland areas involving interventions in the rearing process to enhance production" (FAO, 2018b). Aquaculture can be broken into three main areas namely the aquaculture of (i) finfish e.g. trout, salmon, carp; (ii) shellfish e.g. oysters, mussels, shrimp; and (iii) plants e.g. red alga (Jhingran, 1987). Marine aquaculture involves the culturing of saltwater fish and shellfish, whereas freshwater aquaculture involves the culturing of species in freshwater (Harrell, 2006), with freshwater being less alkaline (Jhingran, 1987). Each species has an optimal temperature for maximising growth and so are reared based on this. For example, the culturing of freshwater salmonids such as rainbow trout is carried out at temperatures of 15°C and below (Schmidt *et al*., 2006; Verner-Jeffreys, 2015).

1.1 Global aquaculture

The aquaculture sector is becoming the fastest-growing food sector in the world (FAO, 2018b). The growing human population, the exhaustion of ocean fisheries stocks and the increased consumption rates *per capita* are an incentive for the aquaculture industry to expand its growth (Crab *et al.*, 2007; Zhang *et al.*, 2011; FAO, 2016). According to FAO (2018), it is the aquaculture industry that is encouraging the remarkable growth in fish production to meet the rapidly increasing demand of fish for human consumption, as presented in **Figure 1.1**, which has now surpassed the demand for meat consumption. During the period 2010 to 2030, global aquaculture production needs to increase threefold in order to meet the demands for fish and

food (DAFM, 2015). The aquaculture industry only accounted for 7% of total fish production worldwide in 1974, which increased to 26% in 1994 and to a further 47% in 2004 (FAO, 2016). Production of fish from aquaculture practices now represents almost half of the total world production of fish for human consumption. Total aquaculture production of food fish has risen from 61.8 million tonnes in 2011 to 80 million tonnes in 2016 (FAO, 2018b).

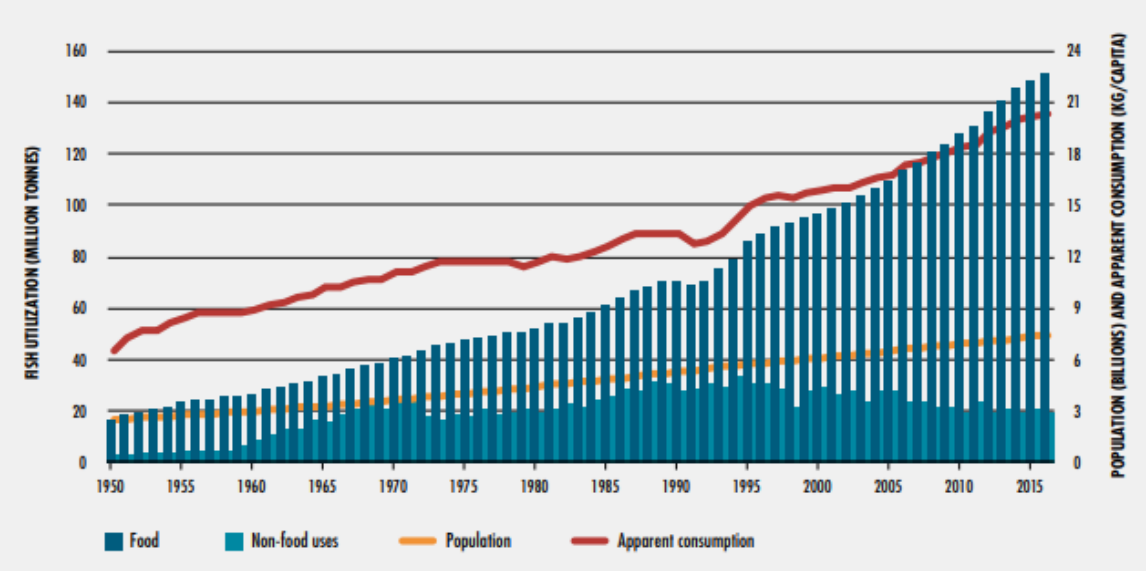

Figure 1.1 World fish utilization and apparent consumption (FAO, 2018).

Although this is a major achievement for aquaculture, this progress in production is unevenly distributed continentally, as Asia is responsible for 89% of the fish produced *via* aquaculture over the last twenty years. East Asia, specifically China, has the greatest input accounting for over 62% of worldwide aquaculture produce (FAO, 2018b). **Figure 1.2** illustrates the influence that Asia has on this increased global production compared to the rest of world and depicts minimal increases in production of fish *via* aquaculture over the last three decades for the remaining continents, highlighting a massive gap in production between Asia and Europe. Another example that portrays the high aquaculture production rate of China is related to filter-feeding finfish production, which, of the 8.2 million tonnes produced globally in 2014, China produced 7.4 million tonnes (FAO, 2016). As China produces the largest amount of fish, it is also the largest exporter of fish globally.

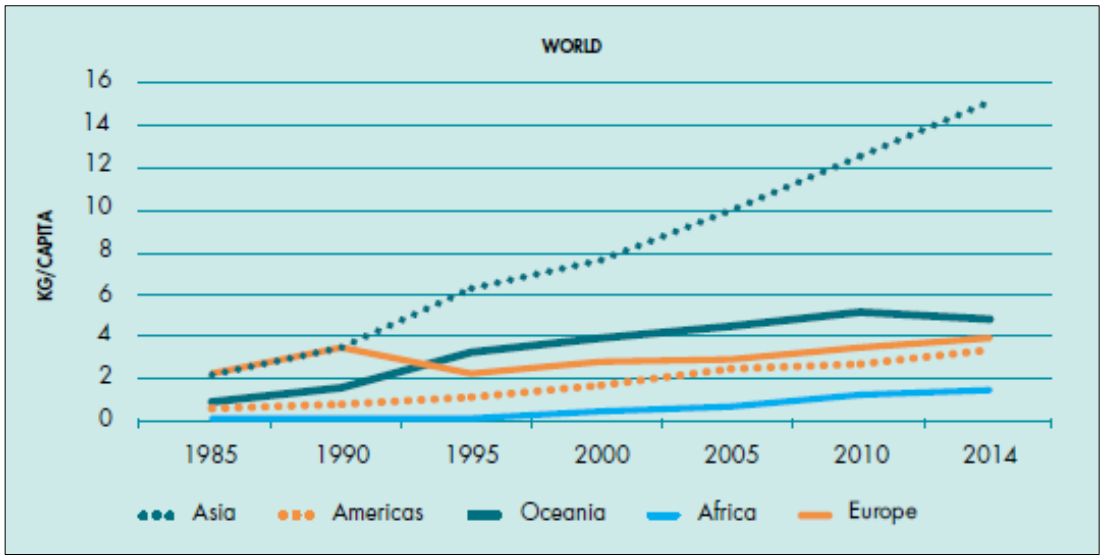

Figure 1.2 The global *per capita* production of aquaculture (kg) from 1985 to 2014 (FAO, 2016).

There are many plans in place to aid in sustainability and food security in terms of fish production such as the 2030 Agenda, the Sustainable Development Goals (SDGs) and the Blue Growth Initiative (FAO, 2018b). The emergence of climate-smart aquaculture (CSA), combining aquaculture and aquaponics, is another innovative approach in the progression of processes, while addressing the economic, environmental and social aspects of sustainability (FAO, 2018b).

1.2 European aquaculture

European aquaculture is a billion dollar industry, which produced 2,327,082 tonnes of fish and shellfish in 2016, a slight drop compared to production in 2015, which was 2,350,278 tonnes (FEAP, 2016, 2017). Norway is the dominant country of production, accounting for 58% of the total tonnage (FEAP, 2017). This production is split up into sectors consisting of marine cold-water production, freshwater production and Mediterranean production. Marine species represent 70% of production, freshwater species represent 16% and Mediterranean produce represents 14% % of the total production (FEAP, 2017). The freshwater aquaculture production in Europe is mainly characterised by the farming of rainbow trout (Donnelly, 2011). When compared to the amount of fish produced in China, Europe produces a considerably lesser amount each year. For example, in 2014 Europe produced 632,000 tonnes of bivalve

molluscs, including clams, mussels and oysters, whereas China produced 12 million tonnes of the same in that year (FAO, 2016).

The EU, being the largest single market for importing fish, relies on the imports from countries such as China, Norway and Thailand in order to satisfy consumer needs in terms of fish consumption (FAO, 2016). The EU also has difficulties competing with cheap imported supplies of aquaculture produce and increased running costs in the aquaculture process (Donnelly, 2011). The low production rates in the EU results in the inability to keep up with the demand for fish (FAO, 2016). This reliance on imported fish to the EU may result in the quality and safety of some fish imports being neglected to fulfil the demand by consumers.

To alleviate the reliance on imports, to meet the fish demand and ensure food security for the rising population, the aquaculture industry in Europe currently aims to introduce sustainable practices to enhance growth and development within the sector. Horizon 2020 provides a huge opportunity to advance towards the enhancement and efficient productivity of the European aquaculture sector, resulting in the overall development of satisfactory food products. Horizon 2020 distributes 20% of the European Maritime and Fisheries Fund (EMFF) across the European countries that partake in aquaculture. The aim of the funding is to tackle aspects of the industry such as disease mitigation, modernisation of practices, support for new farmers, education of staff and innovative investments in equipment (European Commission, 2017). Another strategic EU aquaculture policy developed was a Declaration on ‘Streaming Sustainability in Aquaculture’, which Mr Simon Covney, the acting Irish Minister for Agriculture, Food and the Marine, was an official witness to. This strategy, known as the ‘Dublin Declaration’, was implemented in 2013 and entailed a set of principles to ensure the future of the European aquaculture sector. The core values addressed in this declaration are water, the environment, science, the economy and consumers, and their role in the sustainability of the European aquaculture industry. European aquaculture has the potential to fill the existing gap between the domestic supply of fish and consumer demand levels. However, in order to do so, the industry stakeholders must embrace

and act upon the opportunities provided by schemes such as Horizon 2020 and the Dublin Declaration.

1.3 Irish aquaculture

The aquaculture industry in Ireland employs 1,913 people, with the majority employed in the area of oyster production, a figure that has not shown a significant increase from 2012 to 2017 (BIM (Bord Iascaigh Mhara), 2016, 2018). There has been an increase in aquaculture production since 2015, with total aquaculture production being 47,147 tonnes in 2017, up 7% on the previous year (BIM, 2018). This equates to an increase in the value of the aquaculture industry in Ireland by 24% from 2016 to a total of €208.4 million. In terms of organic status in Ireland, 80% of Irish farmed salmon are certified organic (DAFM, 2015). Marine Harvest is a group comprised of salmon farms from Ireland, Scotland, Norway, France, Chile, Canada and the Faroes. Marine Harvest Ireland (MHI) is the only producer of salmon with organic status in this group and so supplies EU, the United Arab Emirates (UAE), Asia and the US east coast with organic salmon. This makes Ireland the largest global supplier of organic salmon and highlights the importance of the location of Ireland to take advantage of this global supplier opportunity (Marine Harvest Ireland, 2016).

Many Irish policies and schemes have been developed in order to try to increase the productivity and success of the aquaculture sector in Ireland. Harvest 2020, introduced in 2010, is a national strategy introduced by the Irish Government for the "sustainable development of the food industry, including seafood" (DAFM, 2015), the objectives of which are outlined in **Figure 1.3.** This strategy recommended that BIM and the Marine Institute collaborate with research and industry to develop and progress aquaculture *via* green, smart and sustainable methods (DAFM, 2015).

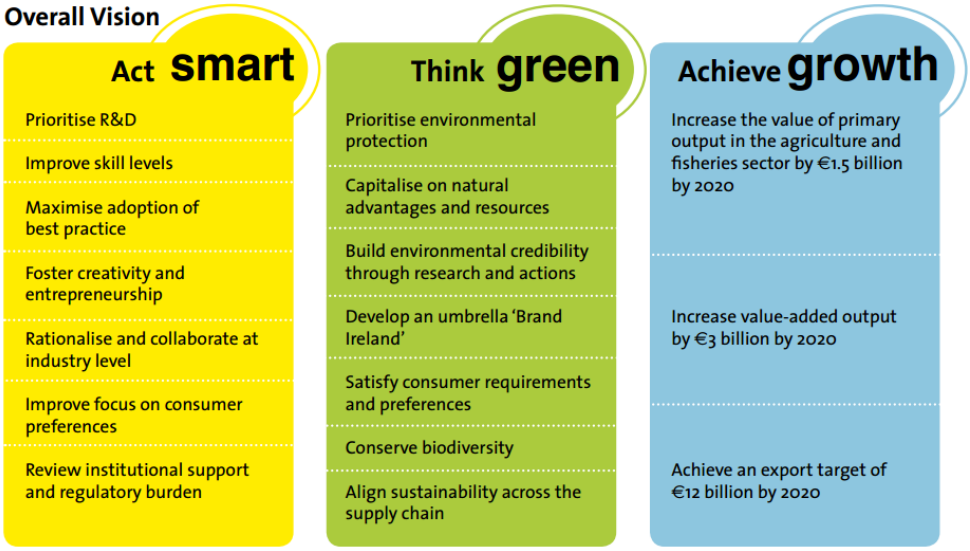

Figure 1.3 An overview of the strategy of Harvest 2020 (DAFM, 2015).

Although the value of the aquaculture industry in Ireland has increased, the freshwater sector remains stagnant in terms of production. The main contributor to the rise in the value and production in the industry in 2016 was due to increased success in salmon and mussel production (BIM, 2018), not freshwater fish, which is the main focus of the MOREFISH project. Perch and rainbow trout are the two main species that contribute to the Irish freshwater aquaculture industry (BIM, 2017). Yet, the production volume of perch and rainbow trout still remain at relatively low levels, with the total value of perch and rainbow trout production in 2016 totalling to just over €2 million in 2016 (BIM, 2017). Rainbow trout production amounted to a total value of €1,961,910 in 2016, whereas perch production resulted in a total value of €266,000 (BIM, 2017); in fact, the production of perch decreased in 2017 (BIM, 2018). Despite such policies including Harvest 2020, there has still been no major increase in freshwater production in Ireland. This project aims to identify the drawbacks in the Irish freshwater industry and introduce innovative techniques that will enhance practices and increase productivity.

Aims & Objectives

This study is based on two inter-related aims. The first aim is the establishment of a baseline of biological and physicochemical parameters in an Irish freshwater pill-pond system in order to develop a greater understanding of the ecosystem dynamic in the rearing water. The second aim is to investigate the use of pulsed ultraviolet (PUV) light as a novel non-chemical disinfection technology to enhance the efficiency of aquaculture practices in Ireland. Therefore, the following related objectives will be addressed:

- Quantification and profiling of the annual phytoplankton and bacterial communities numbers along with relevant physicochemical parameters in an Irish freshwater pill-pond for the culturing of perch.

- Determination of seasonal fluctuations of both phytoplankton and bacterial organisms present a one year monitoring period.

- Correlation of all metadata to establish a baseline of all test parameters in the monitored pill-pond.

- An investigation into the use of *in situ* parameter measurements compared to *ex situ* laboratory analysis.

- Identification of the most commonly used chemical disinfectants in Irish aquaculture.

- Investigation into the use of PUV light in the inactivation of identified pathogens, namely *Aeromonas salmonicida* and *Flavobacterium psychrophilum,* using *Escherichia coli* as a surrogate microorganism.

- Ecotoxicological assessment of selected chemical disinfectants pre and post PUV light treatment *via* the use of a primary producer and two primary consumers, i.e. *Pseudokirchneriella subcapitata*, *Artemia salina* and *Daphnia pulex*, respectively.

Thesis Layout

Chapter 1 introduces the topic of aquaculture, the current status of aquaculture and the rationale behind this research project. **Chapter 2** gives an in-depth insight into the aquaculture process, the biochemistry of the rearing water, problematic pathogens and the most common disinfection processes currently used in the industry. **Chapter 3** describes a long-term study carried out in Keywater Fisheries, a freshwater aquaculture site in the West of Ireland, which focused on obtaining a biological and physicochemical profile for the rearing water in a grow-out pond in the farm. **Chapter 4** describes the use of PUV light as a novel disinfection tool for aquaculture and the outcomes of its use in this study, whilst also comparing it to the more conventional CW UV disinfection currently used in the aquaculture industry. **Chapter 5** details the use of ecotoxicological assays as a useful tool for determining the toxicity of chemicals upon exposure to PUV light, and reveals the results achieved when common aquaculture disinfectants were investigated. **Chapter 6** gives an overall conclusion of the experiments carried out in this project and reflects on how results obtained may influence the freshwater industry in Ireland. It also mentions areas for further development and directions for future projects.

Chapter 2

Aquaculture

The Aquaculture Process

2.1 Flow through aquaculture systems

The primary focus of the MOREFISH project is related to freshwater aquaculture, therefore, any process explained from this point on will be in the context of the freshwater industry. The main system utilised in the freshwater aquaculture industry in Ireland is the traditional flow through (FT) aquaculture system, with the primary freshwater species in Ireland being rainbow trout and salmon smolts (BIM, 2017). A FT system consists of rearing/culture water flowing from a single water body source through a series of long narrow earthen ponds, concrete raceways, troughs or tanks, illustrated in **Figures 2.1** and **2.2**. The water then gets discharged into the receiving water body with very limited or no water treatment prior to this (Váradi, 1984; Schmidt *et al.*, 2006). The incoming water is sourced from streams, springs, rivers or wells (Small *et al*., 2016).

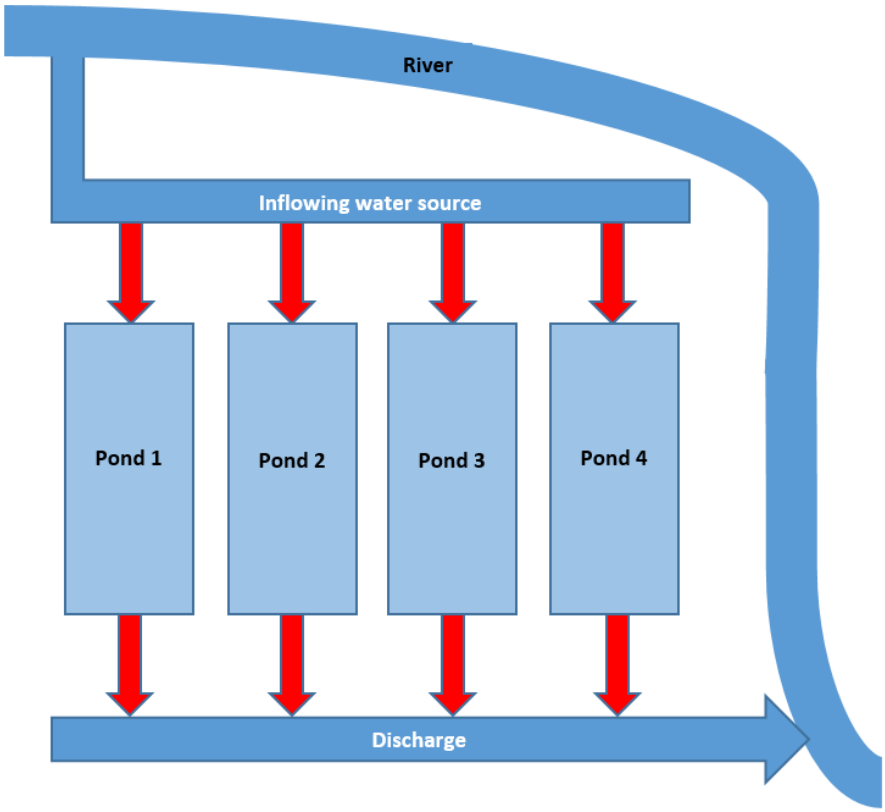

Figure 2.1 Diagrammatic representation of a typical flow through pond system.

Management practices tend to include solids removal from the bottom of the culture unit *via* drainage or suction, to avoid solid build up in the system, as the main source of feed tends to be formulated pelleted food (Váradi, 1984). Minor treatment of culture water may occur as the water passes through the system, however, water does not pass through the same unit more than once (Small *et al*., 2016). The amount of treatment required typically depends on the quality of the water entering the facility. At present in Ireland, the above description of water treatment does not generally apply, as most Irish freshwater fish farms do not currently implement water treatment for flow through systems.

Figure 2.2 A typical aquaculture system - tank setup (left) and pond setup (right), taken by the author (2016).

The abundance of continuously flowing water ensures a sufficient amount of oxygen for the fish and flushes out metabolic wastes that are present, including ammonia, as long as the appropriate flow rate is implemented (Váradi, 1984; Small *et al.*, 2016). The flow rate is calculated based on the intensity of the system i.e. the more fish present the more oxygen required and the greater the volume of water and flow rate needed (Váradi, 1984). If artificial oxygen supply measures are in place e.g. paddle wheels, then the volume of water consumed can be decreased (Váradi, 1984). FT systems are generally used for growing trout and the stages of salmon that are reared in freshwater (cold water species) due to the more convenient availability of cold water all year round (15°C) compared to warm water (Small *et al.*, 2016).

There are some constraints regarding FT systems such as minimal control of the quality of the incoming water. Also, the implementation of an intensification approach to improving aquaculture sustainability may lead to issues regarding water consumption due to constraints on water abstraction levels (Terjesen *et al.*, 2013).

Intensification practices would require increased water consumption, therefore contributing to water shortages, leaving fish farms without an incoming source of rearing water.

2.2 Water re-use systems

In order to support the demand for fish worldwide, intensification practices and a change from typical FT systems must be implemented in a strategic manner. The re-use of water appears to be a solution to aquaculture intensification and could drastically reduce the water consumption from national rivers, lakes and streams, providing more control for farmers on the quality of the water being utilised and enhancing the productivity of the freshwater industry (Jokumsen and Svenden, 2010; Terjesen *et al.*, 2013; Liu *et al.*, 2014). This system is called a recirculated aquaculture system (RAS). In RAS the culture water is either entirely or partially treated within the farm to facilitate the reuse of the rearing water (Sturrock *et al.,* 2008). This avoids the need for the complete reliance of the freshwater aquaculture industry on rivers, lakes and streams as the main water source, as most of the water is reused on the farm (Helfrich and Libey, 1990). Donnelly, (2011) outlines the advantages of a RAS system as the following:

- Reduced land and water use;
- Strict water quality control;
- Lower environmental impact;
- Higher biosecurity;
- Better control of waste production.

There are many other benefits pertaining to RAS systems in terms of a more stable environment and increased process stability. RAS systems increase the opportunity to manipulate production and allow for the culture of a more diverse range of species (Helfrich and Libey, 1990). The majority of perch fry production is done *via* RAS systems in Ireland (Donnelly, 2011). **Figure 2.3** outlines the principal components of a RAS system.

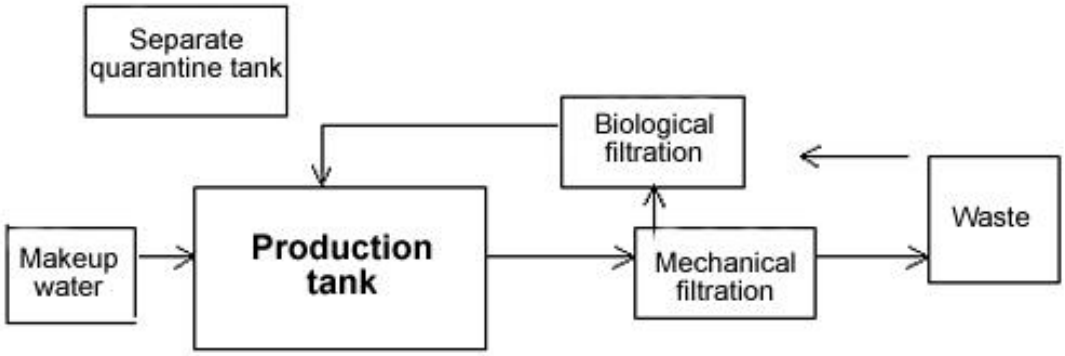

Figure 2.3 Block diagram of a RAS system (Queensland Government, 2015).

Although RAS systems are the way forward, water re-use and recirculation can result in the accretion of high concentrations of organic carbon (Summerfelt *et al*., 2009) which can have a major impact on fish health. RAS systems can also lead to an increase in the pathogenic microbial load, widespread disease and huge economic loss unless effective disinfection measures are in place. Therefore, in order to change from traditional FT aquaculture systems to more intensive RAS systems, the implementation of appropriate water treatment before reuse to maintain a healthy fish stock is essential (Jokumsen and Svenden, 2010).

Firstly, the accumulation of suspended solids (SS) in an aquaculture system may negatively affect system performance. Thus, the addition of a solid/liquid separation step is essential for the collection and treatment of suspended solids and sludge produced (Sturrock *et al.*, 2008). Secondly, the accumulation of toxic compounds such as total ammonia nitrogen (TAN) produced by aquaculture activity can pose lethal effects on fish if left untreated. TAN can pose a risk of toxicity to aquatic species at relatively low concentrations of less than 0.5 ppm for some salmonids (Terjesen *et al.,* 2013). Therefore, a biological nitrification process, which oxidises ammonia, is required (Sturrock *et al.,* 2008) which is explained in more detail in **Section 2.3.1.1**. Thirdly, disinfection measures, *via* chemical or physical methods, are required to kill potentially harmful microorganisms and thus prevent fish diseases and mortality (Sturrock *et al.*, 2008; Jokumsen and Svenden, 2010), which if left untreated can cause huge economic losses due to disease outbreaks. The disinfection measures utilised in the freshwater aquaculture industry are explained in the **Water Treatment**

Section of this chapter. Additional disinfection might be needed for (i) incoming water, (ii) water to be reused in the system and (iii) water to be discharged. This would prevent (i) the introduction of unknown pathogens into the farm, (ii) the accumulation of pathogens in the systems as the water gets recirculated and (iii) the discharge of harmful pathogens into the receiving freshwater environment. The degree to which these measures are utilised is dependent on the volume of water being recirculated. With an increase in the amount of recirculation, there will also be the necessity for an increase in the amount of water treatment steps as outlined in **Figure 2.4** (Pedersen, 2015). In some cases, a final step of denitrification is also required to avoid the accumulation of nitrates but this is mainly in very high rate RAS systems (Sturrock *et al.*, 2008). It must also be noted that fish who spend their entire lifecycle in recirculation systems cannot achieve organic status (Donnelly, 2011). The quality of the water being discharged to the receiving freshwater environment may require substantial treatment, as the Irish Environmental Protection Agency (EPA) become more involved in the aquaculture trade effluent licenses, resulting in policies and standards that are more stringent. The Water Framework Directive (WFD) may also be a driving force that will help the sector expand and develop as a more unified industry in terms of trade effluent licenses, rather than the inconsistency that currently exists with each county council monitoring discharge levels.

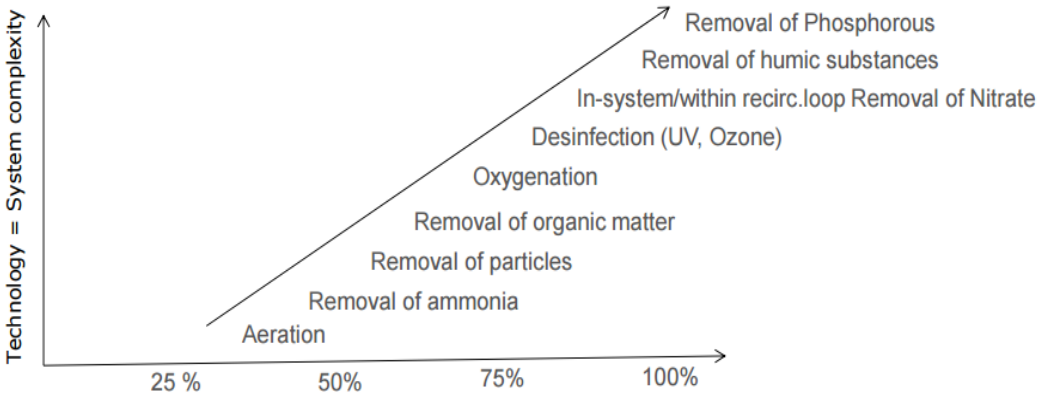

Figure 2.4 Level of aquaculture system complexity based on the percentage of recirculated water (Pederson, 2016).

The main aim of the WFD is to prevent pollution of all rivers, lakes, groundwater, etc. and achieve good quality status for the water in Ireland (EPA, 2017). Both the EPA

and the implementation of the WFD by the EPA may be a potential reason for the enforcement of novel disinfection technologies on discharge water such as PUV, further explained in **Chapter 4** of this document**.**

2.3 The biology and physico-chemistry of a freshwater aquaculture system

In the aquaculture industry, water quality needs to be closely monitored in order to maintain, as closely as possible, the optimal growth conditions for a given species, and consequently to ensure maximised fish production (European Food Safety Authority (EFSA), 2008). This involves a dynamic relationship between a range of biological and physico-chemical parameters including fish, fish biomass, prevailing phytoplankton and bacterial species, dissolved oxygen (DO), carbon dioxide (CO_2), pH and nitrogen levels. None of these entities can be discussed on their own due to the interconnectedness between all the variables, therefore, in this section, the biological aspects of a fish farm are mentioned throughout as each individual physicochemical parameter is outlined. The relationship between these parameters is the basis for one of the aims of this project, with an in-depth investigation presented in **Chapter 3**.

In most outdoor aquaculture settings, a symbiotic relationship between phytoplankton and bacteria occurs (Phang, 1991). This relationship contributes to many useful processes in water treatment such as mineralisation and oxygenation (Thajuddin and Subramanian, 2005). The production of phytoplankton in fish ponds relies on many factors, namely sunlight, pH, temperature and nutrients including carbon, nitrogen, hydrogen, oxygen and phosphorus (Gao and McKinley, 1994; Neori *et al.*, 2004). All of these factors can have a major impact on photosynthesis, cell biomass and metabolism, which in turn influences cell composition dynamics important for individual functionality of each microorganism (Hu, 2004).

2.3.1 Nitrogen

There are many ways in which nitrogen is introduced into freshwater fish farms, e.g. ammonia as an excreted end product of protein catabolism from fish, in the form of dissolved free ammonia from the atmosphere due to precipitation, drainage into the ponds from surrounding lands, and nitrates released from decaying organic matter (Jiménez-Montealegre *et al.,* 2002; Thajuddin & Subramanian, 2005). Accumulation of nitrogen levels can cause toxicity to fish in terms of ammonia and nitrites (Meade, 1985; Helfrich and Libey, 1990; Hargreaves, 1998). High levels of ammonia cause convulsions, lethargy, gill damage, such as hyperplasia, and negatively impacts fish growth (Meade, 1985; Hargreaves, 1998). It is therefore essential to frequently monitor the levels of nitrogen in the culture water.

2.3.1.1 The nitrification process

The oxidation of ammonia by the aerobic bacteria occurs through a process called nitrification (Crab *et al.,* 2007). Ammonia exists in two forms in the water: ionised ammonium (NH_4^+) and unionised ammonia (NH_3). The unionised form is the most toxic, leading to harmful effects at levels above 0.05 mg/l (Helfrich and Libey, 1990). It is therefore essential for fish health and production that ammonia undergoes detoxification such as nitrification. This process requires two types of aerobic bacteria; *Nitrosomonas* species oxidise ammonia to nitrite (NO_2^-), which is then oxidised to practically non-toxic nitrate (NO_3^-) by *Nitrobacter* species (Helfrich and Libey, 1990; Hargreaves, 1998). This is chemically described by the equation $NH_4 \rightarrow NO_2^- \rightarrow NO_3^-$ (Pedersen *et al.,* 2015).

There are contradictory reports on what levels are deemed safe for the culturing of fish. Helfrich and Libey (1990) state that levels of ammonia and nitrates should be maintained at concentrations below 0.05 mg/l and 0.5 mg/l respectively. Yet, studies carried out by Burrows (1964) resulted in toxicity to chinook salmon following exposure to 0.006 mg/l of unionised ammonia for 6 weeks, and Smith and Piper (1975) observed pathological damage in rainbow trout following exposure to 0.0125 mg/l of unionised ammonia after 6 months.

On the contrary to the effect on fish health, nitrogen is very important for the survival of phytoplankton and is essential for building structural and functional proteins (Hu, 2004). It is available in the soil organic matter (SOM); however, nitrogen is not in a bioavailable form for phytoplankton to utilise. Nitrogen-fixing bacteria convert the nitrogen (nitrogen fixation) into a form that can then be utilised by phytoplankton (Thajuddin and Subramanian, 2005; Neospark, 2014). The rate of nitrogen fixation largely depends on the bacterial species present in the water and the concentration of ammonia (Hargreaves, 1998). This nitrogen fixation process highlights the important relationship that exists between phytoplankton and bacteria. Phytoplankton can be considered as a plant biofilter as they uptake the ammonia as well as heavy metals, reducing the availability of toxic substances for fish to consume (Neori *et al.*, 2004). This natural relationship that occurs in aquaculture ponds revolving around the nitrogen cycle is depicted in **Figure 2.5.**

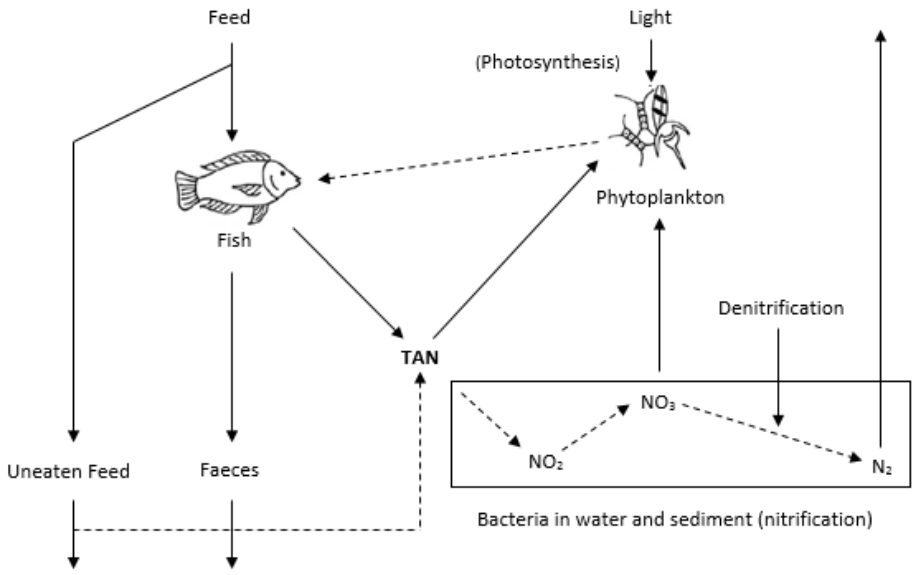

Figure 2.5 The nitrogen cycle in aquaculture ponds excluding respiration at night (adapted from Crab *et al*., 2007).

Phytoplankton populations can produce oxygen enrichment during the daytime *via* photosynthesis (Muller-Feuga, 2004), a process which also reduces toxic gas levels such as ammonia, CO_2 and nitrite (Neospark, 2014). Potent levels of ammonia that increase above 0.05 ppm inhibit the growth of phytoplankton (Davoren *et al.*, 2005) so it is, therefore, important to ensure the monitoring of ammonia levels.

2.3.2 Oxygen

Oxygen supply is essential for fish respiration and for the nitrification process, both for the aerobic bacteria and the oxidation of waste material (Helfrich and Libey, 1990). The main sources of oxygen in aquaculture ponds are from atmospheric diffusion across the surface of the water, production by phytoplankton *via* photosynthesis (Hopkins *et al.,* 1995) and artificial aeration. During the night DO levels decline due to the respiration process carried out by fish, bacteria, plants/phytoplankton and other organisms (Boyd, 1998). However, if the amount of sunlight is sufficient in daylight hours, the amount of oxygen released during the day through photosynthesis tends to supersede the amount utilised during respiration, which helps prevent oxygen depletion (Hopkins *et al.,* 1995). It is usually safe to supersaturate the pond with oxygen, where the water is more highly concentrated with oxygen than normal, as long as the pH is not very high (Phang, 1991). There is an inversely proportional relationship between temperature and DO; as a rise in temperature results in a decrease in DO saturation (Boyd, 1998). The formation of oxygen radicals can also pose a threat should the temperature increase from 20°C to 30°C (Tjahjono *et al.*, 1994).

To some degree, oxygen levels are maintained in aquaculture ponds by the natural phytoplankton/bacterial relationships that exist in water (Phang, 1991). However, the level of oxygen diffusion can be further increased by implementing aeration which generally costs less than pumping in exchange water (Hopkins *et al.*, 1995) and would also help reduce abstraction of large volumes of water from the source. There are many methods of introducing air/oxygen into an aquaculture pond, including paddle wheels, U-tube oxygenation, diffused-air systems and microbubble (MB) devices (Helfrich and Libey, 1990; Boyd, 1998; Hargreaves, 1998). Fish tend to reside in areas that have high DO concentrations, i.e. close to an aerator, when the rest of the pond is oxygen deficient. Therefore, it is important to have a replacement aerator close to the original in the case of power failure, so the fish can avoid passing through low oxygenated waters to reach the replacement source of oxygen (Boyd, 1998).

2.3.3 Carbon

Carbon is sourced from the bacteria, simple carbohydrates and organic acids, and exists in many different forms depending on the pH of the water (Phang, 1991). Aerobic bacteria present in the water body are the main source of breaking down organic matter into CO_2 and ammonia (Phang, 1991). Phytoplankton then utilise the CO_2 for photosynthesis and release oxygen during the process, which in turn oxygenates the water for the fish (Neospark, 2014). The level of CO_2 in the surface waters of aquaculture ponds is between 1-2 ppm (EFSA, 2008). If CO_2 remains in the culture water at concentrations higher than this, it causes a decrease in the pH, which in turn has a negative impact on fish health, as CO_2 accumulates in the blood of the fish, they are unable to utilise DO and they eventually die (Helfrich and Libey, 1990).

2.3.4 Temperature

Freshwater fish such as rainbow trout have an optimal growth temperature of approximately 12 – 14°C (EFSA, 2008). The level of DO in water is regulated by the temperature, and as fish are cold-blooded animals, increased water temperature increases the biochemical oxygen demand (BOD). An increase in water temperature also results in decreased oxygen availability (EFSA, 2008). Therefore, increased oxygen consumption with less oxygen available tends to result in severe stress. According to Hu (2004), temperature is one of the most important influential factors of phytoplankton growth in terms of biochemical composition, carotenoid composition and carbon/nitrogen quotas of the phytoplankton cell.

2.3.5 pH

According to the EFSA (2008), it is important for fish stocks that pH levels remain between 5 and 9 in the culture water. High pH can have adverse outcomes on fish health by damaging the eye cornea and lens, as well as causing gill and skin epithelial injury (Phang, 1991). High pH also affects the oxygen and CO_2 levels in the pond, as well as nitrogen levels, because as the pH rises, the toxicity of ammonia increases

due to a shift in the more toxic unionised form (Hargreaves, 1998; EFSA, 2008). Likewise, water with a low pH can have detrimental effects on fish *via* a number of harmful effects such as acidosis of blood, which can lead to poor lung function, suffocation because of mucous precipitation on the gill epithelium and increased disease susceptibility, with juveniles being more susceptible to these effects. A low pH also slows down the rate of organic matter decomposition (Phang, 1991; EFSA, 2008).

2.4 Feeding practices

Feed is an important area of fish farming as it is the main contributor, alongside water quality, to optimal fish health, growth and development (FEAP, 2015). Feed can cost up to 50% of the total operational costs with fish feed protein constituting the highest cost (Hargreaves, 1998; Neori *et al.*, 2004). Fish feeds are the main source of contributing nitrogen to culture water (Hargreaves, 1998), which if accumulated can lead to toxicity and so alternative non-toxic, sustainable feeding practices are required. To maintain a steady state of water quality, increased feeding times with smaller feed portions and 24-hour lighting is an effective management technique (Summerfelt *et al*., 2009).

The utilisation of algae (phytoplankton and cyanobacteria) as a natural food source, which dates back to the 1970s, has the potential to save on feeding costs (Phang, 1991). Additionally, due to the depletion of global fisheries, which contributes to fish meal (FM), using FM as the prime component of fish feeds is not sustainable (Crab *et al.*, 2007). The phytoplankton species composition of aquaculture ponds determines the different impacts that phytoplankton will have on the aquaculture process, with different phytoplankton species varying in terms of digestibility and nutritional values (Demirbas and Demirbas, 2010). Fish fed on diets consisting of a variety of phytoplankton species with several nutritional attributes rather than just one phytoplankton species is usually better in terms of nutritional balance (Muller-Feuga, 2004). According to Demirbas and Demirbas (2010) algae have great nutritional value in terms of fish food, with high levels of carbohydrates, lipids and proteins (**Table**

2.1). Different phytoplankton species contain different levels of important vitamins such as isoleucine, leucine, aspartic acid, glutamic acid, alanine and valine to name just a few (Phang, 1991).

Cyanobacteria (blue-green algae) have an abundance of high-quality protein, vitamins and essential fatty acids (FA) and also tend to be more digestible e.g. *Microcystis* sp. due to the thin cell wall (Muller-Feuga, 2004). This is an important requirement in terms of high digestibility as chime tends to stay in the fish gut for only a short period of time (Crab *et al.*, 2007). However, cyanobacteria tend to be less tolerable to highly organic conditions than chlorophyta phytoplankton groups (Phang, 1991). Fish also require the inclusion of essential long-chain polyunsaturated fatty acids (PUFAs) and long-chain highly unsaturated fatty acids (HUFAs) in their diet (Muller-Feuga, 2000; FAO, 2016). Certain algae types can act as a substitute for PUFAs (Muller-Feuga, 2000). A deficiency of such fatty acids in the fish diet can lead to negative impacts on fertilisation, hatching rates and fecundity (Muller-Feuga, 2004). It is suggested that light intensity is inversely related to the cellular content of PUFAs in many groups of microalgae (Hu, 2004), highlighting the importance of light exposure during algal growth.

Table 2.1 Chemical composition of algae on the basis of percentage dry matter (Dermirbas & Dermirbas, 2010).

Species of sample	Proteins	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50–56	10–17	12–14	3–6
Scenedesmus quadricauda	47	–	1.9	–
Scenedesmus dimorphus	8–18	21–52	16–40	–
Chlamydomonas rheinhardii	48	17	21	–
Chlorella vulgaris	51–58	12–17	14–22	4–5
Chlorella pyrenoidosa	57	26	2	–
Spirogyra sp.	6–20	33–64	11–21	–
Dunaliella bioculata	49	4	8	–
Dunaliella salina	57	32	6	–
Euglena gracilis	39–61	14–18	14–20	–
Prymnesium parvum	28–45	25–33	22–38	1–2
Tetraselmis maculata	52	15	3	–
Porphyridium cruentum	28–39	40–57	9–14	–
Spirulina platensis	46–63	8–14	4–9	2–5
Spirulina maxima	60–71	13–16	6–7	3–4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrica	43–56	25–30	4–7	–

A study carried out by Nakagawa *et al.* (1987) demonstrated that the addition of as little as 10% of the green algae *Ulva pertusa* resulted in improvements in terms of lipid metabolism and fatty acid composition compared to the control group. Another study carried out by Mustafa *et al.* (1995) demonstrated that supplementation of the red sea bream diet with only 5% of different algal species such as *Porphyra yezoensis*, significantly improved growth, feed efficiency, liver glycogen and metabolism of proteins and lipids, indicating the efficiency of utilising algae in the fish diets. The increased fish production is thought to be due to the greater amount of natural food sources available in the form of algae (Phang, 1991).

The feed additives astaxanthin and canthaxanthin, which provide the pink colouration in the fish flesh to make the fish more appealing to consumers, cost US$3,000 per kilogram (Muller-Feuga, 2000). Phytoplankton are a natural source of this colour enhancement for a variety of fish species, due to the presence of pigments such as chlorophylls and carotenoids, specifically β-carotene, a precursor of Vitamin A (Phang, 1991). This biological source of pigmentation is present in the freshwater chlorophycea *Haematococcus pluvialis* at a rate of 4% and increases the marketability of the product for the producer (Muller-Feuga, 2004). As previously mentioned, temperature greatly affects the cellular composition of phytoplankton cells, and this too occurs in terms of astaxanthin content, which can increase threefold when the temperature rises from 20°C to 30°C.

A portion of capture fisheries is used as a fish feed source in the form of FM and fish oil (FO) to ensure the provision of essential nutrients to fish stock. However, the use of a portion of fish supplies as a feeding resource is questionable in terms of sustainability and best fishing practices. A change in this process regarding the replacement of FM and FO to avoid overfishing is underway by research groups such as PEPPA (Perspectives of Plant Protein usages in Aquaculture) established in 2000 and RAFOA (Researching Alternatives to Fish Oils in Aquaculture) established in 2001. These projects were initiated to investigate the use of plant protein and oil as sources of essential amino acids and fatty acids. Another EU project called ARRAINA aims to reduce the use of FM and FO, whilst also taking life cycle analysis (LCA) into account, a major aim of the overall MOREFISH project. Algal-based feed also has the potential

to replace fish feed protein in the salmon diet, as it can increase the weight of salmon by three times after only three months of feeding (FEAP, 2015).

Freshwater Pathogens in Ireland

A diverse range of viruses, bacteria, fungi and protozoa infect freshwater fish and cause disease outbreaks. Many diseases can be treated; however, some diseases have huge proliferation and dispersal potential, leading to the deaths of large numbers of animals and fish such as rainbow trout and perch. Parasites can enter water supplies in many ways such as wastewater discharges, agricultural runoff and leaking septic tanks (Garvey & Rowan, 2011). In most cases, pathogens can only be identified *via* the use of a microscope or the use of fluorescent *in situ* hybridization (FISH) probes (Bruno *et al*., 2006). It is essential to identify the pathogen, if possible, before treatment with certain antibiotics or chemicals, as repeated exposure to the wrong treatment can be detrimental.

In comparison to mammalian skin, the fish epidermis is composed of living cell layers and lacks the lipid bilayer matrix (*Stratum corneum)* that borders keratinised cells (Cobo *et al*., 2014). Additionally, the gill epithelium consists of a single cell layer which is the major contributor to osmoregulation and gaseous exchange and so the single cell layer epithelium is in direct contact with the blood; thus making the gills very susceptible to invasion by pathogens (Cobo *et al.,* 2014). The defence mechanism against pathogens in rainbow trout relies substantially on the skin and skin mucous (Madetoja *et al*., 2000). Therefore, the infection process of the pathogen can be increased with mechanical injuries on fish skin or mucous abrasions, as it provides a convenient entry point for the pathogen (Madetoja *et al*., 2000).

Common freshwater diseases in Ireland affecting species such as rainbow trout and salmon include viral diseases e.g. infectious pancreatic necrosis (IPN) and pancreas disease; bacterial diseases e.g. rainbow trout fry syndrome (RTFS), enteric redmouth disease (ERD) and furunculosis; parasitic diseases e.g. amoebic gill disease (AGD) and Ich/white spot disease; and fungal diseases such as saprolegniosis (Rodger, 2010).

Ichthyophthirius multifiliis is a ciliated protozoan that is the causative agent of white spot disease or 'Ich' in freshwater fish (Rodger, 2010). This holotrichous parasite is one of the most frequently occurring pathogens of freshwater fish of which the main targets are the gills and skin (Zheng *et al.*, 2014). White spot disease, found in varying regions of the world from the tropics to the Arctic, can infect fish for human consumption as well as ornamental fish (Shinn *et al.,* 2001). The life cycle of Ich is temperature dependent, for example, at 15°C the parasite takes 12 to 15 days to mature, whereas it will mature within three to six days when the water temperature is at 25°C (Shinn *et al.*, 2001; Sudová *et al.*, 2010). Direct sunshine can increase the outbreak of the Ich infection due to increased temperature in recirculating systems (Jørgensen *et al.*, 2009).

The remainder of this section focuses on two diseases in detail, namely, furunculosis and RTFS as these diseases tend to pose problems for the collaborating fish farms involved in the MOREFISH project.

2.5 Furunculosis

A major disease of farmed salmonids, called furunculosis, is caused by the gram-negative bacterium *Aeromonas salmonicida* (McCarthy, 1975). *A. salmonicida* is one of the oldest known fish pathogens worldwide (Morgan *et al.*, 1993; Austin, 1997; Hidalgo and Figueras, 2012). Symptoms of the disease present as furuncles or boils on fish flanks (**Figure 2.6 (A)**). Other early signs include lethargy, loss of appetite and hyperpigmentation of the skin. Once the infection has developed, it can result in small haemorrhagic lesions, anaemia, septicaemia and swelling of the eyes, known as exophthalmia. The gills, fins, muscles and internal organs may also be affected by haemorrhages (Hidalgo and Figueras, 2012). The main targets of the pathogen are understood as being the gills, anus, mouth and passages where any mechanical damage to the epithelial surface may have occurred, weakening the protective barrier (Austin, 1997). The internal necrosis caused by this pathogen is shown in **Figure 2.6 (B)**. *A. salmonicida* has a diverse range of hosts that includes fish other than salmonids (Morgan *et al.*, 1993; Hidalgo and Figueras, 2012). These bacterial

species have the ability to survive various temperatures and water conditions, including brackish, fresh and seawater. However, the pathogenicity of the bacteria seems to be reduced greatly once it leaves its host and enters the aquatic environment (Austin, 1997).

A. salmonicida infectivity studies carried out on rainbow trout by Ellis *et al.* (1981) suggest that the pathogenic effects of this pathogen are due to the release of toxins and aggressins by the bacteria *in vivo*. These proteins can, however, be neutralized by serum factors, specifically α-globulin, from normal healthy fish. If these serum factors are low or if the immune system of the fish is impaired the bacteria will prevail and cause furunculosis (Ellis *et al.,* 1981). Agglutinating bacterial strains of *A. salmonicida* are pathogenic to the host fish, whereas the non-agglutinating strains are non-pathogenic as discovered by Sakai (1986). This may be due to the net negative charges attributed to the living cells of the virulent strains in contrast to the net positive charges on the non-agglutinating cells (Sakai, 1986).

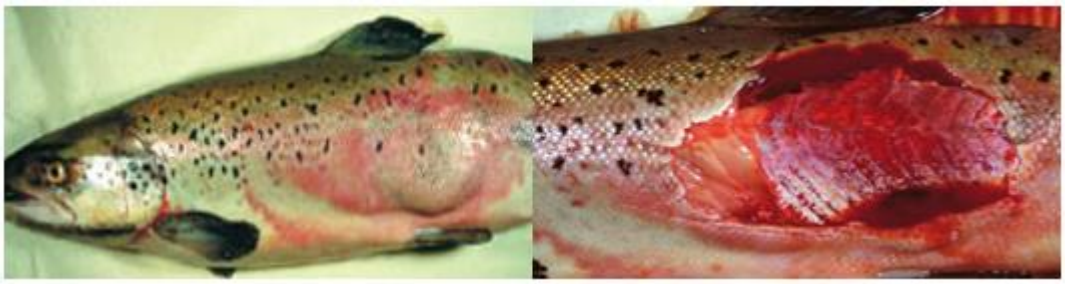

Figure 2.6 (A) Furunculosis disease on salmon; **(B)** Furunculosis disease on Atlantic salmon showing the underlying necrotic tissue following the removal of the furuncle (AGDAFF, 2008).

While early detection of furunculosis is critical, this can be difficult as some clinical signs may go unnoticed, yet carrier fish still have the ability to shed thousands of bacterial cells (Hidalgo & Figueras, 2012). There is also the chance that *A. salmonicida* bacteria may be in the viable but non-culturable state (VBNC), where they are still capable of causing disease but undetectable by normal diagnostic and culturing methods (Morgan *et al.*, 1993). Methods of controlling this disease include the use of probiotics, vaccines, chemicals, altered fish diets and effective husbandry practices. One product, an iron-regulated outer membrane protein-based vaccine (IROMP) was developed for use against furunculosis, which was the probable basis for a reduction in the disease outbreak in the UK in 1992 (Austin, 1997).

Currently, the four subspecies of *A. salmonicida* that affect fish in the aquaculture process, as well as the wild, are *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp*. achromogens*, *A. salmonicida* subsp. *smithia* and *A. salmonicida* subsp. *masoucida* (Fernández-Álvarez *et al.*, 2016). Typical furunculosis disease is caused by *A. salmonicida* subsp. *salmonicida*, whereas the atypical form of the disease is due to the other subspecies (Miyata *et al.*, 1996; Austin, 1997; Fernández-Álvarez *et al.*, 2016).

2.6 Rainbow trout fry syndrome

Flavobacterium psychrophilum, previously known as *Cytophaga psychrophila* and *Flexibacter psychrophilus*, mainly affects salmonid fish in freshwater farms (**Figure 2.7 (A)**) as the causative agent of two diseases namely bacterial cold water disease (BCWD) and RTFS (Madetoja *et al.,* 2000). Rainbow trout fry syndrome is commonly associated with fry fish and internal pathological symptoms, whereas BCWD is the disease name given to table size fish infected with this pathogen presenting external symptoms (Lorenzen and Olesen, 1997). *F. psychrophilum* is a gram-negative, yellow-pigmented bacterium in the form of long slender rods; younger cells being longer and thinner (Lorenzen and Olesen, 1997; Rodger, 2010).

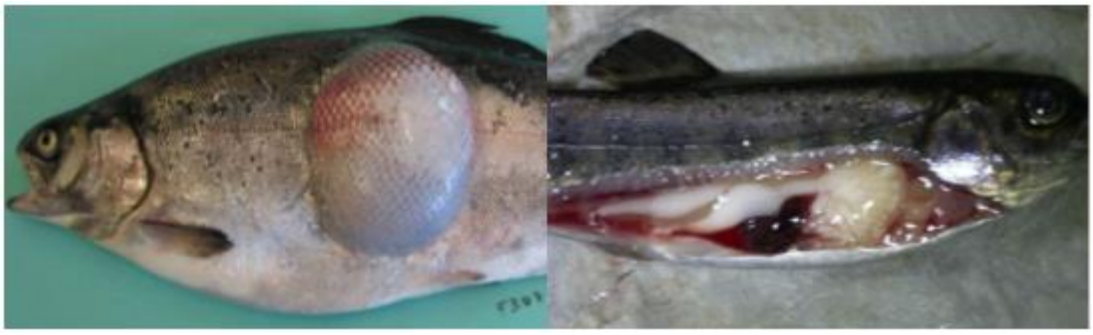

Figure 2.7 (A) Rainbow trout with *F. psychrophilium* infection (Johansen, 2013); **(B)** Fish necropsy on rainbow trout displaying the swollen spleen typical in RTFS (Roger, 2010).

Many symptoms have been associated with this disease such as anorexia, lethargy, pale gills, exophthalmos and trailing mucoid casts as described by Branson (1998). Other recognised symptoms include darkened skin, ascites, skin ulcerations, exophthalmos, high mortalities, pathological necrosis of red pulp in the spleen,

swelling of the spleen (**Figure 2.7 (B)**) and epidermal hyperplasia (Rodger, 2010). Fish also tend to acquire corkscrew swimming or hang at the surface of the water when infected (Branson, 1998). Lorenzen and Olesen (1997) proposed that the ability of the pathogen to partially lyse and agglutinate host erythrocytes might contribute to the marked anaemia that develops in rainbow trout fry following infection.

F. psychrophilum can originate as emitted cells from infected fish (alive or dead) or as growing and dividing cells present in the water, possibly attached to plankton particles, surviving on environmental nutrients (Madetoja *et al.*, 2000). There are many incidents of RTFS in farmed trout in Ireland, which can be difficult to deal with due to robustness and the ability of the pathogen to be transmitted vertically and horizontally (Rodger, 2010). Madetoja *et al*. (2000) explain that vertical transmission occurs when the offspring acquire the disease from the infected parent, whereas horizontal transmission involves the transferal of the disease from infected fish to non-infected fish *via* pathogen encounter. Vertical transmission is a significant means of infection in young fish as it is transmitted from infected broodstock where it has been located in ovarian fluid (Branson, 1998).

Oxytetracycline is one of the antibiotics used against *F. psychrophilum*, however, it has become less effective due to increasing antimicrobial resistance and now treatment includes the use of amoxicillin, and florfenicol in Ireland (Branson, 1998). The latter has been found to be a very effective means of disease control with administration orally in a dose of 10 mg/kg body weight/day for 10 days (Branson, 1998).

With the many freshwater diseases that have the potential to cause immense disease outbreaks, huge mortalities and in return huge economic losses for fish farmers, adequate management and control of a fish farm must be implemented in all areas. It is important to utilise the correct measures to prevent diseases before they arise i.e. sufficient water treatment. If the disease cannot be prevented it is important to use a treatment method, such as a chemical disinfectant or an antibiotic that is specific to that disease. The next section includes a review of the preventative and treatment measures for freshwater finfish pathogens in Ireland.

Water Treatment

Many factors can contribute to the contamination of water sources such as sewage from livestock, which results in the accumulation of nitrates, ammonia and numerous water pathogens (Wang *et al.,* 2005). In order to maintain fish stock in a manner which is suitable for human consumption, diseases need to be eradicated to avoid accumulation into the human food chain. There are many methods which are currently used to keep the pathogenic load under control. Most of these methods are chemical, however, disinfection technologies such as UV light may also be used. There are three general methods of water treatment in aquaculture: physical (sedimentation or mechanical filtration), chemical (disinfectants or ozonation) and biological (biofilters). One of the main objectives of this project is the rearing water dynamic and natural disinfection. With the influence of the natural ecosystem dynamic already discussed in **Section 2.3**, the basis of this section on water treatment is focused on chemical and non-chemical disinfection measures, with a brief mention of biological filtration.

2.7 Biological filtration

Every component of culture water in a fish farm requires monitoring so that disease outbreaks, oxygen depletion and ammonia levels are managed to ensure production is maximised and profitable. Hopkins *et al.* (1995) state that nitrifying bacteria and phytoplankton are almost certainly the primary mechanisms of ammonia recycling in fish ponds. However, aside from the natural nitrification process that exists in aquaculture ponds, depicted in **Figure 2.5**, in some cases more efficient methods of water treatment are required to purify the water, especially if the water is recirculated as in RAS systems.

The biological nitrification process is the most important water treatment process (Crab *et al.,* 2007). The general biological filtration principle involves the fixation of a film of bacteria on an active or inert support such as sand, beads, plastic sheets or fluidised filters i.e. moving bed bioreactors, through which the water is passed

(Helfrich and Libey, 1990; Pedersen *et al.*, 2015). The culture water contains two main pollutants that need to be removed especially in the case of a RAS system, namely undigested fish feeds and excreted fish biomass (Helfrich and Libey, 1990). The 'polluted' water is introduced into the system and the fixed bacterial biomass can oxidise nitrogen ammonia to less toxic nitrite and nitrate forms (Poquillon and Petit, 1989; Pedersen *et al.*, 2015).

2.8 Chemical disinfection

Many chemicals are used in the aquaculture industry to reduce fish pathogens. The chemicals used in various farms depends on licensing in that country which determines the chemicals permitted for use. Common treatments include bronopol (Pyceze™), chloramine-T, hydrogen peroxide, sodium chloride, copper sulphate, formaldehyde (Formalin™), sodium percarbonate (SPC) and iodine (Marine Institute, 2017). Malachite green was the traditional treatment for controlling infections, however, it has been banned for use since 1983 on food fish by the Food and Drug Association (FDA) due to its carcinogenicity (Shinn *et al.*, 2012). A common practice with chemical disinfection involves the treatment of eggs or fish in baths separate to the normal culture tanks, ponds or raceways, where the concentration and duration of exposure are the two critical parameters. The fish are treated with the recommended dose of the selected chemical depending on the disease. The duration for which the bath occurs can be a dip, short bath (1 hour) or continuous/prolonged (indefinite). The concentration of the chemical differs for each treatment, becoming less concentrated the longer the bath duration (Francis-Floyd, 1996). For example, in the case of formalin, the concentrations recommended for a dip, short-term bath and prolonged bath are 500 ppm, 250 ppm (30-60 minutes) and 15-25 ppm, respectively (Francis-Floyd, 1996). Following treatment, it is essential that a withdrawal period is adhered to, i.e. "the period of time between the last administration or exposure of fish to a veterinary drug and point of harvest to ensure that the concentration of the veterinary drug in their edible flesh complies with the maximum permitted residue limits for human consumption" (ASEAN, 2013).

Egg disinfection is of utmost importance in terms of prevention of diseases in fry and fingerlings (Wagner *et al.,* 2008). Iodine is commonly used for egg disinfection at concentrations between 100 to 500 ppm (Schmidt *et al.*, 2006). Formalin is also effective at reducing bacteria and fungi present on eggs, however, it fails to completely eliminate external bacteria (Wagner *et al.,* 2008). The World Health Organisation (WHO) classified formaldehyde/formalin as "carcinogenic to humans" in 2004, and so new methods of parasitic control and prevention are urgently required (Picón-Camacho *et al.,* 2012).

Hydrogen peroxide is used for treatment of external fungal, parasitic and bacterial infections of fish species worldwide, including freshwater fish (Schmidt *et al.*, 2006). The advantage of using hydrogen peroxide is the degradation by-products of the chemical as it dissociates into water and oxygen, posing minimal environmental toxicity risks (Heinecke and Buchmann, 2009). Wagner *et al.* (2008) found that concentrations of hydrogen peroxide at 0.2% are effective at significantly reducing the bacterial load on eggs, however, at concentrations of 2.0% the mortality of eggs increases. The chemical disinfectant formalin is commonly used to treat furunculosis disease, however, repeated and even short-term treatment with formalin can alter the fish epithelium resulting in injuries (Jørgensen *et al.,* 2009). Copper sulphate, chloramine-T, formalin and hydrogen peroxide are commonly used bath treatments for controlling RTFS (Henriksen *et al.,* 2013). Malachite green was the traditional treatment for controlling the parasitic infection Ich before it was banned (Shinn *et al.,* 2012). Sudova *et al*. (2010) investigated peracetic acid (PAA) as a treatment for Ich as it has broad antimicrobial properties and found that a 4-day continuous exposure treatment plan was not sufficient to eradicate all of the life stages of Ich.

Copper sulphate is mainly used for treating phytoplankton and external parasites (Klinger and Floyd, 1998). However, the algicidal activity of this chemical can cause oxygen depletions in water, especially in warmer months, when the temperature rises above the optimal 15°C and is therefore toxic to fish. Having the correct disinfection concentration is essential and is based upon the alkalinity of the water.

The greater the alkalinity of the water the higher the concentration of copper sulphate needed (Klinger and Floyd, 1998).

As evident from the literature, there are several chemicals used in the aquaculture industry for disinfection and treatment purposes. Two main chemicals were focused on for this project namely, bronopol and chloramine-T, both of which are discussed in **Sections 2.8.1** and **2.8.2**, respectively. These two chemicals were selected for investigation as they are currently used for disinfection and treatment in the collaborating fish farms on the MOREFISH project.

2.8.1 Bronopol (Pyceze™)

Bronopol (2-bromo-2-nitropropane-1, 3-diol), is a microbial disinfectant, which goes under the trade name of Pyceze™ and is considered as the chemical disinfectant replacement of malachite green (Cawley, 1999; Shinn *et al.*, 2012). It is a dehydrogenase enzyme inhibitor and works by inhibiting membranous thiol enzymes causing membrane leakage and damage to microbes such as *E. coli* (Madsen *et al.*, 2001; Noga, 2010; Picón-Camacho *et al.*, 2012). Bronopol, the structure of which is illustrated in **Figure 2.8**, is considered as a broad spectrum disinfectant for use in both the freshwater and marine aquaculture industry for both antibacterial and antifungal treatment (Saito and Onoda, 1974; Treasurer *et al.*, 2005; Picón-Camacho *et al.*, 2012).

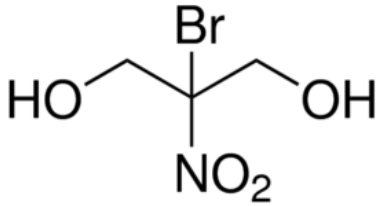

Figure 2.8 Structure of Bronopol .

The antibacterial properties extend to the treatment of both gram-negative bacteria e.g. *Pseudomonas aeruginosa*, *E. coli*, *Salmonella typhosa*; and gram-positive bacteria e.g. *Staphylococcus aureus*, *Bacillus subtilis*, and opportunistic pathogenic yeast e.g. *Candida albicans* (Cawley, 1999; Birkbeck *et al.*, 2006).

Continuous/prolonged (30 days) in-bath applications of bronopol to rainbow trout at low doses of 1, 2, 5 and 10 ppm carried out by Shinn *et al.* (2012) demonstrated no histopathological gill toxicity and as bronopol does not accumulate in fish tissues, no withdrawal period is required (Noga, 2010). It degrades rapidly in the environment; this degradation can be enhanced following exposure to high-intensity UV light (Noga, 2010; Picón-Camacho *et al.*, 2012; Kapupara *et al.*, 2016). The degradation of bronopol in natural waters (**Figure 2.9**), as investigated by Cui *et al*. (2011) demonstrated that the breakdown products caused higher toxicity to aquatic animals than the parent compound. The major degradation products, namely bromonitromethane (BNM) and 2-bromo-2-nitroethanol (BNE), persisted in the environment despite complete and rapid degradation of bronopol itself(Wang *et al.*, 2002; Cui *et al.*, 2011). It is, therefore, important to assess this characteristic upon exposure of bronopol to PUV light, which is a major investigation of this project.

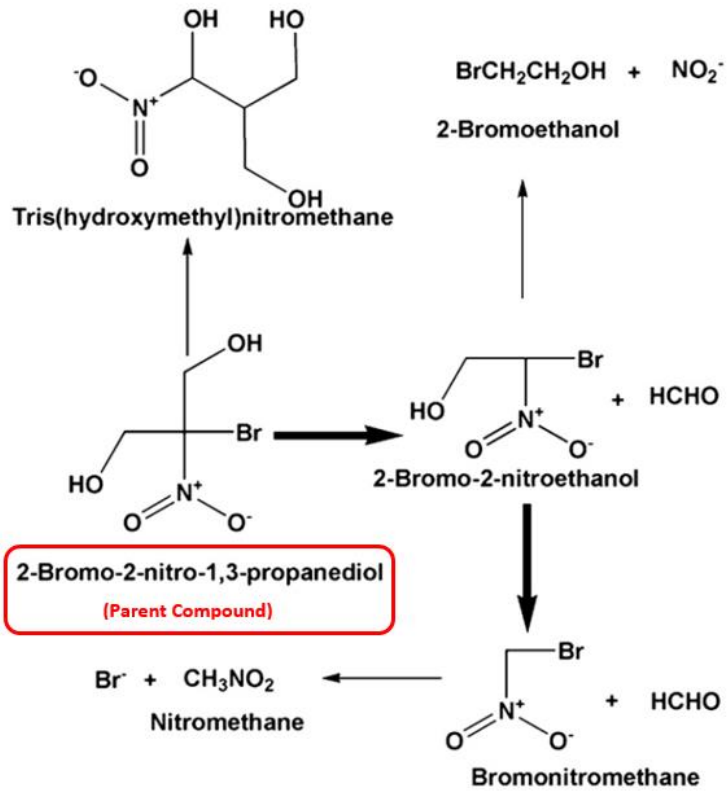

Figure 2.9 The proposed degradation pathway for bronopol in natural waters (Cui et al., 2011).

Bronopol is authorised by the EU for use as a fungal treatment and control measure for infected fertilised salmonid eggs, mainly against *Saprolegnia* sp. (Treasurer *et al.*,

2005; Oono *et al.*, 2007), and is also safe for use on salmonid fish, namely Atlantic salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss* (Cawley, 1999). Picón-Camacho *et al*. (2011) discovered that a dose of 2 ppm reduced the number of trophonts present in the gills of rainbow trout infected with white spot disease by up to 40% compared to the control after 10 days of administration. Oono *et al*. (2007) found that a 30-minute treatment of 100 ppm of bronopol was effective against fungal infection in rainbow trout eggs. In terms of aquatic organisms, bronopol causes toxicity at concentrations lower than 1 ppm for phytoplankton and crustaceans (Madsen *et al*., 2001), and this should be considered when administering doses for treatment of diseases, as natural food sources may be affected.

2.8.2 Chloramine-T

Chloramine-T (sodium *N*-chloro 4-methylbenzenesulfonamide trihydrate) is an organic, chlorine releasing biocide, **Figure 2.10**, commonly referred to as its trade name Halamid® (Schmidt *et al.*, 2007; Noga, 2010). It is widely used in the aquaculture industry as a bath treatment for bacterial diseases such as BGD and white spot disease (Noga, 2010; Verner-Jeffreys and Taylor, 2015). It is also effective at treating many types of viruses, water moulds and parasites (Noga, 2010). Bath treatments are typically applied at concentrations of 4 to 25 parts per trillion (ppt) for a duration of 30 to 60 minutes (Verner-Jeffreys and Taylor, 2015). The mode of action of chloramine-T for eradicating microbes is through oxidation and/or irreversible binding to microbial structures (Noga, 2010).

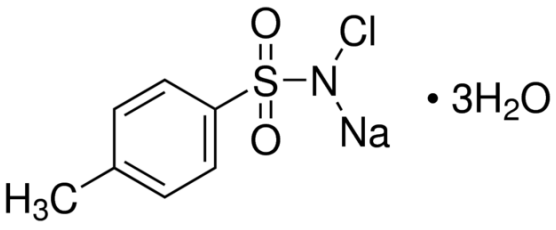

Figure 2.10 Structure of chloramine-T (Sigma Aldrich, 2016b).

Once administered in an aquaculture site, there are many fates of chloramine-T, illustrated in **Figure 2.11**. Once chloramine-T reacts with oxidizable materials

including amino acids, humic substances and inorganic or organic material, the degradation process of the chemical initiates (Verner-Jeffreys and Taylor, 2015). The chloramine-T anion is firstly converted to hypochlorite ion (OCl^-) and then to hypochlorous acid ($HOCl^-$) upon being dissolved in water; $HOCl^-$ is a strong disinfectant (Noga, 2010). The relatively stable para-toluenesulfonamide (p-TSA) is produced during the process, as the primary degradation product of chloramine-T.

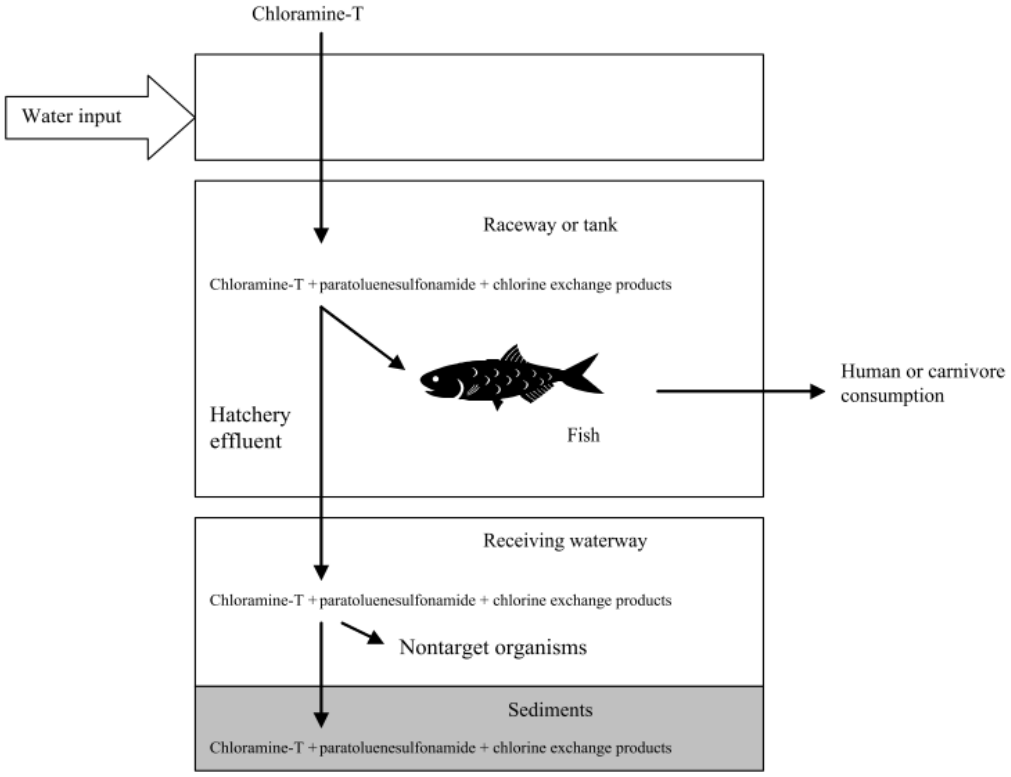

Figure 2.11 Conceptual model of the fate of chloramine-T used in intensive aquaculture (Schmidt *et al*., 2007).

It is important to administer the correct dose of chloramine-T, as outlined in **Table 2.2**, as overdosing with this chemical can damage fish gill epithelia, causing intercellular oedema and respiratory distress (Noga, 2010).

Table 2.2 Concentration of chloramine-T to use at various pH and hardness levels of water (Noga, 2010).

pH	Dose (mg/L)	
	Soft water	Hard water
6.0	2.5	7.0
6.5	5.0	10.0
7.0	10.0	15.0
7.5	18.0	18.0
8.0	20.0	20.0

2.8.3 Impact of chemical disinfection

Although chemicals have proved efficient in many cases as a disinfection treatment for finfish pathogens (Shepherd *et al.*, 1988; Yisa *et al.*, 2014), there are other effects which lead to the questionability of the usage of chemicals on fish farms. Use of biocidal chemicals can pose an occupational hazard for users and can also lead to a large volume of waste that requires treatment to avoid adverse impacts on the environment (McDonald *et al.*, 2000). The potential for carcinogenic by-products formed with the use of particular chemicals, such as formalin, is of toxicological concern if chemicals are released into the receiving water body, be it a lake or a stream, where both short-term and long-term effects may be exerted on resident biota (Schmidt *et al.*, 2006).

There are also disadvantages associated with their usage in terms of the impacts on fish. In the case of parasites, controlling the external stages requires multiple bath treatments, which can be stressful to fish and is quite costly (Shinn *et al.*, 2001). According to Madetoja *et al.* (2000), the use of chemicals can increase the potential for diseases such as RTFS caused by *F. psychrophilum*. For example, repeated use of formalin baths can cause thinning of the epidermis promoting invasion of pathogens such as *F. psychrophilum* (Madetoja *et al.*, 2000). A study carried out by Buchmann *et al.* (2004) showed that exposure to formalin at 50 ppm initiated plasma membrane blebbing (swelling/bulging) in rainbow trout after 1 hour. At concentrations higher than 50 ppm over a period of 24 hours, damage and disorganisation of cell layers and plasma membranes was evident. Buchmann *et al.* (2004) also found that high concentrations and long exposure of the rainbow trout epithelium to formalin caused a decrease in mucous cell density, compromising an integral support component of the epidermis. The effects of this may be lethal for fish as it presents easy invasion access from opportunistic pathogens.

Another disadvantage associated with chemical disinfection methods is the ability of some microorganisms to develop resistance to the effects of a specific chemical. Many microorganisms have developed such resistance, for example,

Cryptosporidium and *Giardia* oocysts have developed resistance to chlorination, which prevents them from being eliminated (Garvey and Rowan, 2011).

2.9 Ultraviolet light disinfection

The need for alternatives to chemical treatments for the elimination of pathogenic microorganisms arises due to the many disadvantages associated with chemical disinfection, including microbial resistance to the chemicals (Pedersen *et al.,* 2010), increased disease susceptibility (Heinecke and Buchmann, 2009) and lack of efficiency to properly eliminate pathogens (Wagner *et al.*, 2008). UV light treatment is one such alternative antimicrobial technology (Sharifi-Yazdi and Darghahi, 2006). UV lamps have many applications in sterilisation ranging from use in clean rooms to hospitals to food processing areas (Bank *et al.,* 1990). UV lamps come in the form of low-pressure UV (LP-UV) or medium pressure UV (MP-UV) mercury-based bulb systems and are commonly referred to as CW UV lamps or static UV lamps. Although the LP-UV lamps are more electrically efficient, the MP-UV lamps emit a much higher light intensity and so, to achieve disinfection, shorter irradiation times are required (Wang *et al*., 2005).

UV light treatment, based on the UV region of the electromagnetic spectrum consists of three segments of UV light, namely, UVA, UVB and UVC (**Figure 2.12**). Wavelengths ranges for these three segments are 315 to 400 nm for UVA, 280 to 315 nm for UVB and 200 to 280 nm for UVC. UVC light is considered the most important wavelength for inactivation of microorganisms due to their high absorbance of light at 254 nm and is therefore known as germicidal wavelength (Walker *et al.*, 2013; Zhang *et al.*, 2015).

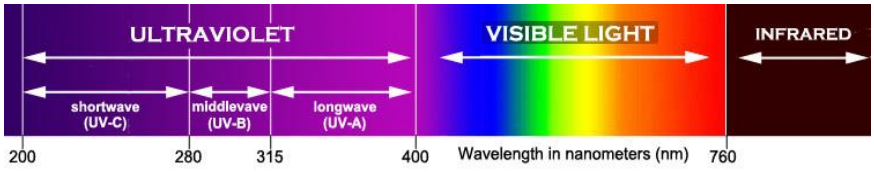

Figure 2.12 The ultraviolet light spectrum (United Nuclear Scientific, 2016).

Exposure to damaging doses of UV light inhibits the normal replication mechanism of the genome and so the microorganisms become inactivated (Zhang *et al.,* 2015). Photochemical damage arises when the biomolecular chemical bonds have the same energies as the photons irradiated (Xenon Corporation, 2006b). The photons are absorbed by deoxyribose nucleic acid (DNA) molecules and inactivation of microorganisms is mainly caused due to the formation of DNA mutations (Sharifi-Yazdi & Darghahi, 2006).

Upon exposure of microorganisms to the germicidal UVC light, the primary damage induced involves biomolecular damage to DNA and ribose nucleic acid (RNA). Irreversible nucleic acid damage occurs when carbon, nitrogen, oxygen and hydrogen bonds dissociate once UV photons are absorbed (Xenon Corporation, 2006b). The damage continues with the formation of cross-links between pyrimidine bases, thymine and cytosine, on the same strand due to absorption of the UV light in microbial DNA, blocking transcription and so replication (Wang *et al.,* 2005). This leads to the development of mutagenic lesions due to covalent bond formation between pyrimidine bases on the same strand of DNA. Pyrimidine-pyrimidone 6-4 photoproducts (6-4PP) and cyclobutane pyrimidine dimers are the two main types of mutations formed when microbes are subjected to UVC, which prevents DNA transcription (Maclean *et al.*, 2008). The most common type of dimer formed is a thymine-to-thymine dimer on the same strand, which gives rise to a cyclobutyl ring, inhibiting DNA replication (Garvey and Rowan, 2011). The end result of this photoinactivation property for microorganisms is mortality (Wang *et al.,* 2005).

Depending on the pathogen being eradicated, the required dose of UV radiation will vary, but the most common dose for viruses and bacteria is 30 mJ/cm^2 at 254 nm. Many studies have proved the efficacy of conventional UV light as a disinfection mechanism of pathogens. Zhang *et al.* (2015) demonstrated that UV light effectively reduced the culturability of *E. coli* from $2.0x10^9$ colony forming units (CFU)/ml to $1.0x10^7$ CFU/ml and *P. aeruginosa* from $1.5x10^9$ CFU/ml to $3.2x10^4$ CFU/ml at a dose of $50mJ/cm^2$. The inactivation efficiency further increased when the dose of UV light increased to $100mJ/cm^2$ (Zhang *et al*., 2015). Garvey *et al.* (2014) also demonstrated

the effectiveness of CW UV light on spore-forming bacteria. A complete 7 $\log_{10}$ inactivation of *Bacillus cereus* and *Bacillus megaterium* occurred following exposure to LP-UV at a dose of 163.5 mJ/cm^2.

Although successful at eliminating pathogens, UV light has some drawbacks to its application. One such drawback is the induction of a VBNC state in bacteria, during which the bacteria fail to form colonies *via* normal plating methods, yet remain viable and can retain virulence as mentioned in **Section 2.5**. These cells retain the ability to initiate infections once returned to a metabolising state, which can be activated by temperature or nutrient changes (Zhang *et al*., 2015). A study carried out by Zhang *et al.* (2015) reported that *E. coli* and *P. aeruginosa* did not form colonies when cultured on agar plates following treatment with a dose of 300 mJ/cm^2. However, high transcriptional levels of genes involved in pathogenesis were observed after molecular analysis, highlighting how this VBNC state does not necessarily result in the loss of pathogenicity (Zhang *et al*., 2015). UV lights also need an external power source that can be inconvenient for field analysis. UV bulbs need to be replaced and extrinsic safety procedures need to be put in place for the handling of UV light due to the risks high doses of UV and handling mercury can pose to users (Walker *et al*., 2013).

2.9.1 Photoreactivation

Exposure to UV light has great microbial inactivation potential, however, limitations remain due to the photo repair mechanisms of microorganisms and depend on the penetration depth of the UV light (Garvey and Rowan, 2011). These microorganisms can become reactivated when subjected to visible light of wavelengths ranging from 300 to 500 nm; the greater the light intensity the greater the photoreactivation. This photo repair mechanism is enzyme-mediated, with photolyases being the principal catalysts of the reaction. The photolyase enzyme serves to direct any DNA damage which has occurred to split the dimer back to monomers enabling the microbes to replicate once again (Lamont *et al.,* 2004; Maclean *et al*., 2008).

Chapter 3
Rearing Water Profiling and Characterisation in the Irish Freshwater Aquaculture Industry

Introduction

Aquaculture is of growing importance in terms of food sustainability, with ongoing challenges from depletion of world fish stocks and an increasing human population as outlined in **Chapter 2**. It is also a more eco-friendly method of food production, which results in 50% less greenhouse gas emissions compared to meat production processes (Bentzon-Tilia *et al.*, 2016). To try to meet the growing demand, fish tend to be reared at high densities for increased productivity, which can lead to the build-up of inorganic nutrients, excreted waste and feed remnants; all factors that contribute to eutrophication (Bentzon-Tilia *et al.*, 2016). Inorganic phosphorus and nitrogen tend to be the most influential parameters linked with self-pollution of the water (Bentzon-Tilia *et al.*, 2016). When the conditions are optimal, i.e. high nutrient loads, high temperature and sunlight, phytoplankton can grow to problematic levels, forming unwanted blooms in the rearing water (Drikas *et al.*, 2001), which can have detrimental effects on the fish. With high density rearing practices, harmful pathogens also have a greater chance of rapid circulation and persistence, therefore, resulting in the potential of a serious disease outbreak.

Due to these facts, bacteria and phytoplankton are often associated with the infection and ultimately killing of fish. However, they have many other roles to play in the aquaculture setting, as mentioned in **Chapter 2**, **Section 2.3**, such as nutrient recycling, degradation of organic matter, and even the protection against disease (Bentzon-Tilia *et al.*, 2016). Phytoplankton such as *Chlorella* sp. and *Scenedesmus* sp. provide great opportunities for the treatment of wastewater due to their efficiency at nutrient removal and selective antibiotic and heavy metal removal (Nurdogan and Oswald, 1995; Min *et al.*, 2011; Chen *et al.*, 2012; Choi and Lee, 2012; Godos *et al.*, 2012; Delgadillo-Mirquez *et al.*, 2016; Delrue *et al.*, 2016). The same benefits can be applied to the rearing water in an aquaculture setting. A biotic balance must be achieved to ensure that the bacterial and phytoplankton numbers and populations are having this positive influence on productivity, rather than negatively influencing rearing water quality and consequently the production and efficiency of an aquaculture farm. It is therefore of utmost importance that the microbiomes of

aquaculture settings are more fully understood. It is the lack of knowledge regarding microbial interactions, and ecology of these systems, that prevents the utilization of microbial communities in the assessment, improvement and control of aquaculture farms (Bentzon-Tilia *et al.*, 2016). To begin to address this knowledge gap a long-term study (10 months) was carried out on a freshwater fish farm in the West of Ireland. The main aim was to establish a profile of the different bacterial and phytoplankton trends over the duration of the study, which could then be investigated for correlations with any influencing physiochemical conditions of the water from week to week. The succeeding sections in this chapter discuss the important interactions in aquaculture rearing water, the techniques deployed for community profile development in this project, with an insight into alternative approaches and a description of the collaborating aquaculture site. This is followed by the main findings of the study in terms of physicochemical, bacterial and phytoplankton trends on the aquaculture site and the general conclusions from the data gathered.

3.1 Rearing water dynamic

The microbial processes that occur in the rearing water affect many water quality parameters, such as oxygen, carbon dioxide and ammonia content (Moriarty, 1997). A co-dependent relationship exists between phytoplankton and nutrients in rearing water, as phytoplankton abundance depends on nutrient availability and nutrient cycling depends majorly on the presence of phytoplankton (Li *et al.*, 2012). This highlights how important the phytoplankton balance is in rearing water, as not only do the phytoplankton produce oxygen during daylight (Moriarty, 1997), but also recycle metabolites that would otherwise build up in the water. Similarly, bacteria and nutrients have a co-dependent relationship. Heterotrophic bacteria require oxygen for the oxidation of organic matter while releasing carbon dioxide; autotrophic bacteria then use carbon dioxide for the oxidation of ammonium and nitrites (Moriarty, 1997). Muñoz and Guieysse (2006) demonstrated this concept in the cycle depicted in **Figure 3.1**.

Bacteria thrive on organic matter (Baines and Pace, 1991; Amon and Benner, 1996; Blancheton *et al.*, 2013). One of the principal sources of organic matter in the rearing water is primary production by phytoplankton, followed by excreta and feed pellets (Baines and Pace, 1991; Moriarty, 1997). This emphasises another aspect of the relationship that exists between bacteria and phytoplankton in rearing water, aside from the symbiotic association in terms of oxygen and carbon dioxide production.

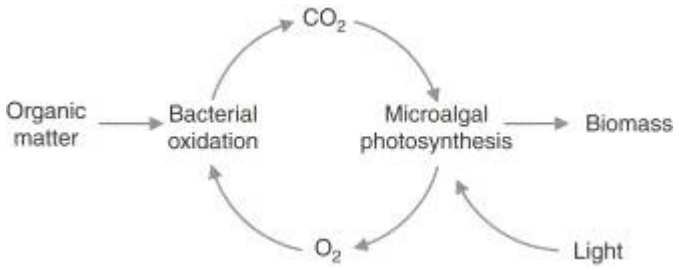

Figure 3.1 Principle of photosynthetic oxygenation in BOD removal processes (Muñoz & Guieysse, 2006).

While it is important to determine the biological composition of the water, it is the physicochemical parameters, such as salinity, pH and oxygen concentration, that tend to frame the biological communities, with compounds such as nitrogen and phosphorus also having a huge impact on the condition of the rearing water (Bentzon-Tilia *et al.*, 2016). According to Moriarty (1997), temperature and readily available organic material are two of the most influential factors in terms of bacterial growth in the pond rearing water. Moriarty (1997) also highlights that the two most important nutrients for phytoplankton productivity, i.e. the main source of the organic material for phytoplankton, are nitrogen and phosphorus. A study carried out by Mostert and Grobbelaar in 1987 demonstrated the importance of both nutrients for the successful production of phytoplankton in mass culture. This highlights how important it is to investigate and determine the link between both the physicochemical and biological water parameters in terms of an early indication system of the rearing water condition so that alterations can be put into place if needs be.

With the increasing rates of antibiotic resistance, more sustainable and preventative measures need to be implemented, such as vaccination procedures, to decrease the use and in some cases misuse of antibiotics (Bentzon-Tilia *et al.*, 2016). The use of

probiotics is another alternative, where certain bacteria such as *Roseobacter* clade produce antimicrobial compounds (Bentzon-Tilia *et al.*, 2016). Nevertheless, for the above measures to be implemented to improve culturing conditions, an understanding of the underlying rearing water ecosystem dynamic is required. A characterised baseline including the phytoplankton, bacterial and physicochemical influences is necessary and is a focus of this project, to ascertain key interactions between all parameters and relevant species. Without this information, the required knowledge to inform prevention measures rather than undergoing treatment processes would be unfeasible. Having an in-depth knowledge of this characterisation would also be the basis for future diagnostic applications such as the design and development of diagnostic molecular kits.

3.2 Techniques for biological community profile development

Many techniques have been used for determining community profiles in a variety of environments. In order to achieve an in-depth characterisation of such environments, a combination of methodologies is required. The approaches involved can range from the utilisation of metabolic properties to microscopy to metagenomics (Øvreås, 2000; Bourne *et al.*, 2004; Riesenfeld *et al.*, 2004; Wegley *et al.*, 2007; Ray *et al.*, 2010). The techniques used in this study included epifluorescent microscopy, flow cytometry (FCM) and DNA fingerprinting using 16S rRNA, which are introduced in this section.

3.2.1 Epifluorescent microscopy

Common methods used for microbial enumeration include agar plating methods, transmission electron microscopy (TEM) and epifluorescent microscopy. The use of agar for bacterial counting has been regularly practised in many fields and continues to be a major method of enumeration (Massa *et al.*, 1998; Borges *et al.*, 2008; Ajayi and Okoh, 2014). However, plate count techniques tend to be selective for particular organisms (Amann *et al.*, 1995), therefore, when analysing unknown mixed populations these methods are less informative and non-selective. Also, less than 1%

of microorganisms will grow *via* the typical plating procedure (Torsvik *et al.*, 1990; Amann *et al.*, 1995; Øvreås, 2000; Riesenfeld *et al.*, 2004; Schloss and Handelsman, 2005). TEM has been recognised as a standard procedure in many studies for bacterial and viral enumeration, as well as phytoplankton enumeration (Bergh *et al.*, 1989; BØrsheim *et al.*, 1990; Proctor and Fuhrman, 1990, 1992; Wommack *et al.*, 1992; Weinbauerl and Suttle, 1997; Nozaki *et al.*, 2014). Often, results obtained *via* TEM and epifluorescence for enumeration have been well correlated. For example, the enumeration of bacteria carried out by BØrsheim *et al.* (1990) *via* the use of TEM showed no difference in estimations when compared to epifluorescence using DAPI (4', 6-diamidino-2-phenylindole) as a stain. However, this method has certain disadvantages; enumeration analysis for microorganisms *via* TEM methods requires expensive equipment and is quite time consuming (Hennes and Suttle, 1995).

To get a real-time measure of the numbers of bacteria present in the culture water a direct count is required. With a direct count, all species present can be determined, and the problems associated with plating methods, in terms of selective growth, are avoided. Epifluorescent microscopy has been used as the standard method for the enumeration of aquatic bacteria since the 1970s (Daley and Hobbie, 1975; Allen-Austin *et al.*, 1984; Morgan *et al.*, 1991; Bloem and Vos, 2004; Lunau *et al.*, 2005). It has proven to be a more reliable method of enumeration than TEM, where even distribution of the bacteria on the filter is achieved (Lunau *et al.*, 2005). Moriarty (1997) stated that epifluorescent microscopy revealed an increased abundance of bacterial numbers compared to traditional methods in the range of 10 to 100,000 times greater. The direct counting method is only one way in which epifluorescence can be used in terms of community profiling analysis. It has also been implemented in the detection of VBNC cells in aquaculture (Morgan *et al.*, 1991). Additionally, Ray *et al.* (2010) used epifluorescence for the quantification of photosynthetic pigments in eukaryotic phytoplankton and cyanobacteria to estimate phytoplankton abundance.

Many stains are used for epifluorescence including DAPI, a double-stranded DNA stain, acridine orange (AO) and Yo-Pro {4-[3-methly-2, 3-dihydro-(benzo-1, 3-oxazole)-2-methlymethlyedene]-1-(3'-trimethlylammoniumpropyl)-

quinoliniumdiiodide}, a cyanine-based nucleic acid stain (Hobbie *et al.*, 1977; Morgan *et al.*, 1991; Hennes and Suttle, 1995; Weinbauerl and Suttle, 1997). Enumeration of microorganisms including viruses and bacteria, are less expensive using these staining methods over TEM methods (Hennes and Suttle, 1995). A study carried out by Hennes and Suttle, in 1995 based on viral and bacterial enumeration of fresh and marine waters, compared the TEM method with the staining method followed by epifluorescent microscopy and found that enumeration with the use of Yo-Pro simplistic and very precise compared to TEM methods. In fact, they found that the TEM method underestimated viral abundance in some natural waters compared to staining with Yo-Pro.

Much consideration is required when choosing the most suitable stain for the application i.e. the sample type being analysed, as field samples contain much more than viable bacterial cells, including dead organic particles, dead cells, phytoplankton, viruses, rotifers, clay *etc*. Therefore, it is important to choose the stain that will provide the most accurate total counts possible, with the least amount of background staining. The type of stain chosen can also depend on the organism for enumeration, as Hennes and Suttle (1995) found that the use of DAPI for enumeration of viruses fluoresced much less than staining with Yo-Pro. According to Bloem and Vos (2004), because DAPI is a double stranded DNA stain, it tends to be more specific in the staining process, resulting in much less non-specific background staining.

The Yo-Pro stain does not interact well when samples are fixed in aldehydes and the binding of the stain to the nucleic acids can be affected (Hennes and Suttle, 1995; Weinbauerl and Suttle, 1997) and therefore samples have to be filtered and stained after collection. It also takes 2 days for the preparation of a slide using the Yo-Pro stain (Weinbauerl and Suttle, 1997) compared to 30 minutes with DAPI or 5 - 10 minutes with SYBR Green. For this reason, it was not suitable to use Yo-Pro for this study as the samples obtained from the fish farm were fixed in formaldehyde and the method is too time consuming. High humic content in a water sample can also cause background fluorescence when using Yo-Pro (Hennes and Suttle, 1995), which would also have been a disadvantage for this study as the samples were sourced from an

earthen pond. Morgan *et al.* (1991) used AO for the staining and successful detection of VBNC *A. salmonicida* cells from freshwater lakes. However, Bloem and Vos, (2004) stated that AO can also result in background fluorescence as the dye binds to other components that may be in the sample such as clay and detritus, therefore, AO was not suitable for use either.

A study carried out by Shibata *et al.* (2006) which compared the use of SYBR Gold to SYBR Green discovered that there was no significant difference between the two stains upon enumeration of bacteria from seawater samples where the ratio was 0.99 ± (mean ± SD). However, upon excitation and thus exposure to the blue illumination, samples stained with SYBR Green decreased to a greater extent than those exposed to SYBR Gold after a time period of 15 minutes. There tends to be very little difference between the results obtained for bacteria in terms of counts between SYBR Gold and SYBR Green (Carreira *et al.*, 2015). However, the other findings suggest that SYBR Gold is a more effective and stable staining method, even upon excitation, offering prolonged endurance of fluorescence.

3.2.2 Flow cytometry

Since the introduction of the Beckman Counter in the 1970s, automated cell counting has overtaken manual cell counting as the preferred method for phytoplankton enumeration when it comes to quick results, minimising human error and improving the statistical significance of results, as stated by Marie *et al.*, (2005). The Beckman Counter is composed of an electric current flowing through two electrodes, which are separated by a small gap, and works on the basis of a signal in the form of a pulse each time a particle passes through the gap (Marie *et al.*, 2005). There are some limitations to this method described by Marie *et al.* (2005); it is not suitable for picoplankton counting, distinguishing phytoplankton from other particles such as debris or bacteria can be difficult, and analysing mixed cultures may be problematic as different phytoplankton species of similar size can overlap. Another automated instrument for phytoplankton counting is the HIAC counter by Pacific Scientific Instruments. This uses a light beam for particle differentiation, however, due to the

fact that it operates on a single cell parameter basis, it also has limitations when it comes to cell differentiation and mixed populations (Marie *et al.*, 2005).

A more advanced technique, FCM, allows for the analysis and measurement of several properties, such as size and fluorescence intensity, of a single cell flowing in a fluid stream passing through a laser light, through which thousands of cells can pass per second (Marie *et al.*, 2005; Ozbay and Jackson, 2010). FCM was selected as the best method for phytoplankton enumeration in this project based on the several advantages it possesses compared to the earlier techniques mentioned. For example, the fact that several parameters can be measured simultaneously for each cell allows for sub-population sorting (Marie *et al.*, 2005; Cunningham and Leftley, 1986). Larger species up to 200 µm can be analysed, as well as the smaller picoplankton at the other end of the cell size spectrum (Marie *et al.*, 2005). It is also more suitable than epifluorescence in the case where some cells may be too dim to be analysed in this way (Marie *et al.*, 2000). FCM involves the passing of a liquid suspension of the sample to be analysed through a narrow stream, upon which a light source, such as a laser, is focused (Marie *et al.*, 2005). The passing of each cell scatters the light differently, depending on the cell size, shape and fluorescence property (Marie *et al.*, 2005), with a typical throughput of 1000 cells/s depending on the parameters in place (Cunningham and Leftley, 1986). The pulses of light generated as each cell passes in front of the laser, known as an event, are detected by photomultipliers or photodiodes, depending on the machine, and are displayed as mono-parametric histograms or bi-parametric cytograms (Marie *et al.*, 2005). The detectors are typically located at 180°, known as forward scatter (FSC), which is mainly related to cell size and shape, and at 90°, known as side scatter (SSC), which is also related to cell size, but is more so based on the cell surface and internal structure (Cunningham and Leftley, 1986; Marie *et al.*, 2005).

Originally developed for analysis of biomedical samples, the flow cytometer has become more frequently used for phytoplankton analysis since the 1980's (Cunningham and Leftley, 1986; Li and Wood, 1988; Olson *et al.*, 1988; Rutten *et al.*, 2005; Tijdens *et al.*, 2008; Graff *et al.*, 2012; Read *et al.*, 2014; Thomas *et al.*, 2018). The applications of FCM for phytoplankton reach far beyond cell enumeration, sizing

and sorting. Currently due to the use of molecular probes, phylogenetic (DNA-related) and physiological information can also be uncovered (Marie *et al.*, 2000). One of the most valuable properties is the utilisation of the pigment content of the cells, which can group the species based on their fluorescing pigments, such as chlorophyll and phycocyanin (PC) (Marie *et al.*, 2005; Rutten *et al.*, 2005). The presence of phycoerythrin (PE) in Cyanobacteria and Cryptophyceae emits a strong orange/red fluorescence, which can be used to differentiate these phytoplankton from other eukaryotic phytoplankton (Marie *et al.*, 2005). The pigment PC can be utilised to represent the presence of cyanobacteria, along with its derivative allophycocyanin (APC) (van Vuuren *et al.*, 2006; Brient *et al.*, 2008; Read *et al.*, 2014; Thomas *et al.*, 2018). This natural property can also be used to differentiate phytoplankton from detritus, suspended sediments and other phytoplankton cells (Cunningham and Leftley, 1986; Marie *et al.*, 2000; Ozbay and Jackson, 2010).

According to Marie *et al.* (2005), fluoresceinisothiocyanate (FITC) is the most commonly used stain for FCM in regards to phytoplankton analysis. In fact, up to three different stains can be used to analyse different features of the cell depending on the fluorochrome absorption and emission spectra (Cunningham and Leftley, 1986), which can generate a significant amount of information regarding the sample. To further explore heterotrophic cells and aspects of the cell cycle, the use of nucleic acid stains can be employed such as DAPI or SYBR™ Green (Marie *et al.*, 2000).

In terms of preservation of phytoplankton, Lugol's iodine can affect the cell shape and fluorescence properties, however, formalin fixation is unstable for long term preservation, also affecting cell shape (Marie *et al.*, 2005). Propidium iodine (PI) is commonly associated with live/dead analysis of cells, however, in the case of phytoplankton, it tends to interfere with chlorophyll and cannot be used for staining phytoplankton (Marie *et al.*, 2005). A preservation method used by Marie *et al.* (2000) involved the addition of 10% paraformaldehyde and 0.5% glutaraldehyde to marine samples in a cryovial, followed by storage in liquid nitrogen until required for further use. Issues that can interfere with staining include inadequate fixative solutions and contaminated solutions, such as buffers (Marie *et al.*, 2000), therefore it is important to consider these facts when preparing for the analysis of samples.

3.2.3 Molecular methodologies

There is great potential in the aquaculture industry for the use of high-throughput sequencing (HTS) technologies for monitoring the performance of an aquaculture system. This involves studying the genomic make-up of microorganisms present in a single water sample (Bentzon-Tilia *et al.*, 2016), which would allow for real-time identification of the microbiome present. However, this approach to characterisation of bacteria, both pathogenic and non-pathogenic, is at very early stages, therefore, resulting in the lack of knowledge that exists concerning ecosystem interactions in rearing water (Bentzon-Tilia *et al.*, 2016). Metagenomic approaches are becoming more widely used to investigate microbial communities in different environments (Venter *et al.*, 2004; Wegley *et al.*, 2007; Dinsdale *et al.*, 2008; Rodriguez-Brito *et al.*, 2010; Durso *et al.*, 2012; Segata *et al.*, 2012; Huang *et al.*, 2016; Lyons *et al.*, 2017). However, this approach can involves many stages, such as shotgun sequencing and the establishment of a DNA library (Riesenfeld *et al.*, 2004; Thomas *et al.*, 2012), which is quite expensive and time-consuming (Knight *et al.*, 2016) and therefore was not suitable for the duration of this study.

Second-generation sequencing based on 16S rRNA genes has been used for identification of microbial species from common environments (Zwart *et al.*, 2002) to extreme environments such as Lake Radok in East Antarctica (Karlov *et al.*, 2016). The homologous and ubiquitous nature of the 16S rRNA gene shared amongst microorganisms allows for the exploitation of the gene as an indicator of microbial diversity (Pace *et al.*, 1986; Muyzer *et al.*, 1993; Ortiz-Estrada *et al.*, 2019). This small subunit has nine variable regions (V1 – V9) comprised of highly conserved areas of DNA among semi-conserved and non-conserved areas, which allows for the analysis of mixed and diverse populations (Pace *et al.*, 1986; Edwards *et al.*, 1989; Schloss and Handelsman, 2003; Ortiz-Estrada *et al.*, 2019). Polymerase chain reaction (PCR), using specific primers for the 16S rRNA sequence allows for the amplification of microorganisms in a sample, even those present at low numbers (Muyzer *et al.*, 1993; Øvreås, 2000). The amplified products can then be used in DNA fingerprinting analysis methods, such as denaturing gradient gel electrophoresis (DGGE) and

terminal-restriction fragment length polymorphism (T-RFLP), to provide a rapid reproducible view of microbial diversity.

DGGE PCR provides a gene fragment approach to the speciation of organisms of interest (Li *et al.*, 2012). DGGE separates DNA fragments based on different nucleotide composition rather than fragment length (Muyzer *et al.*, 1993; Bukowska *et al.*, 2014), which is the basis for standard agarose gel electrophoresis. DGGE tends to be a more sensitive method of analysis for speciation of organisms compared to traditional methods (Li *et al.*, 2012), such as plate counts for example. It provides an immediate profile of the microorganisms present in a sample providing both qualitative and semi-quantitative information (Muyzer *et al.*, 1993).

This method of analysis is based on DNA melting behaviour which allows for the separation of DNA fragments in acrylamide gels (Børresen *et al.*, 1988). The acrylamide gel consists of an increasing gradient of two DNA denaturants, urea and formamide, from top to bottom. As amplified double-stranded DNA samples are loaded onto the gel, DNA fragments move at a constant rate until they reach the lowest melting domain specific to each fragment, after which they melt resulting in partially degraded DNA (Myers *et al.*, 1985; Børresen *et al.*, 1988). The unravelling of the DNA helix causes a decrease in the mobility of the DNA fragment (Fischer and Lerman, 1983; Myers *et al.*, 1985). DNA fragments with differences as little as a single-base substitution have different melting domains and so this partial denaturation will occur at different positions within the gel, resulting in separation of DNA molecules of identical sizes but differing sequences (Myers *et al.*, 1985; Børresen *et al.*, 1988). Therefore, each band present on the gel represents a different bacterial taxon. DGGE was first used in the analysis of whole bacterial communities by Muyzer *et al.* (1993) and has since been used in a range of areas such as ophthalmology (Schabereiter-Gurtner *et al.*, 2001), oral biology (Rasiah *et al.*, 2005) and in water ecosystem biodiversity (Van Hannen *et al.*, 1999; LaPara *et al.*, 2002; Zwart *et al.*, 2002; Goddard *et al.*, 2005; Tijdens *et al.*, 2008; Li *et al.*, 2012; Martins *et al.*, 2013; Tang *et al.*, 2014; Karło *et al.*, 2015; Olsen *et al.*, 2017).

3.3 Keywater Fisheries perch farm

Keywater Fisheries (KWF) is a freshwater perch farm located in Boyle, Co. Sligo. It is a low production perch farm, mainly utilised for pilot studies in order to determine optimal conditions for a successfully operating pill-pond system. It contains broodstock tanks, a hatchery for eggs and larvae and a nursery for juvenile growth. All of these indoor systems are based in tanks and operate under a RAS system. There are three grow out ponds for the larger fish. These are earthen pill-pond systems based on low surface flow water sourced from the local stream. The focus of this study was based on one of the grow out ponds, a.k.a. pond 1, denoted by the red outline in **Figure 3.2** of the farm.

Figure 3.2 Keywater Fisheries outgrow ponds - aerial view.

The pond is divided into two sections, one section that is stocked with the adult perch known as the fish pond (FP) and the other section that is not stocked, known as the treatment pond (TP). The flow is circulated in and out of the FP region using paddle wheels guided by walls. The growth of phytoplankton in the TP supplements oxygen delivery at certain times of the year. **Figure 3.3** depicts this concept, indicating the dimensions of pond 1. All sampling for this project was carried out in pond 1, with all noted alterations to the process recorded and included in the analysis of results.

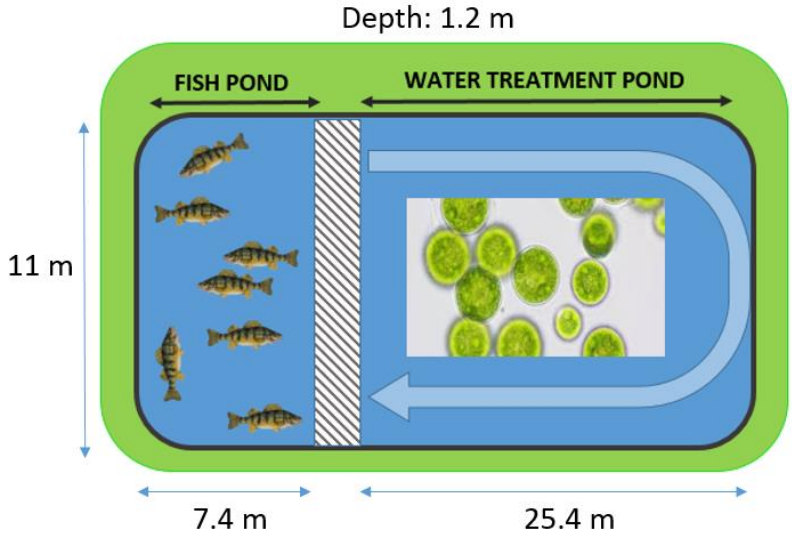

Figure 3.3 Layout and details of Pond 1 at Keywater Fisheries.

Methodologies

Identification of the microbial community structure over an annual period provides an indication of the peak seasons for certain phytoplankton, important variations in bacterial diversity and how this influences the physicochemical parameters or *vice versa*. A schematic of the overall project objectives is presented in **Figure 3.4**.

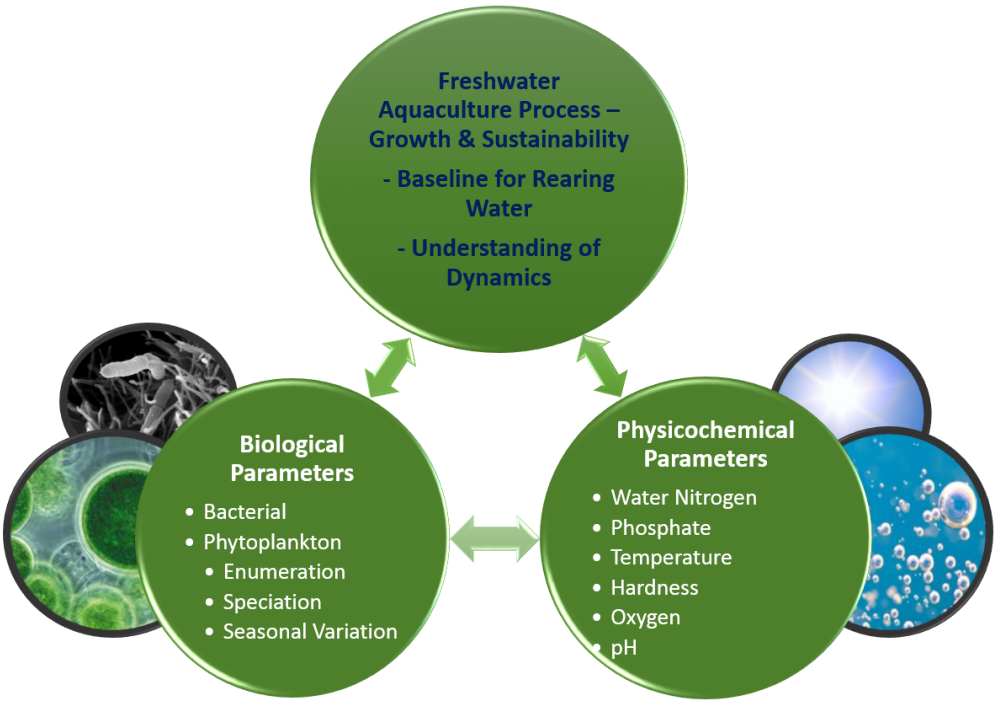

Figure 3.4 Schematic identifying the objectives in the determination of a profile of the rearing water.

Determination of a baseline profile of the rearing water conditions, followed by correlation of the results to periods of the most successful production and profit has the potential for expanding the industry, allowing for the establishment of systems with defined favourable microbial communities. While it is important to establish sustainable water quality for successful production, it is also important to ensure that the intensive rearing process has minimal impact on the wild biota in the surrounding environment and on human health as consumers.

A number of processes were investigated to obtain the endpoint profile of the correlation between biota and physicochemical parameters. A short-term pilot study was previously carried out in 2017 to ascertain the parameters that were necessary for analysis. The outcomes from the pilot study informed the necessity of the following physicochemical parameters: oxygen, temperature, pH, nitrate, nitrite, ammonium and phosphate concentrations, carbonate hardness and turbidity. To correlate these parameters with potential biological influencers i.e. bacteria and phytoplankton, a study based on PCR, DGGE, FCM and microscopy as the main analytical tools was chosen. These methodologies allow for both enumeration and seasonal pattern determination of bacteria and phytoplankton, with selective speciation in cases of recurring or unusual patterns. A sampling regime was prepared from March 2018 to March 2019 to ensure consistency of sampling and is briefly outlined in **Table 3.1.** Due to aquaculture schedules, the sampling ended in November as there were no fish in the pond following that time period**.**

Samples were obtained from both the FP and TP of pond 1 in KWF in all cases, at the same location of the pond each time. Samples were initially obtained using a 25 L container to ensure a representative sample, from which smaller volumes were transferred to the respective sterile polyethylene containers. To maintain close to *in situ* conditions certain preservative measures were necessary on-site. The bacterial samples were preserved in a final concentration of 2% formaldehyde as per Noble and Fuhrman (1998). The phytoplankton samples were preserved in a final concentration of 1% Lugol's iodine (Guillard and Sieracki, 2005) and the samples for physicochemical analysis were adjusted with 10% sulphuric acid until the pH was lowered to 2 or below to halt further biological activity. All samples were stored at

4°C as described by Bloem and Vos (2004) and Noble and Fuhrman (1998) until required for subsequent use.

Table 3.1 Sampling regime for obtaining rearing water profile at KWF.

Sample Type	Volume	Application	Frequency	Preservative
Bacterial	100 ml	Enumeration	Biweekly	Formaldehyde
Phytoplankton	500 ml	Enumeration, Identification & Profile Development	Biweekly	Lugol's Iodine
Physicochemical	500 ml	Parameter Measurement	Biweekly	Sulphuric Acid
Bacterial	10 L	Profile Development *via* Molecular Methods	Fortnightly	N/A

Upon receipt of the water samples from KWF as per **Table 3.1**, the samples were transported in a cooler box to the lab. The preserved samples were stored at 4°C until further analysis was carried out. The large 10 L samples were concentrated immediately via initial filtration, followed by a second filtration step prior to extraction of the DNA from the samples using a DNA extraction kit. Gel electrophoresis was carried out to confirm the presence of sample DNA. PCR steps were carried out using primers specific for bacterial 16S ribosomal DNA (rDNA), followed by nested DGGE PCR, the products of which were used for biological profile analysis with DGGE. Bands of interest, such as bands common to many lanes or bands increasing in strength, were excised from the gel, re-amplified with appropriate primers and sent for sequencing for species identification. The aforementioned steps for both biological and physicochemical analysis are explained in more detail in the succeeding pages.

3.4 Physicochemical parameter measurements

The physicochemical parameters that were measured in the lab included nitrates, nitrites, ammonium and phosphate concentrations, pH and carbonate hardness. Whilst nitrates, nitrites, ammonia, phosphate, pH, oxygen and turbidity were measured *in situ* on the farm by standard methods. All parameters measured in the lab were done so using individual test kits for each specific parameter outlined in **Table 3.2**. After initial preservation with sulphuric acid, the pH was increased to

between 6 – 7 which was required for all tests. Each test was carried out as per the individual kit instruction manual in duplicate. A Jenway UV-Vis Spectrophotometer was used for the spectrophotometric tests.

Table 3.2 Test kits used for the physicochemical analysis of rearing water samples.

Parameter	Test	Method Basis
Nitrates	Spectroquant® Nitrate Test – 1.09713	Photometric
Nitrites	Spectroquant® Nitrite Test – 1.14776	Photometric
Ammonium	Spectroquant® Ammonium Test – 1.14752	Photometric
Phosphate	Spectroquant® Phosphate Test – 1.14848	Photometric
pH	pH 1100 L Meter - VWR	pH electrode
Carbonate Hardness	MColortest™ Carbonate Hardness Test – 1.08048	Titration

3.5 Epifluorescent microscopy enumeration of bacteria

The methodology used for the preparation of the filter on the slide for enumeration was as per Bloem and Vos (2004). SYBR Gold was used in this study to stain the microbial cells. It was supplied in 10,000X form and diluted to a 10X working concentration using distilled deionised water. Bacteria in freshwater tend to be quite small and so the 0.22 µm isopore membrane filters were used, as this proved successful for retaining 99% of freshwater bacteria for enumeration by Hobbie *et al.* (1977). Cellulose filters can be inadequate for smaller bacteria as they can become trapped inside the filter (Hobbie *et al.*, 1977), and therefore counts can be underestimated. A support Whatman™ filter was moistened and placed beneath the isopore membrane filter to enhance vacuum distribution. For each filter, 10 ml of the rearing water sample was added to the reservoir and 200 µl of 10X SYBR Gold was added. The sample was stained for a period of 5 minutes after which the sample was filtered through using the Sartorius filtration apparatus connected to a vacuum. The sample was filtered to dryness and washed with 5 ml of distilled deionised water to remove any residual background staining. The filter was removed with sterile forceps and placed on a glass slide onto a drop of immersion oil. A drop of oil was placed on top of the filter for adherence of a cover slip. Slides were counted immediately to avoid fading of the stain, which occurs if kept in contact with light for long periods (Kumaravel *et al.*, 2009).

For each filter, a selection of random fields was counted until a total of at least 300 cells were counted over a minimum five fields. Counting was facilitated with the use of an epifluorescent microscope under oil immersion (100x objective lens) resulting in a magnification of 1000x. The samples were observed under a blue optical filter on which has an excitation from 465 to 505 nm, 510 nm cut-off; emission from 515 to 565 nm as adopted from Shibata *et al.* (2006). Counts were carried out within 15 - 20 seconds of commencement of excitation to minimise the fading of the stain. Bacterial cells were visualised as tiny green dots or lines. Phytoplankton were also present in the sample, which were visualised under a different focus and could be easily distinguished from the bacterial cells due to the size difference and brightness. Once a count was obtained, the following formula was used for enumeration of bacterial cells per millilitre:

$$\frac{Count\ x\ 176}{Dilution\ factor\ x\ No.of\ fields\ counts\ x\ 0.186}$$

where 176 mm was the area of the field of view on the microscope, the dilution factor accounts for the 10 ml analysed in the filtration of the sample, and 0.186 mm^2 was the filter area. Each sample was counted in triplicate and where numbers exceeded 100 cells per field of view, a minimum of 4 counts was obtained or a further dilution was made.

3.6 DNA extraction from rearing water samples

An initial step in the bacterial DNA extraction protocol was carried out to concentrate cells *via* tangential flow filtration, followed by further filtration onto cellulose acetate membranes. The DNA was extracted from the filters using a DNA isolation kit from QIAGEN until required for subsequent analysis.

3.6.1 Sample filtration – Tangential flow filtration

In order to initiate molecular analysis on water samples of large volumes, 10 L in this case, concentration of the water sample to a smaller volume was a requirement. Based on numerous studies carried out on phytoplankton and bacterial filtration

(Rossignol *et al.*, 1999; Zhang *et al.*, 2010; Sun *et al.*, 2013), ultrafiltration was the method of choice for filtration of the water samples obtained from KWF in this project. Initial filtration of the water samples from KWF was performed using a tangential flow/crossflow filtration device, the Vivaflow® 200, sourced from Sartorius. This reusable device has been used in many applications relating to aquaculture (Tijdens *et al.*, 2008; Daud *et al.*, 2015). The molecular weight cut off point (MWCO) i.e. the pore size, of the membrane selected was 100,000 Da with a polyethersulfone (PES) material as recommended by the supplier for bacterial filtration and phytoplankton filtration. This MWCO has been used in many studies in terms of both bacterial and phytoplankton ultrafiltration in aquaculture and waste water treatment plants (WWTP) (Liang *et al.*, 2008; Lou *et al.*, 2010; Sun *et al.*, 2013).

The apparatus was set up as displayed in **Figure 3.5** from the Vivaflow® 50/50R/200 Datasheet, with the two cassettes utilised to maximise sample concentration time. The whole Vivaflow® 200 system consists of a peristaltic pump, tubing, flow restrictor, pressure control gauge, Y-connector, collection vessel, filtrate vessel and two 100,000 MWCO PES membrane cassettes.

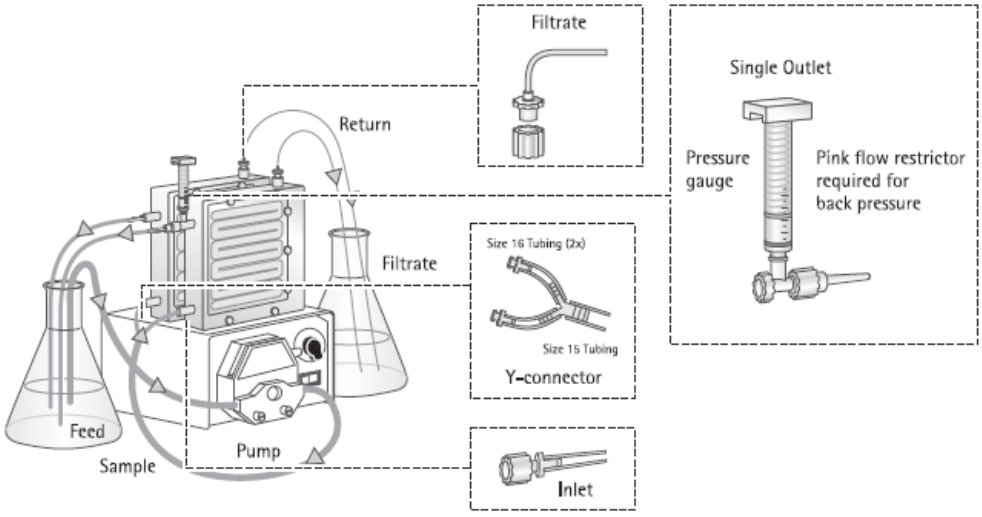

Figure 3.5 Set up of the Vivaflow 200 apparatus (Sartorius, 2018).

The peristaltic pump allows the recirculation of the sample through the membrane. The flow restrictor in the outlet tubing generates back pressure allowing for sample concentration and the separation of unwanted constituents as the sample flows across the membrane. The sample is continuously concentrated as it is recirculated

back into the collection vessel, whereas the filtrate is collected in a separate vessel. Once the desired volume is achieved the process can be stopped.

The apparatus was operated at a moderate to high velocity, as Elcik *et al.* (2016) discovered when cultivating *Chlorella vulgaris*, that a higher crossflow velocity (CFV) resulted in less accumulation of phytoplankton cells on the surface of the membrane, potentially increasing the longevity of membrane usage. The sample was concentrated down to a volume of approximately 500 ml, where a notable increase in colour intensity was visualised compared to the original sample, especially in summer months (**Figure 3.6**). The sample was further concentrated onto 0.45 µm Whatman® cellulose acetate filters using a Sartorius glass vacuum filtration system and stored at -20°C prior to extraction of the DNA from the filter using the DNeasy Powerwater Kit.

Figure 3.6 Vivaflow 200 system set up, with colour intensification of the sample during concentration.

3.6.2 DNeasy Powerwater kit

In order to utilise the DNA collected on the cellulose acetate membrane for PCR studies, the DNA was extracted using the DNeasy® PowerWater® Kit (QIAGEN). The cellulose acetate membrane filters were removed from the freezer and allowed to defrost for approximately 20 minutes to avoid brittleness when rolling up for insertion into the extraction tubes. The protocol was followed as per the DNeasy® PowerWater® Kit Handbook centrifugation protocol. DNA was eluted in a final volume of 100 µl and stored at -20°C for subsequent PCR amplification.

3.6.3 Microbial DNA extraction from a pure culture

In order to extract the DNA from cultured bacterial samples for use in PCR as a positive control, the MO BIO UltraClean® Microbial DNA Isolation Kit was used and followed as per maufacturer's instructions. This kit involved several centrifugation steps (all at 10,000 x *g*) and washing/binding steps to ensure isolation of a high-quality genomic product from all test bacterial samples. DNA was eluted in a final volume of 50 µl and stored at -20°C for subsequent PCR amplification.

3.7 Agarose gel electrophoresis of bacterial samples

As defined by Reed *et al.* (1998) "electrophoresis is a separation technique based on the movement of charged molecules in an electric field". In this project, agarose gel electrophoresis was utilised as a method to confirm the presence of DNA following extraction procedures and following PCR to ensure the formation of a high quality PCR product.

3.7.1 Preparation of agarose gel

In all cases, a 2% gel was prepared by adding 1.2 g of agarose powder (Sigma Aldrich) to 60 ml of 1X Tris-acetate EDTA (TAE) buffer. The 50X TAE buffer (Sigma Aldrich) was diluted to 1X concentration with distilled deionised water followed by autoclaving at 121°C for 15 mins to ensure sterility. The agarose and 1X TAE solution was heated until the agarose had dissolved, forming a clear solution. The solution was cooled to approximately 60°C and 6 µl of Invitrogen™ SYBR® Safe DNA gel stain (ThermoFisher Scientific) was added to allow visualisation of the DNA in the agarose gel. The gel was poured into the casting tray and allowed to solidify for 15 to 30 minutes. The tank was filled up to the allocated mark with 1X TAE buffer.

3.7.2 Preparation of samples for gel electrophoresis

In order to analyse DNA extracts/PCR products, 5 µl of each sample was added to 3 µl of 6X DNA loading dye in sterile eppendorfs. The samples were added to each

respective well. In each case, a DNA ladder was used for identification of the DNA fragment size. In the case of the initial bacterial DNA extracts a 1kb ladder was used as bacteria range from approximately 112 kb (Bennett and Moran, 2013) to 140 Mbp (Han *et al.*, 2013). A 100 bp ladder was used for PCR and nested PCR products as the 16S rRNA was the target gene. Upon completion of sample loading, the tank was connected to the power supply and set at 80 mV for a duration of 1 hour. All gels were analysed under UV transilumination with the Azure c600 Gel Imaging System from Azure Biosystems.

3.8 Polymerase chain reaction of bacterial samples

The BIOTAQ™ DNA Polymerase Kit from Bioline was used for the PCR amplification of 16S rDNA. PCR reactions were carried out in 50 µl volumes for each sample reaction as per (Li *et al.*, 2012). Each reaction contained 10X NH_4 reaction buffer, 50 mM $MgCl_2$ solution, 100 mM of dNTPs, 25 µM of the forward and reverse primers, 0.2 µl of BIOTAQ, 26.3 µl of molecular grade water and 5 µl of the DNA template extracted (described in **Section 3.2**) in a total volume of 50 µl. In order to increase the PCR yields, 10 mg/ml of Bovine Serum Albumin (BSA) was also added to each reaction. Two sets of primers were required for the PCR reactions, details of which are outlined in **Table 3.3**. The initial 16S rDNA PCR was carried out using the universal bacterial 16S rDNA primers 27F and 1492R for amplification of full length 16S rDNA (Wilson *et al.*, 1990; Lane, 1991; O'Brien *et al.*, 1994; Frank *et al.*, 2008; Piterina *et al.*, 2010; Kumar *et al.*, 2011; Martins *et al.*, 2013). This was followed by a nested PCR amplification of the V3 region of the full length 16S rDNA PCR products using the 357F and 519R primers (Muyzer *et al.*, 1993; Lai *et al.*, 2006; Li *et al.*, 2009; Tang *et al.*, 2014; Karło *et al.*, 2015). The 357F primer contained a 40 bp GC-rich clamp, which is commonly used for optimal resolution of the DNA fragments (Myers *et al.*, 1985; Muyzer *et al.*, 1993). The products of the nested PCR were analysed by DGGE. The conditions for the initial full length 16S rDNA PCR reactions, carried out in the Labcycler by SensoQuest, were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 40 seconds denaturation at 95°C, 1 minute annealing at 55°C, 1 minute extension at 72°C, and a final extension step of 5 minutes at 72°C. For the

nested DGGE PCR the conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 30 seconds denaturation at 95°C, 1 minute at the annealing temperature, 1 minute extension at 72°C, and a final extension step of 10 minutes at 72°C. During the reaction the annealing temperature decreased by 1°C every cycle from 65°C to 56°C, remaining at 55°C for the last 10 cycles. PCR amplification was carried out twice for each sample in an attempt at improving the strength of the products. All PCR products (5 µl) were analysed through 2% gel electrophoresis as described in **Section 3.4**.

Table 3.3 Nucleotide sequences of primers used in the PCR reactions.

Reaction	Primer
16S rDNA PCR	**27F** 5'-AGAGTTTGATCATGGCTCAG-3'
	1492R 5'-GGTTACCTTGTTACGACTT-3'
Nested DGGE PCR	**357F** 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3'
	519R 5' ACCGCGGCTGCTGGCAC 3'

3.9 Denaturing gradient gel electrophoresis

The DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc.) was used for DGGE analysis of nested PCR products described in **Section 3.5**. Acrylamide-bis 30% (37.5:1) was used to create the 0.8 mm gel by using a high strength (70%) and a low strength (35%) solution to create a denaturing gradient for optimisation of product separation. The two solutions were prepared as described in **Table 3.4**. Once the solutions were dissolved, 20 ml of each solution was poured into two universals. Catalysts were added to initiate polymerisation, which included 0.09% v/v (90 µl) of 10% ammonium persulfate and 0.09% v/v (90 µl) of tetramethylethylenediamine (TEMED). Once the solutions were prepared for pouring, two syringes attached to the Gradient Former device were used to draw up the solutions immediately, as the polymerisation process occurs within 5 – 7 minutes. The gels were poured between two glass plates separated by two spacers. The resultant gel contained a denaturing gradient (35% – 70% from top to bottom). The gel electrophoresis tank was filled with 7 litres of 1X TAE running buffer and pre-heated to 60°C.

Table 3.4 Preparation of low and high strength denaturants for DGGE.

Chemical	Low Strength – 35% (200 ml)	High Strength – 70% (200 ml)
Formamide	28 ml	56 ml
Urea	29.4 g	58.8 g
30% Acrylamide-bis	40 ml	40 ml
50x TAE	4 ml	4 ml
Glycerol	4 ml	4 ml
ddH_2O	Add to make 200 ml	Add to make 200 ml

The gel was inserted into the tank and the system was switched back on to allow the upper buffer chamber between the glass plates to be filled with running buffer. PCR products (45 µl) were mixed with 40 µl of 6X DNA loading dye (Fisher Scientific) and were loaded into the wells. Gels were run at 60V for 16 hours, with the temperature remaining constant at 60°C (Fischer and Lerman, 1983), as illustrated in **Figure 3.7.**

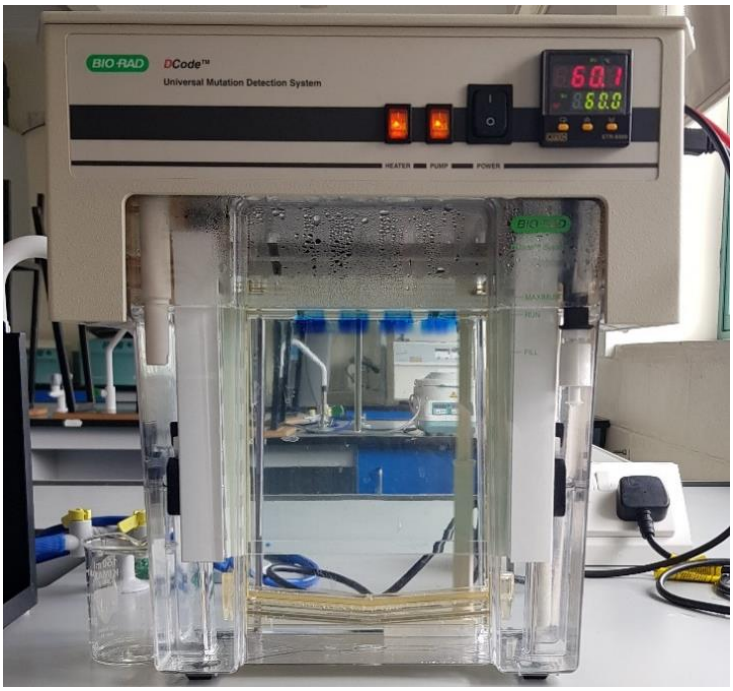

Figure 3.7 DCode Universal Mutation Detection System as used for DGGE analysis.

Prior to UV visualisation, gels were placed in a staining tank with 500 ml of 1X SYBR Gold in water for 30 minutes to 1 hour. A de-staining step was using distilled water was carried out for 5 minutes prior to viewing with the Azure c600 Gel Imaging System from Azure Biosystems under UV fluorescence. Distinctive bacterial V3 region

banding patterns were analysed to select relevant bands for excision and sequencing. A selection of bands was excised, including very bright bands, bands that were common across all lanes, bands that appeared across from the *A. salmonicida* marker and bands that were unique to only certain lanes. Once image analysis was complete, the bands of relevance were excised from the gels and stored in TE buffer at 4°C. The eluent was reamplified using the 357F (without the GC clamp) and 519R primers using the PCR conditions described in **Section 3.8**. Once the presence of DNA was confirmed *via* gel electrophoresis, the PCR products were purified using the Wizard® SV Gel and PCR Clean-up System (Promega), sourced from MyBio Ireland, before being sent for sequencing to Sequiserve GmbH, Germany.

3.10 Bacterial sequence analysis

V3 region PCR products amplified from selected bands excised from DGGE gels were analysed by Sequiserve GmbH, Germany, using short-run sequencing with editing. Results were received in the form of individual sequence files and electropherograms. Sequences were input into the online BLAST (Basic Local Alignment Search Tool) web server, hosted by the National Centre for Biotechnology Information (NCIB) to determine significant matches with other sequences for in the GenBank sequences database. As the fragment length was quite short for the PCR products analysed (< 161 bp), the cut-off point of > 80% match with possible organisms was employed. The most relevant organisms from the top BLAST matches were selected for phylogenetic analysis. The sequences for relevant matches were downloaded from BLAST and stored in a Notepad file with the excised band sequences for phylogenetic analysis.

3.10.1 Phylogenetic analysis of bacterial sequences

To elucidate the relationships between the excised sequences, phylogenetic analysis was carried out using MEGA 7.0 (Molecular Evolutionary Genetic Analysis), a phylogenetic analysis tool. Comparative relationships following phylogenetic analysis are usually depicted on the phylogenetic tree, a branching, tree-like diagram. A

number of steps were taken to create this phylogenetic tree, with the molecular marker for analysis, in this case, being the nucleic acid sequences. Firstly, all sequences from the previously created Notepad file were uploaded to MEGA 7.0 and aligned using the Muscle Alignment Tool (Hall, 2013). The alignment of sequences is the most important part of the phylogenetic process as it identifies conserved regions between species. Once the alignment was complete, columns predominantly composed of gaps, which indicate insertions/deletions during evolution, were removed to achieve a better alignment of the sequences.

Selection of a model of evolution was the next stage in the process. This determines the best method for analysis of the selected data. Each model simplifies the complexity of the biological mutation process and predicts relationships based on computational statistical methods. Two models were used in this analysis; the Jukes-Cantor (JC) one-parameter model and Kimura's two-parameter model, with gamma distribution in some cases, which accounts for variations in the rate of mutation among sites in the alignment. Both models assume that all nucleotides occur in equal frequency (25%). However, the JC model assumes that all nucleotides are substituted with equal probability, i.e. the number of transition mutations (interchange of a purine for a purine or a pyrimidine for a pyrimidine) is equal to the number of transversion mutations (interchange of a purine for a pyrimidine or *vice versa*). The Kimura two-parameter model takes into account that transition mutations are more common than transversion mutations (Choudhuri, 2014; Weyenberg and Yoshida, 2015). MEGA 7.0 calculates the best fit model to use for each particular set of data for analysis.

Two methods were used for the construction of the phylogenetic tree for each data set; the distance-matrix method in the form of neighbour joining (NJ) and the character-based method in the form of maximum likelihood (ML). The distance-matrix method is based on the genetic distance between sequences. NJ assumes that all sequences radiate from one internal node to which another node is added by joining the most closely related sequences from the data, as per the distance matrix computed. This is continued until all possible pairs have been examined and branch lengths between all pairs have been calculated. The whole process is repeated using

a new interior node until the tree is fully constructed (Weyenberg and Yoshida, 2015). ML estimates the unknown parameters of the data based on probability models (Choudhuri, 2014). More complicated statistical calculations are used compared with NJ (Weyenberg and Yoshida, 2015), resulting in a tree topology with the highest accuracy based on the aligned sequences and evolutionary model used (Choudhuri, 2014). The reliability of the phylogenetic tree is then established by analysing the bootstrapping values to determine whether the topology of the tree is reliable. The higher each individual bootstrapping value, the more reliable the topology of that branch/node on the tree. In this study, the 500 resamplings were used for the bootstrapping with the NJ method, whereas 100 were used for the ML method.

3.11 Flow cytometry enumeration of phytoplankton

The FCM instrumentation used in this project was the MACSQuant® Analyzer 10 sourced from Miltenyi Biotec (**Figure 3.8**). This instrumentation has three lasers with eight different channels for use with several different dyes, as well as the FSC and SSC channels, outlined in **Table 3.5**. The main lasers used for analysis in this study were FSC, SSC, B1 laser for SYBR Gold fluorescence, B3 laser for chlorophyll fluorescence and R1 laser for PC fluorescence.

Figure 3.8 MACSQuant 10 Flow cytometry analyser (Milteny Biotech).

Table 3.5 Lasers and channels available on the MACSQuant 10 analyser.

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

3.11.1 Preparation of phytoplankton samples

As all the samples sourced from KWF contained mixed populations of organisms, certain steps were taken in order to analyse and obtain a cell count on the desired population i.e. phytoplankton. The samples were preserved in Lugol's iodine in order to preserve the phytoplankton population at the time of sampling. However, other organisms may survive in this preservative and debris is also be visible or considered as an 'event' during flow cytometry analysis. Protocols for analysing a mixed freshwater phytoplankton population from aquaculture sites, that require long-term (1-2 years) preservation before analysis, have not been thoroughly developed. Therefore, limited knowledge is available on the most suitable method of obtaining an accurate representation of the phytoplankton population in a fish farm over a long-term period. Various preparation techniques were tested in order to obtain the most appropriate one for this type of sample analysis. Firstly, sedimentation of the phytoplankton sample for 24 – 72 hours with subsequent removal of the top 80 % of water proved ineffective for capturing all species in the volume of sample. This was evident from the large volume of algae still present in the supernatant when viewed under the microscope. This process would, therefore, have resulted in an underestimated phytoplankton count.

Although centrifugation of phytoplankton can harm the cells (Marie *et al.*, 2005) other researchers used centrifugation in a manner by which cells were unaffected. Suman *et al.* (2015) collected *C. vulgaris* cells by centrifugation at 3500 g for 10

minutes. Bellinger and Sigee, (2015) suggested centrifugation of phytoplankton cells at 1000 g for 20 minutes for concentration prior to enumeration. A combination of these settings was investigated to determine the optimal rpm that resulted in the concentration of cells, without loss in the supernatant and which ensured the cells remained structurally intact and undamaged. These settings involved centrifugation at 3500 x g for 20 minutes; all samples were initially prepared in this manner in 10 ml volumes and the cell pellet was then resuspended in flow buffer. Flow buffer was prepared by adding 1 mM EDTA (ethylenediaminetetraacetic acid), Tween (0.2%) and sodium azide (0.1%) to 1 litre of phosphate buffered saline (PBS); the buffer was passed through a 0.2 µm filter prior to use to remove impurities. The 10 ml resuspended sample was then divided into two 5 ml aliquots, one for unstained samples, which was ready to use, and the other for stained samples. Staining with SYBR Green was used to separate the DNA-containing cells from debris, such as organic matter and sedimentation that would be present in the pond. An unstained phytoplankton representative was required for each sample in order to eliminate natural auto fluorescence from occurring at the same wavelength as that of the blue B1 laser used to analyse the SYBR Green stained cells, which other may have led to false positive results. For the stained samples, SYBR Green was added to the 5 ml aliquot at a final concentration of 1:10,000. For each sample analysed four wells were utilised (200 µl per well); the first well consisted of the unstained sample and the following three wells contained the stained sample in triplicate to ensure appropriate distribution and representation of each species present.

3.11.2 MACSQuant instrument settings & phytoplankton 'gating' procedure

In order to ensure the reliability of results, the flow cytometer was calibrated prior to phytoplankton analysis using MACSQuant calibration beads. The machine was set up for sample analysis at a medium flow rate with high mixing before uptake of 90 µl of each sample to ensure sufficient mixing. A trigger point of 1.0 for the FSC laser was used to eliminate material that was considered debris in the sample. The overall gating method to obtain and enumerate the desired population, i.e. phytoplankton

excluding cyanobacteria, was adapted from Moorhouse *et al.* (2018), Haynes *et al.* (2015) and Read *et al.* (2014) for phytoplankton and plankton analysis. This process is explained over the following pages in **Figures 3.9** to **3.13**. The first step required the running of the unstained representative for each sample. **Figure 3.9** illustrates the establishment of a SYBR Green positive gate, denoted 'SYBR Pos' for the elimination of as much natural autofluorescence as possible that may otherwise occur for the stained representative samples leading to false positive results.

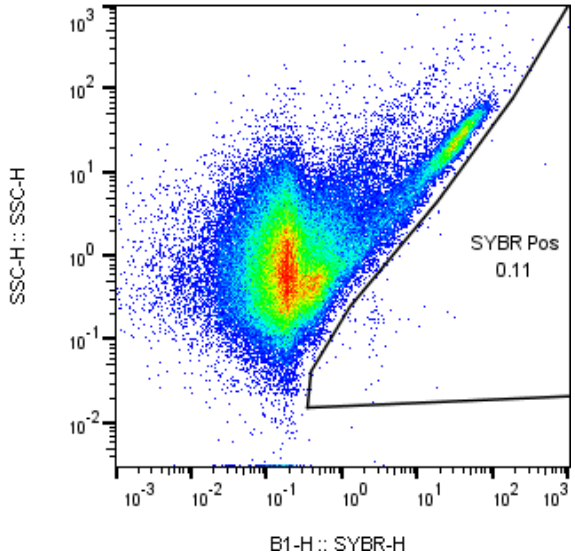

Figure 3.9 Gating for auto-fluorescence in the unstained sample.

This gate was then used to determine the actual DNA-containing cells in the stained samples, without natural auto-fluorescence interference. **Figure 3.10** illustrates the distribution of living cells present with all auto-fluorescent cells or unwanted material falling outside of the area/gate of interest. The organisms of interest i.e. phytoplankton in this case, all contain chlorophyll, therefore this was the next population to isolate. This was achieved by acquiring the cells that fluoresced once excited due to chlorophyll fluorescence. Chlorophyll fluoresces red and absorbs light very well in the blue regions of the visible spectrum (Moorhouse *et al.*, 2018), therefore the blue B3 laser was used, with a filter range between 655-730 nm.

Figure 3.11 illustrates the chlorophyll positive population with FSC on the x-axis relating to cell size and SSC on the y-axis relating to cell granularity and complexity. Cyanobacteria were present in this population as they also possess chlorophyll. In

order to eliminate this group of cells from the phytoplankton count, the blue B3 laser versus the red R1 laser, with a filter range of 655-730 nm, was used as PC fluoresces within this range. As previously mentioned in **Section 3.2.2** cyanobacteria populations can be represented by PC. The only other two populations which possess PC are red phytoplankton and those belonging to the *Cryptophyta* phylum (van Vuuren *et al.*, 2006; Brient *et al.*, 2008). As these two groups of phytoplankton were not identified microscopically as being present in the samples, the PC positive populations were assumed to mainly to be representative of cyanobacteria present.

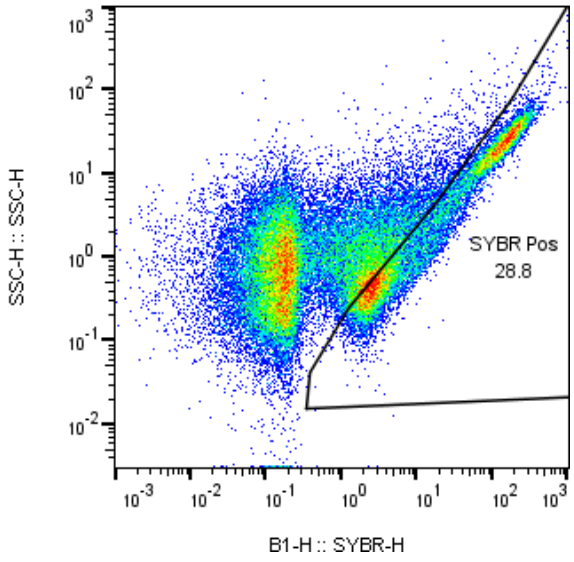

Figure 3.10 Gating for living organisms in the SYBR Green stained sample.

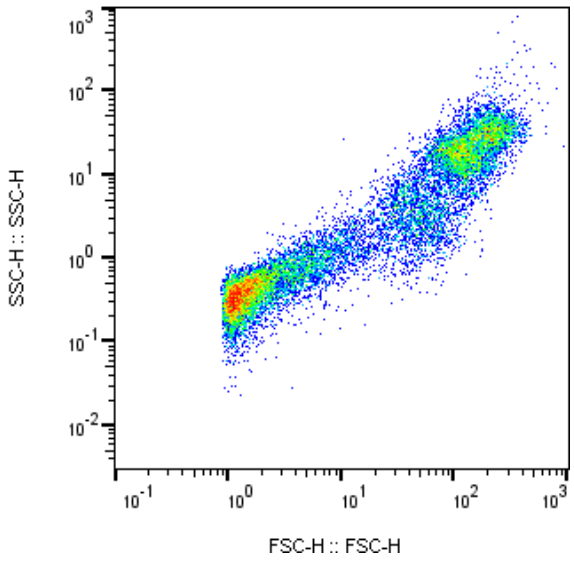

Figure 3.11 Chlorophyll positive population of the living cells viewed under FSC vs SSC.

Figure 3.12 illustrates how the phycocyanin positive population, a.k.a. the cyanobacteria, were eliminated *via* gating around the chlorophyll positive/ phycocyanin negative population. The final population depicted in **Figure 3.13**, is the population of interest viewed under FSC and SSC. The isolated cyanobacterial populations from each sample were also analysed and enumerated to determine the trends over the duration of the study. All flow cytometry data gating was carried out using the FlowJo software package.

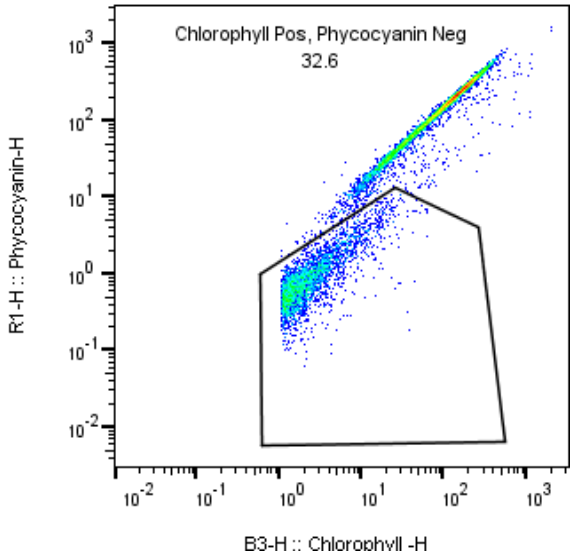

Figure 3.12 Gating to eliminate cyanobacteria population.

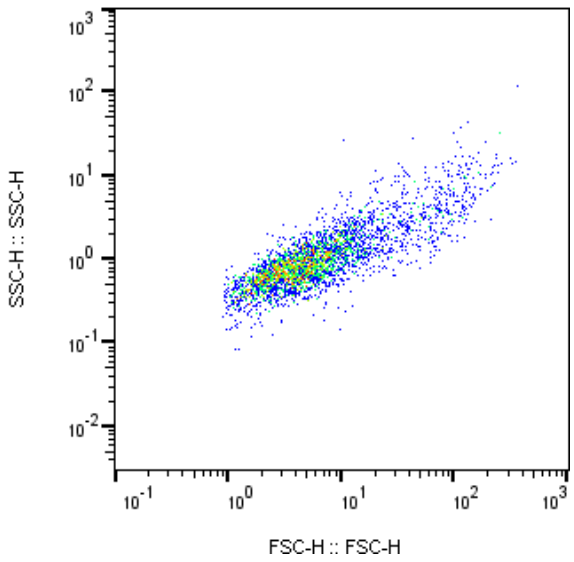

Figure 3.13 Phytoplankton population of interest for enumeration viewed under FSC vs. SSC.

3.12 In situ AlgaeTorch monitoring

In addition to FCM analysis of phytoplankton populations, the chlorophyll and Cyanobacteria populations were also measured *in situ* using the AlgaeTorch (**Figure 3.14**) sourced from bbe Moldaenke. The AlgaeTorch is based on real-time *in vivo* fluorescent measurement upon excitation of the algal cells in response to six LEDs of three different wavelengths, 470, 525 and 610 nm. The measurement analysis carried out is in accordance with ISO 10260:1992 Water quality – Measurement of biochemical parameters – Spectrometric determination of the chlorophyll-a concentration and DIN 38412/16:1985 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 (L 16). Both chlorophyll and cyanobacterial content were measured in µg chl-a/l (bbe Moldaenke, 2017).

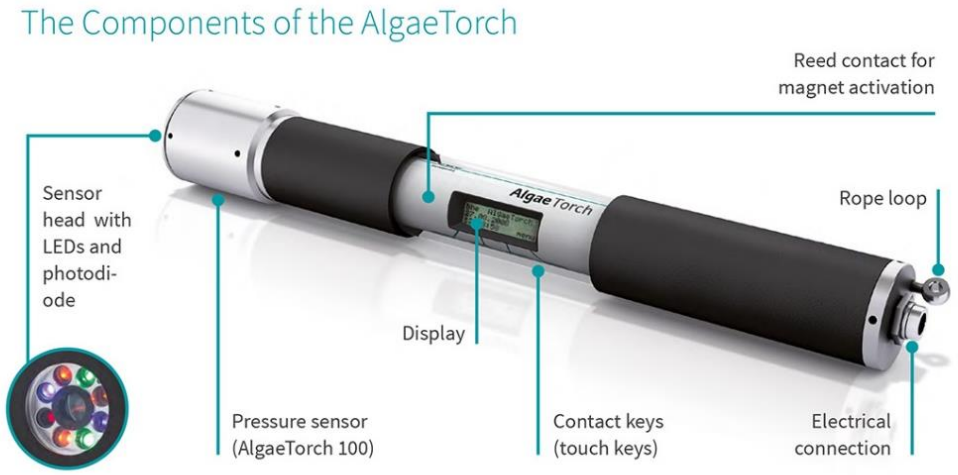

Figure 3.14 AlgaeTorch diagram outlining the main components for operation (Source: bbe Moldaenke).

3.13 Microscopic analysis of phytoplankton samples

Biomass analysis tends to be an insufficient method for speciation of phytoplankton that lack distinctive features (Li *et al.*, 2012), which highlights the importance of traditional microscopic analysis, complementing the emerging techniques, such FCM, in aquaculture research. There are many ways of counting phytoplankton through the traditional slide methods such as the Sedgewick-Rafter slide, the Palmer-

Maloney slide and the standard haemocytometer (Guillard, 1978). The most common method for phytoplankton enumeration is the Utermöhl method, however, this requires an inverted microscopic with sophisticated optics in order to ensure reliable results (Vuorio *et al.*, 2007). The requisite microscope was not available for this project. Variation between operators and between microscopes used are also factors in this type of analysis (Vuorio *et al.*, 2007). Therefore, identification using a standard inverted microscope for morphological analysis and photographic identification keys (van Vuuren *et al.*, 2006; Bellinger and Sigee, 2010) were used in conjunction with flow cytometry for enumeration to maximise the information obtained.

Results & Discussion

3.14 Physicochemical parameter analysis

All physicochemical parameters displayed from **Figures 3.15** to **3.23** include the standard curve for each parameter (excluding hardness), followed by the respective parameter trends as determined *via* kit format as mentioned in **Section 3.1**. Nitrates, nitrites, ammonium and phosphate results were expressed in mg/l, with water hardness expressed in the most common form as calcium carbonate hardness ($CaCO_3$) as per Wurts and Durborow (1992). In all cases an R^2 value of > 0.99 was achieved for the standard curve, indicating the reliability of the test method with very little variation.

The first parameter analysed was the nitrate concentration in **Figure 3.16**. The nitrate concentration peaked in October reaching levels of 3.27 mg/l and 3.25 mg/l for the FP and TP, respectively, which are well below the 50 mg/l maximum acceptable limit of SI No. 77 of 2019. The levels were lowest at the end of May with levels below the limit of detection achieved for both the FP and TP. However, on this date there was a water change in the pond, therefore the fish were removed. This process may have reduces the level of nitrates, with one of the main sources being fish waste, as well as decaying organic matter in the water (Jiménez-Montealegre *et al.*, 2002;

Thajuddin and Subramanian, 2005), which would have also been partially removed in the process.

Nitrites were the second parameter measured, levels of which ranged from below the limit of detection in March to 0.118 and 0.103 mg/l for the FP and TP, respectively, in June. The temperature was highest at this point ranging between 19 and 21°C and the bacterial counts were also highest in June overall, which may have increased nitrification processes. As initial steps in this process involves the oxidation of ammonia to nitrite (Helfrich and Libey, 1990; Hargreaves, 1998), **Figures 3.20** and **3.18** illustrate the decrease of ammonium levels at the start of June, followed by an increase in the nitrite levels, respectively, which may in part account for this increase in nitrite concentrations.

The ammonium trends are illustrated in **Figure 3.20** with the lowest levels of 0.12 and 0.08 mg/l for the FP and TP in April, respectively. The highest ammonium concentration levels were reached in June for both ponds, with a concentration of 1.89 mg/l in the FP and 1.69 mg/l in the TP. The partial change of water in the pond at the end of May could have contributed to the rise in ammonium with a sudden decrease in both the bacterial and phytoplankton numbers (**Figures 3.28** and **3.50**, respectively). The ammonium ion tends to be harmless to fish unless extremely high concentrations are reached (Boyd and Lichtkoppler, 1979).

Phosphate concentrations ranged from 0.17 to 1.66 mg/l in the FP, and from 0.10 to 2.20 mg/l in the TP as depicted in **Figure 3.22**. Lund (1965) stated that phosphorus levels can decrease in the summer, and as the summer months progressed, the phosphate concentrations measured during this study did in fact decrease. This may in part be due to the high levels of bacteria in the summer which are major competitors of phytoplankton for the uptake and utilisation of inorganic phosphorus (Lund, 1965).

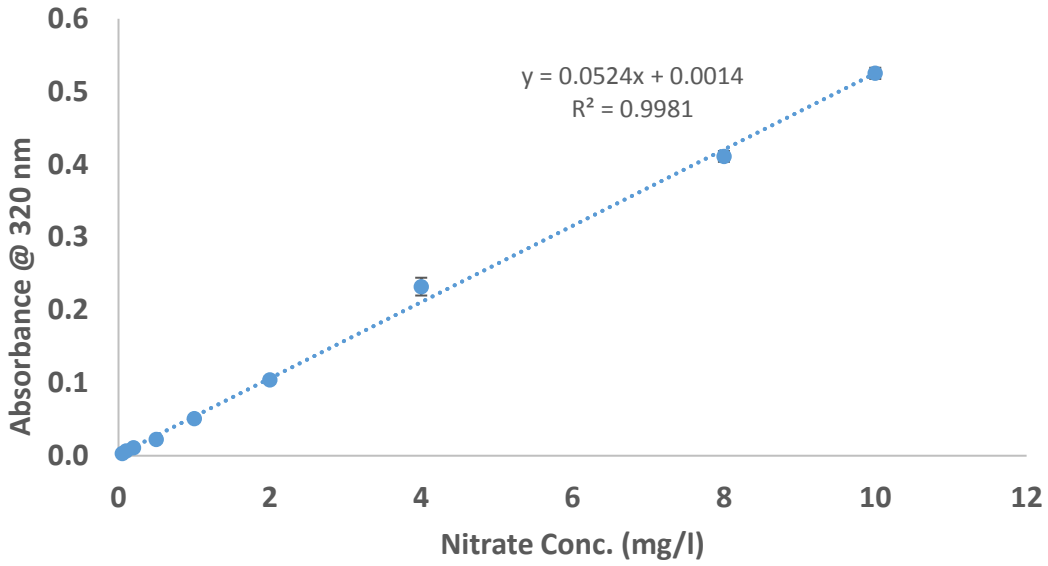

Figure 3.15 Standard curve for nitrate concentration in mg/l at an optical density of 320 nm.

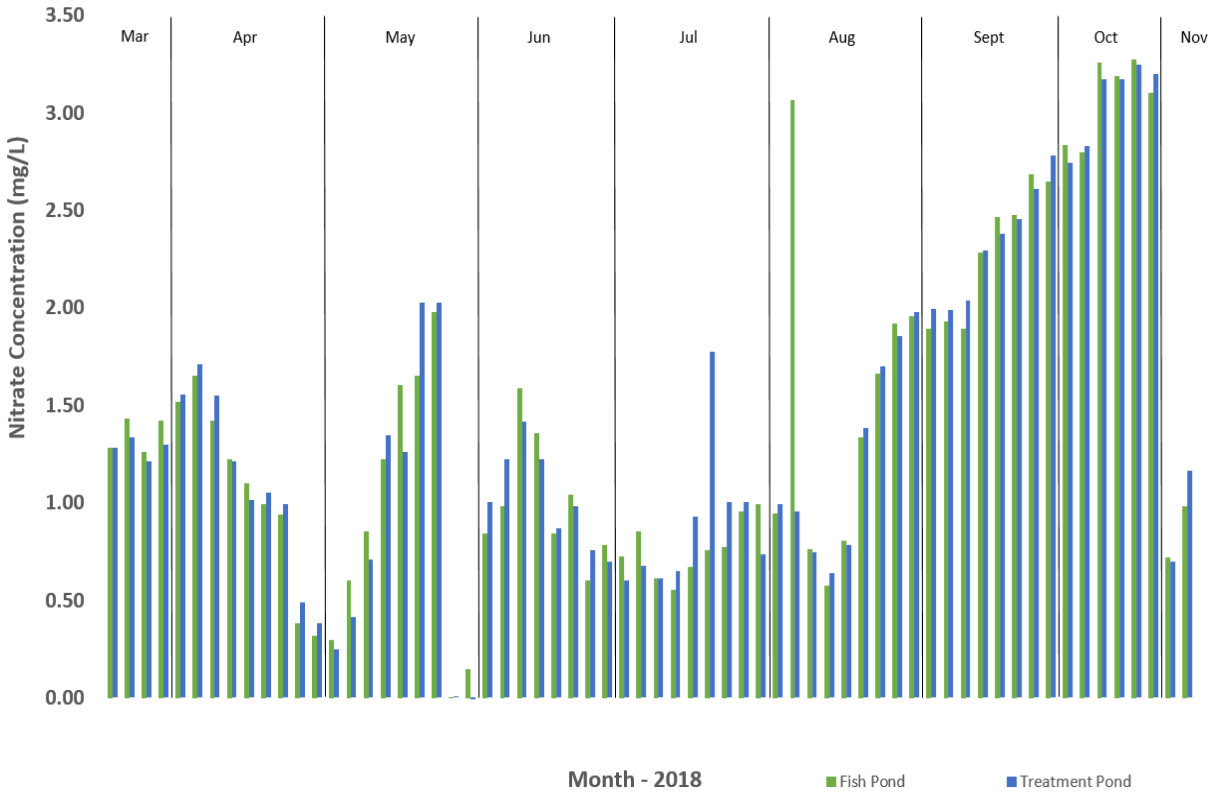

Figure 3.16 Nitrate concentration (mg/l) from March to November 2018 in Pond 1 at KWF.

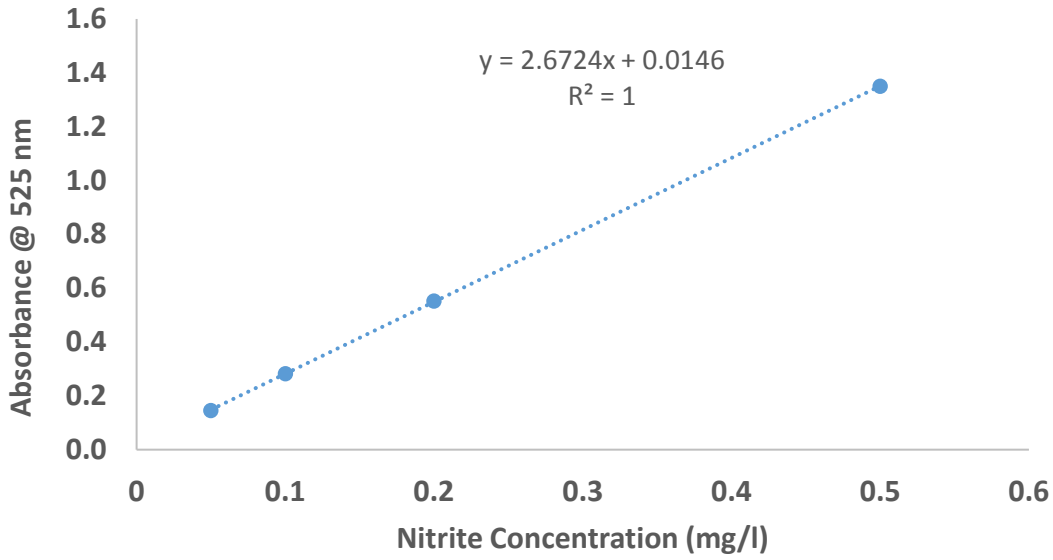

Figure 3.17 Standard curve for nitrite concentration in mg/l at an optical density of 525 nm.

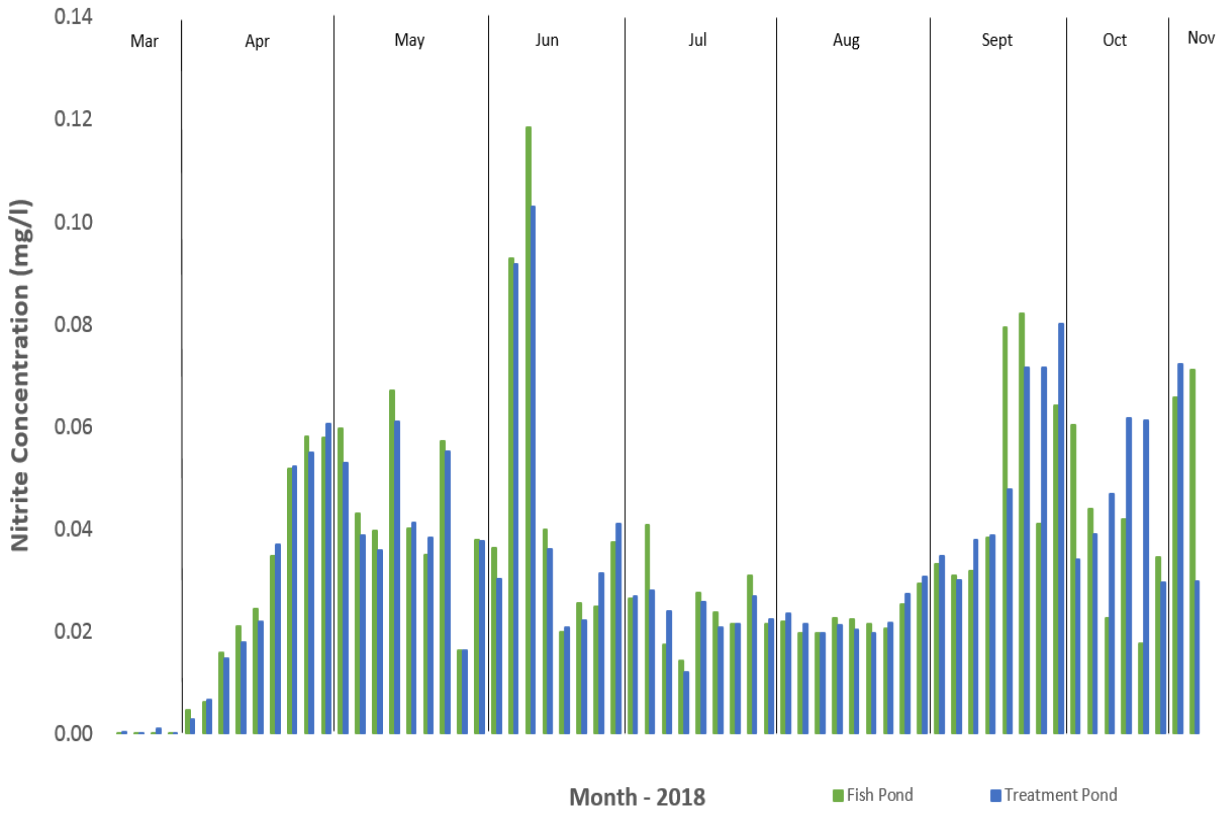

Figure 3.18 Nitrite concentration (mg/l) from March to November 2018 in Pond 1 at KWF.

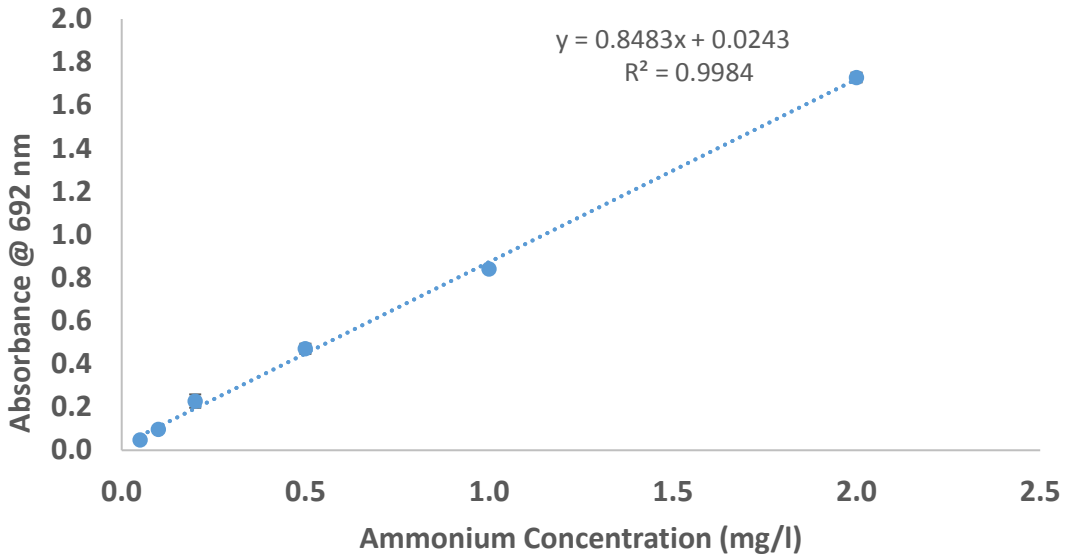

Figure 3.19 Standard curve for ammonium concentration in mg/l at an optical density of 692 nm.

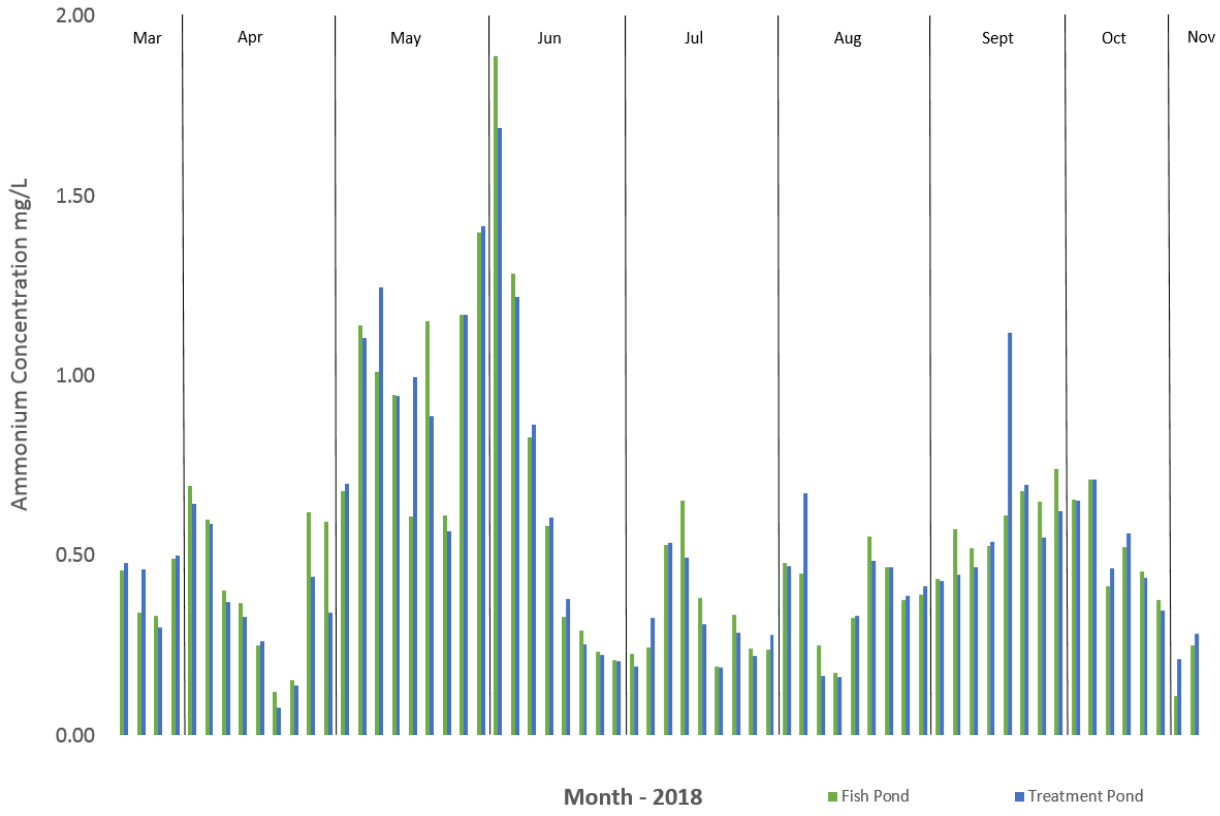

Figure 3.20 Ammonium concentration (mg/l) from March to November 2018 in Pond 1 at KWF.

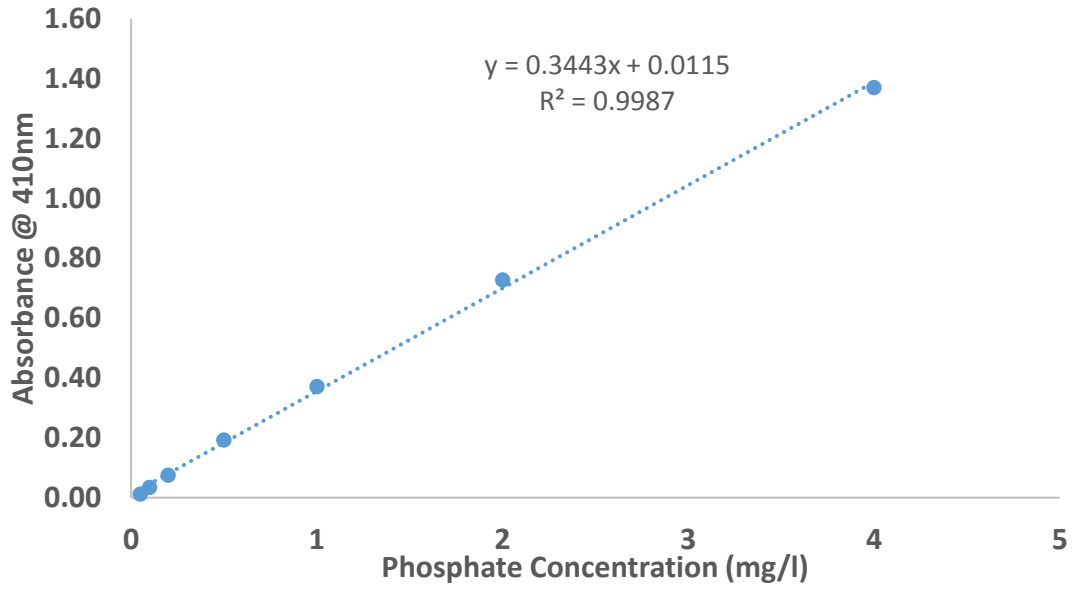

Figure 3.21 Standard curve for phosphate concentration in mg/l at an optical density of 410 nm.

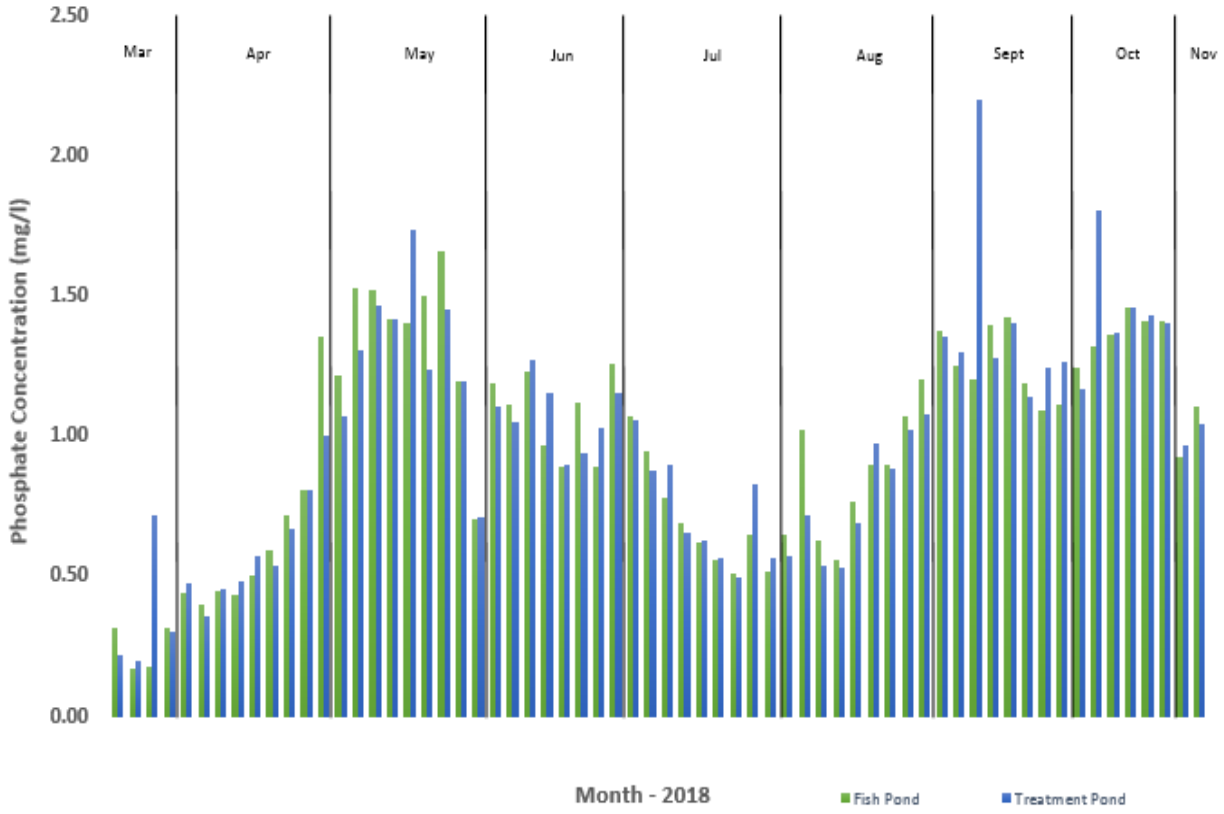

Figure 3.22 Phosphate concentration (mg/l) from March to November 2018 in Pond 1 at KWF.

Water hardness concentrations, depicted in **Figure 3.23**, were expressed as milligrams of calcium carbonate hardness ($CaCO_3$) per litre. The levels ranged from 70.06 to 650.57 mg $CaCO_3$/l in the FP and 50.04 to 630.55 mg $CaCO_3$/l in the TP. The water hardness was highest in September, with average hardness levels reaching 157.95 mg $CaCO_3$/l in FP and 129.53 mg $CaCO_3$/l in TP, when the two high values reached in September were excluded. The two high results may be outliers or there may have been a change in levels of calcium or magnesium at this time of sampling. With the exclusion of these high results, the overall water hardness classification in pond 1 of KWF was slightly hard to moderately water. It is important to have a sufficient level of water hardness as the minerals contributing to the hardness levels are essential for many biological processes in fish such as bone formation and blood clotting (Wurts and Durborow, 1992).

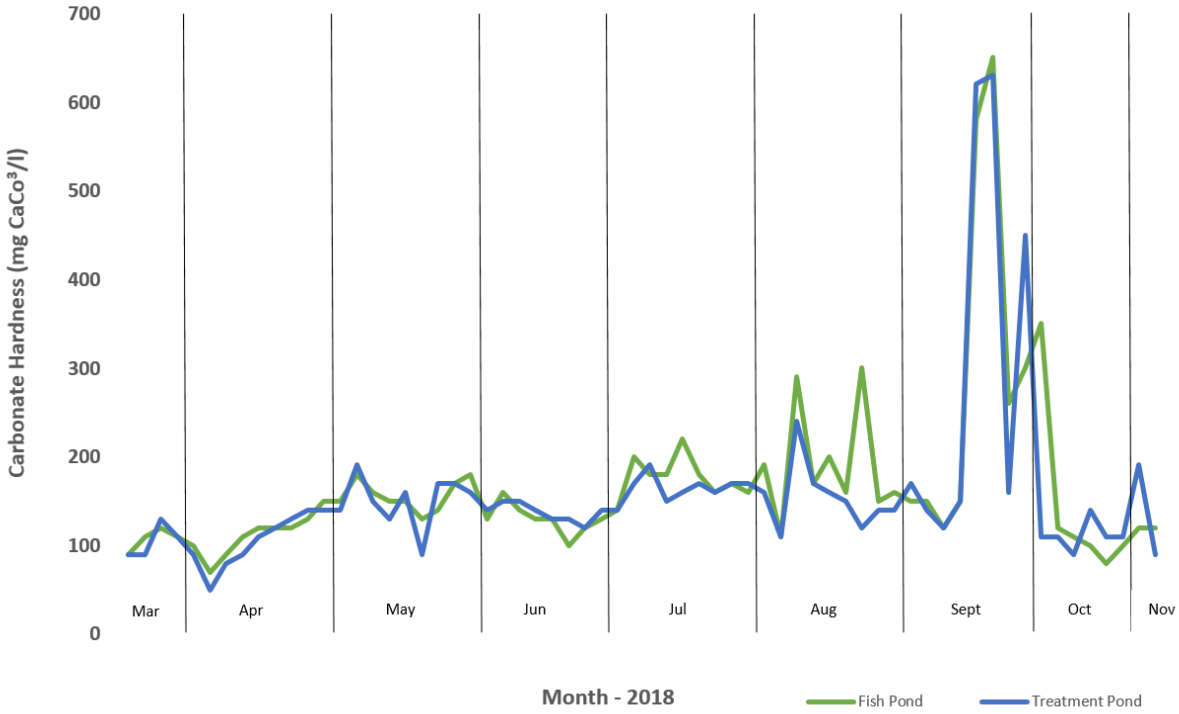

Figure 3.23 Calcium carbonate hardness concentration (mg CaCO3/l) from March to November 2018 in pond 1 at KWF.

Turbidity of samples is a factor that may affect results by interfering with the spectrophotometric method of analysis, providing false high positives in cases where the mg/l of the parameter in question may be low. To investigate the impact of this

on the results from this study, two samples were filtered for the nitrite test, one which turned very pink during the initial test (prior to filtration), giving a high absorbance value, and another which remained clear, resulting in a low absorbance value. Following filtration, the test was carried out again and the same results were observed. Therefore, it was determined that turbidity had no impact on the measurement process.

One main observation from the physicochemical analysis is that the results obtained for both the FP and the TP were very similar. A D'Agostino and Pearson normality test indicated that the data for nitrate results were normally distributed; $p > 0.05$, where $p < 0.05$ implies a statistically significant difference at the 95% level of confidence. An unpaired t-test was used to determine differences between the nitrate concentrations for the FP and TP. The data was not normally distributed with $p < 0.0001$ in the case of nitrite, ammonium, phosphate and carbonate hardness. Therefore, rather than using a parametric test to analyse the data, a nonparametric test in the form of a Mann-Whitney test was used. There were no statistically significant differences between the data obtained for both sections of pond 1 (p . 0.05) for nitrate, nitrite, ammonium, phosphate and carbonate hardness, regardless of the test used for analysis, with the p values displayed in **Table 3.6**. These results may indicate that there was a sufficient flow and circulation of water within the entire pond for the duration of the study.

Table 3.6 P values for determining significance between the data obtained for the FP and TP in KWF for nitrate, nitrite, ammonium, phosphate and carbonate hardness concentrations.

Parameter	P Value (where p < 0.05 = significant)
Nitrate	0.9445
Nitrite	0.9347
Ammonium	0.7567
Phosphate	0.9215
Hardness	0.0619

In terms of the concentration levels reached for all of the parameters, there are many recommended limits for good water quality in the cultivation of freshwater fish by EC 2006/44 Directive, the EPA and the FAO. Boyd (1998) suggested that the most acceptable nitrogen levels in aquaculture ponds are 0.2 to 2.0 mg/l of ammonium, <

0.1 mg/l of ammonia, 0.2 to 10 mg/l of nitrate and < 0.3 mg/l of nitrites. With nitrate and ammonium levels below 2 mg/l and nitrites below 0.14 mg/l, the parameters measured in KWF rearing water were all within the desirable limits. The estimated ammonia levels which are discussed further on in this section were also below 0.1 mg/l. In terms of carbonate hardness, Wurts and Durborow (1992) suggested a range of between 100 – 250 mg/l $CaCO_3$. For the majority of the time points analysed in this study, the carbonate hardness fell within these limits. Many studies have been carried out that conclude the most acceptable levels of each water quality parameter for successful freshwater fish production. However, the levels vary depending on the aquaculture set up, i.e. tanks, ponds, RAS systems, the intensity of the practice, as well as the type of fish being cultured. In addition, the unique pill-pond system at KWF, which is also impacted by the presence of the nutrients in the soil, may require the establishment of revised acceptable water quality parameter limits for this type of aquaculture set up.

The same trends for nitrates, nitrites, ammonium and phosphates were repeated in **Figures 3.24** to **3.27** for comparison with KWF analysis of the same parameters. The kits used on-site at KWF were based on colorimetric analysis, which provides an estimate of the concentration of each relevant parameter. The kits used in the laboratory were Spectroquant® and MColortest™ as previously mentioned, which require the use of a photometer or a spectrophotometer producing more precise measurements for each parameter. One main consideration regarding the nitrate and nitrite analysis is that the estimated levels provided by on-site colorimetric kit analysis compared to kits used in the laboratory can lead to a large variation in results compared to the laboratory analysis. The results for the on-site analysis was a value of zero for the majority of measurements in the case of both nitrates and nitrites. However, there were also sudden peaks in both parameter measurements using the on-site analysis, reaching high concentrations of 10 and 2.0 mg/l for nitrates and nitrites, respectively, which were not observed in the laboratory results.

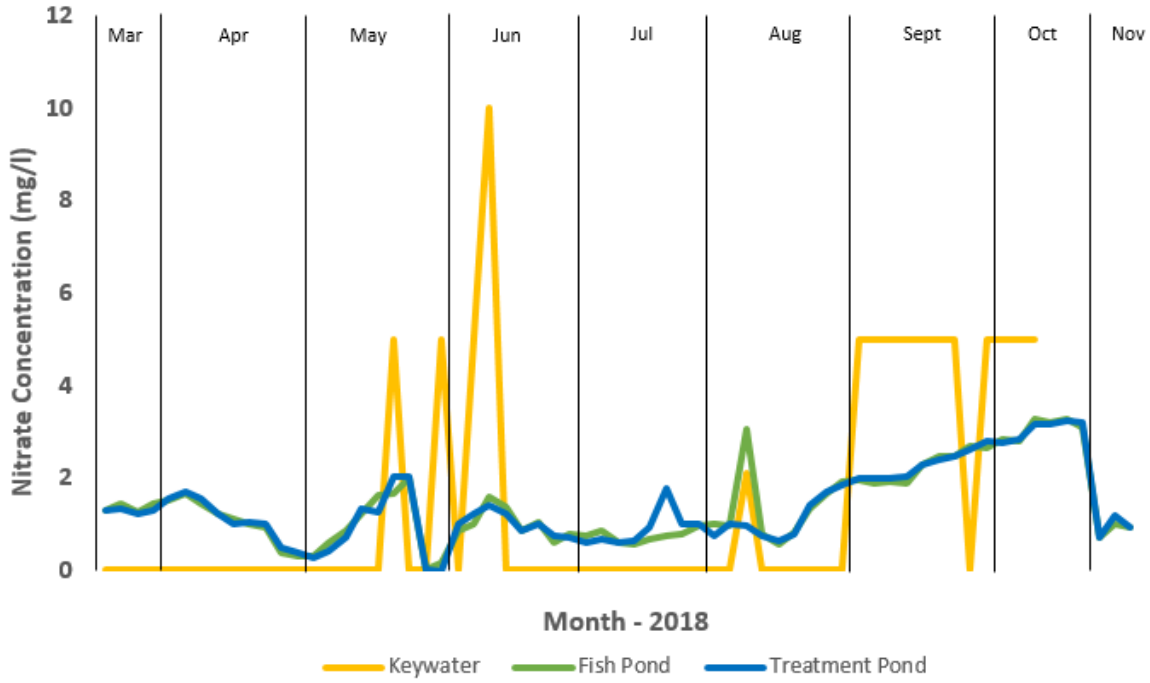

Figure 3.24 Nitrate trends *via* lab analysis for pond 1 in KWF from both the FP and TP from March to November 2018 compared to on-site analysis of same.

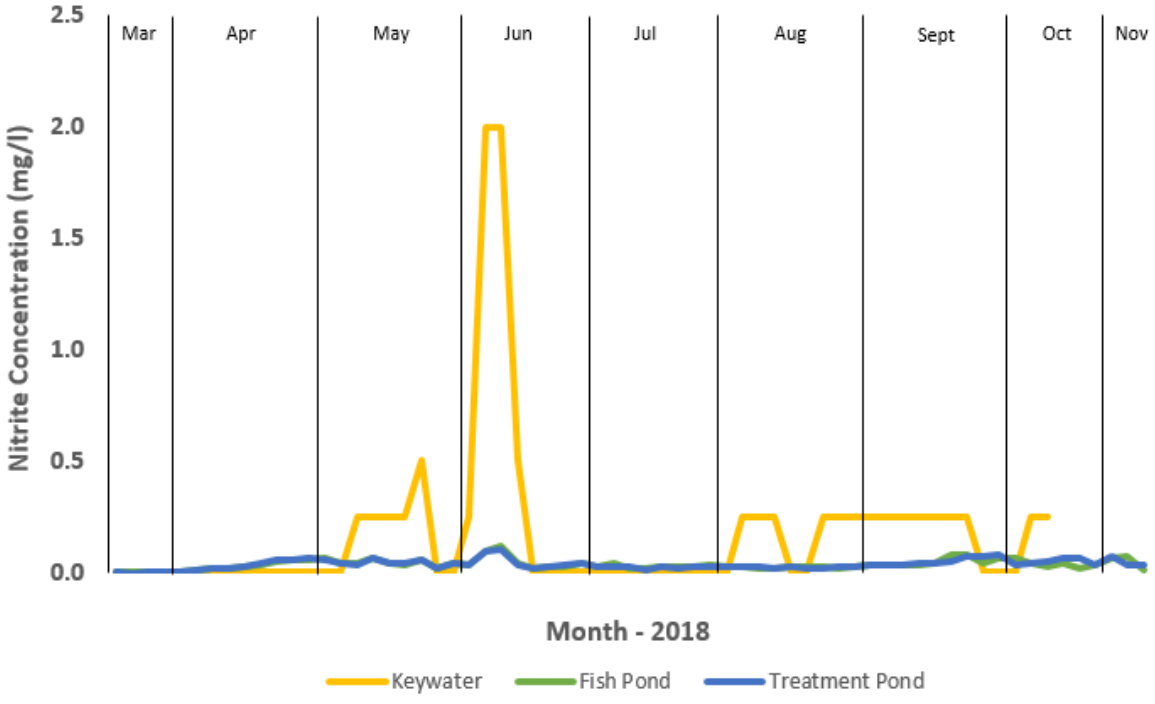

Figure 3.25 Nitrite trends *via* lab analysis for pond 1 in KWF from both the FP and TP from March to November 2018 compared to on-site analysis of same.

In the case of ammonium trends, similar results between on-site measurements and laboratory measurements were obtained as illustrated in **Figure 3.26**. The laboratory

kit measured ammonium, whereas TAN was measured on-site. However, as approximately 90% of the water at pH 7.4 (the average pH of the rearing water in KWF) is in the form of ammonium, results obtained *via* both kits are still relatively representative of the same parameter. A parameter table by Boyd (1982) based on pH, water temperature and TAN concentration of the water in mg/l, allows the estimation of the more toxic, unionised form of ammonia. Based on this table the values for unionised ammonia in the rearing water were estimated and did not exceed a concentration of 0.01 mg/l in any case. The fact that this only accounts for 10 % of the TAN measured indicates that the low ammonia levels estimated would not have a negative effect on fish health or production.

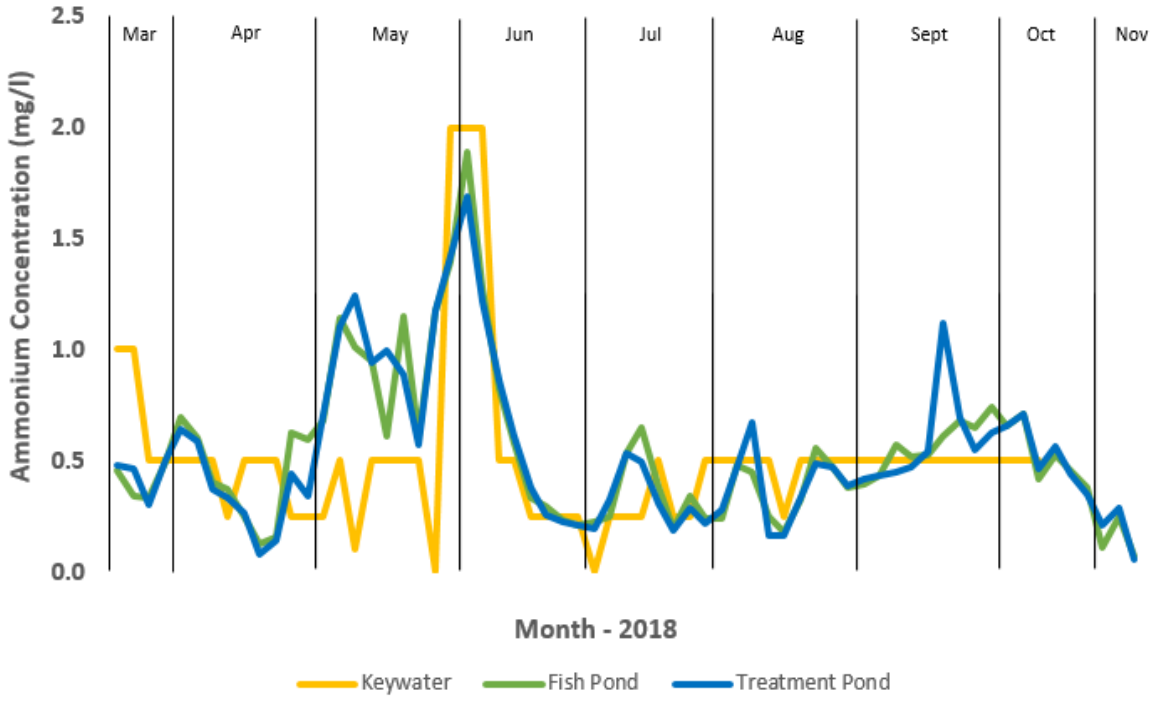

Figure 3.26 Ammonium trends *via* lab analysis for pond 1 in KWF from both the FP and TP from March to November 2018 compared to on-site analysis of same.

In terms of phosphate concentrations measured, the results obtained with the laboratory method were higher than those obtained on-site for both the FP and TP for almost every sample. This may lead to an underestimation of the phosphate levels present in the rearing water which can have a major effect on phytoplankton growth. As demonstrated by comparing both the on-site and *ex situ* techniques for physicochemical parameter analysis certain parameters were overestimated e.g.

nitrites, whereas others were underestimated e.g. phosphates, thus ease of use of on-site analysis must be weighed against inaccuracy of results.

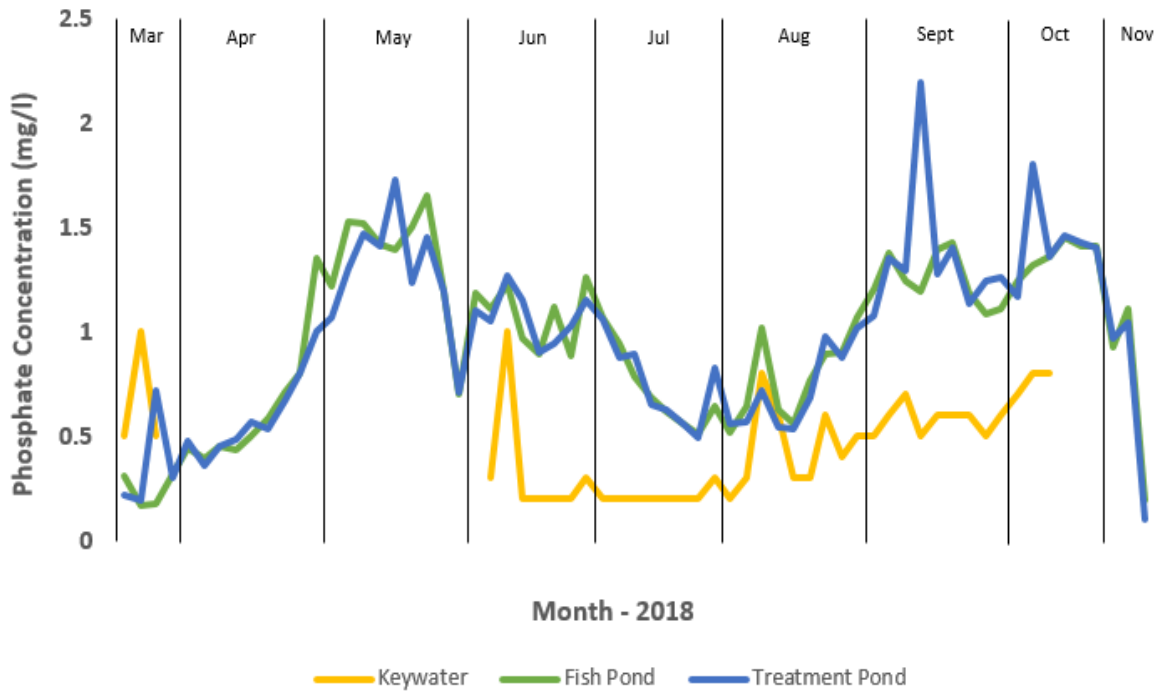

Figure 3.27 Phosphate trends *via* lab analysis for pond 1 in KWF from both the FP and TP from March to November 2018 compared to on-site analysis of same.

In general, the lab analysis produced slightly higher results than those obtained *via* on-site methods, with the exception of the high peaks obtained with the on-site kit between the end of May and the start of June. These peaks were marginally reflected by the lab results, and in some cases were not reflected at all. This demonstrates the difference in the specificity of both methods and so further research into the sensitivity limits of all kits is required as well as determining the specificity of parameter concentrations required for successful production on the farm.

3.15 Bacterial enumeration

As mentioned in the methods section of this Chapter, biweekly bacterial counts were obtained *via* the use of the epifluorescent microscope for both the FP and TP. The trend for this analysis from March to November 2018 is displayed in **Figure 3.28** expressed as $\log_{10}$ cells/ml. The overall average count of total bacteria for pond 1 was 6.33×10^6 cell/ml. It is difficult to ascertain whether this count is problematically high

or not, as the method of enumeration involves total bacterial counts, including both beneficial and pathogenic species. The temperature was unusually high for a long period during the summer months, which has a massive influence on bacterial growth (Tang *et al.,* 2014). Based on the literature, bacterial numbers in freshwater aquaculture environments range from 3.01 x 10^7 CFU/ml (Banu *et al.*, 2001) to 3.5 x 10^9 cells/ml (Qin *et al.*, 2016). Other authors suggest that levels of *E. coli* ranging from 1 x 10^3 to 1 x 10^4 CFU/ml are critical levels for certain fish species (Pal and Das Gupta, 1992; Guzmán *et al.*, 2004). Therefore, the diversity analysis and speciation of prominent members of the bacterial community through DGGE is a key step in this research for elucidating baseline information about microbial diversity in the fishponds.

A D'Agostino-Pearson normality test carried out on the bacterial enumeration data established that the data was not normally distributed for the FP or the TP. In comparison to the physiochemical analysis, a Mann-Whitney test indicated that there was no significant difference between the data obtained for the FP and the TP with a p value of 0.6987, with significance present when $p < 0.05$ (95% level of confidence).

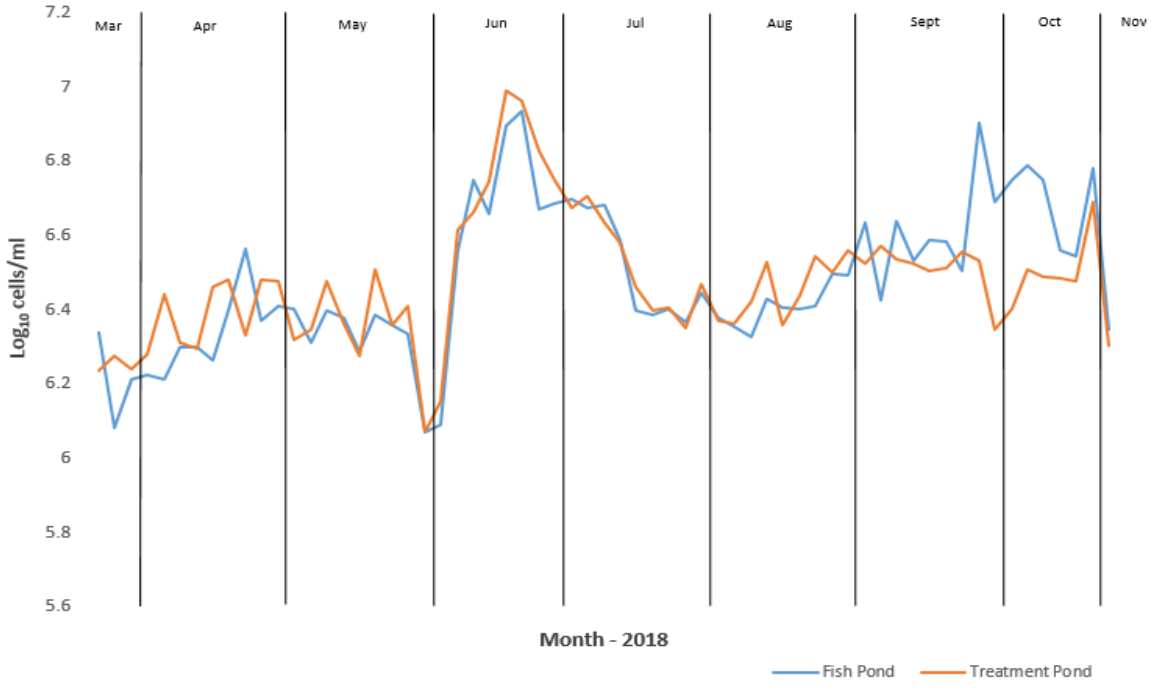

Figure 3.28 Bacterial enumeration (log_{10} cells/ml) from March to November 2018 in pond 1 at KWF.

3.16 Bacterial community profiling

Bacterial community profiling was carried out, as one of the key aims for the EcoAqua project was to develop an early warning system for problematic bacterial pathogens *e.g. A. salmonicida*, before clinical manifestations occur. This section outlines and discusses the results obtained for the various stages of this analysis, including gel electrophoresis results for the DNA extraction, PCR and nested PCR, DGGE analysis and sequencing of relevant bands from bacterial community profiles.

3.16.1 DNA extraction – Confirmatory gel electrophoresis

As mentioned in the methods section of this Chapter, gel electrophoresis was initially carried out to confirm the successful extraction of DNA from the KWF water samples using the PowerWater DNA extraction kit. **Figures 3.30** to **3.31** confirm the presence of high molecular weight DNA from all samples following DNA extraction. The DNA samples appeared as bright bands on an agarose gel under UV trans illumination. A 1kb ladder was used in the initial gel electrophoresis experiments displayed in **Figure 3.29**. A 100 bp Plus ladder was also used for later gels after PCR amplification where smaller size products were expected and is also displayed in **Figure 3.29**.

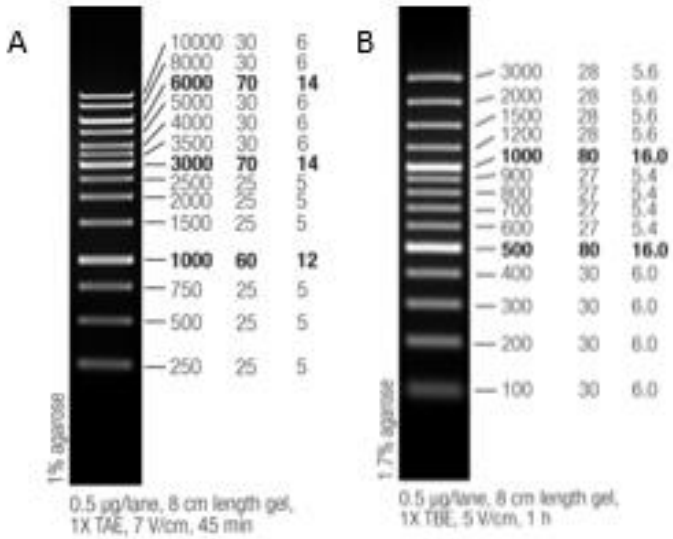

Figure 3.29 Band sizing for **A)** Thermo Scientific GeneRuler 1 kb DNA Ladder and **B)** Thermo Scientific GeneRuler 100 bp Plus DNA ladder.

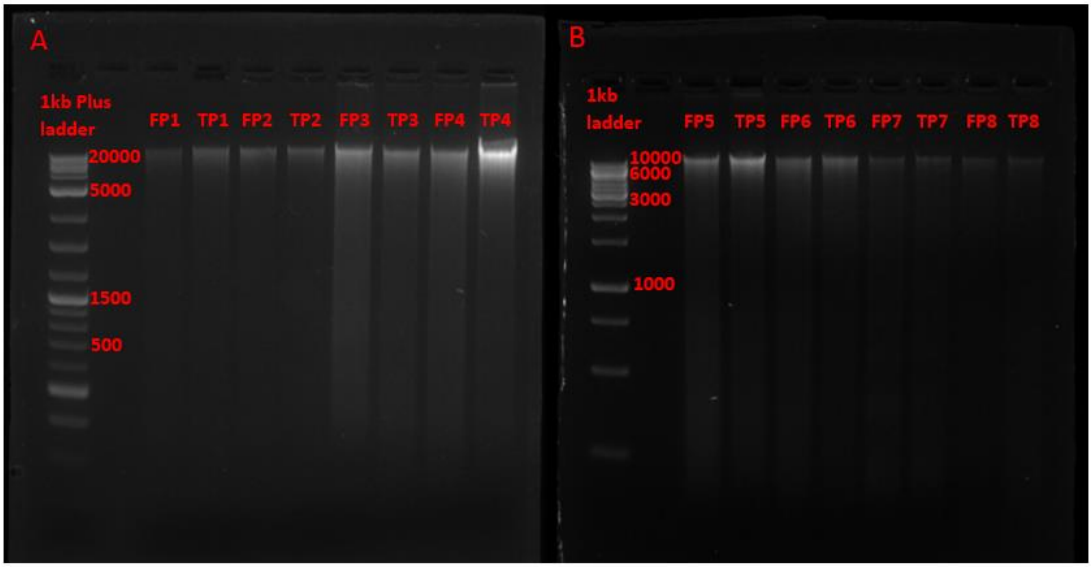

Figure 3.30 Agarose gel of DNA extraction from KWF (**A.** Samples 1-4 and **B.** samples 5-8 of FP & TP).

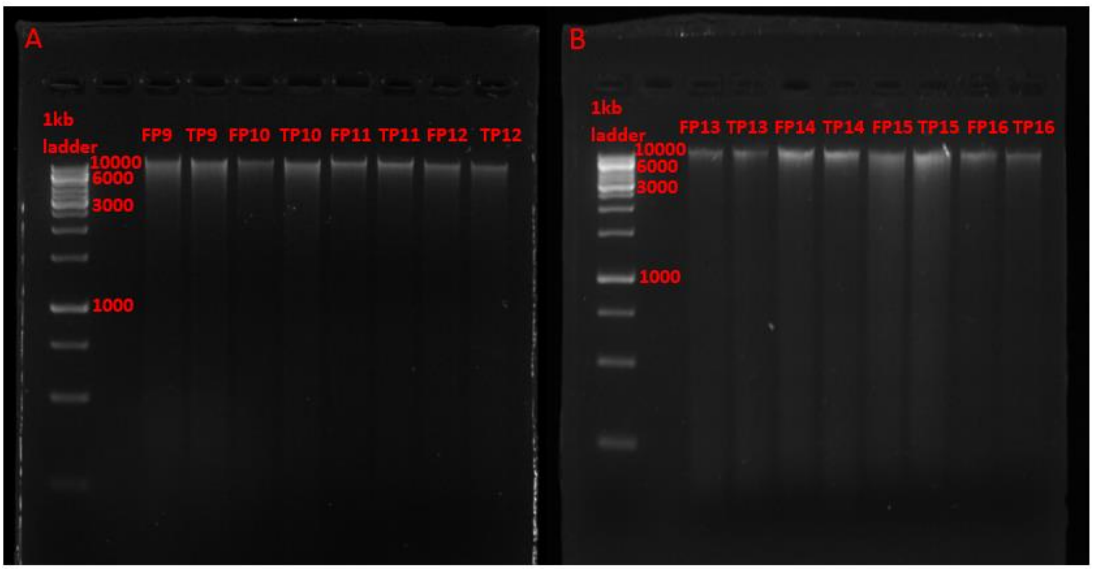

Figure 3.31 Agarose gel of DNA extraction from KWF (**A.** Samples 9-12 and **B.** samples 13-16 of FP & TP).

3.16.2 16S PCR – Confirmatory gel electrophoresis

Following DNA extraction of the samples from KWF, PCR was carried out using the 16S rRNA gene as the target. The products of the 16S rDNA PCR are displayed in **Figures 3.32** and **3.33** on an agarose gel under UV trans illumination. In this case, a 100 bp Plus ladder was used for samples 9 to 12, rather than a 1 kb ladder. In all cases, the products were just under 1,500 bp in size.

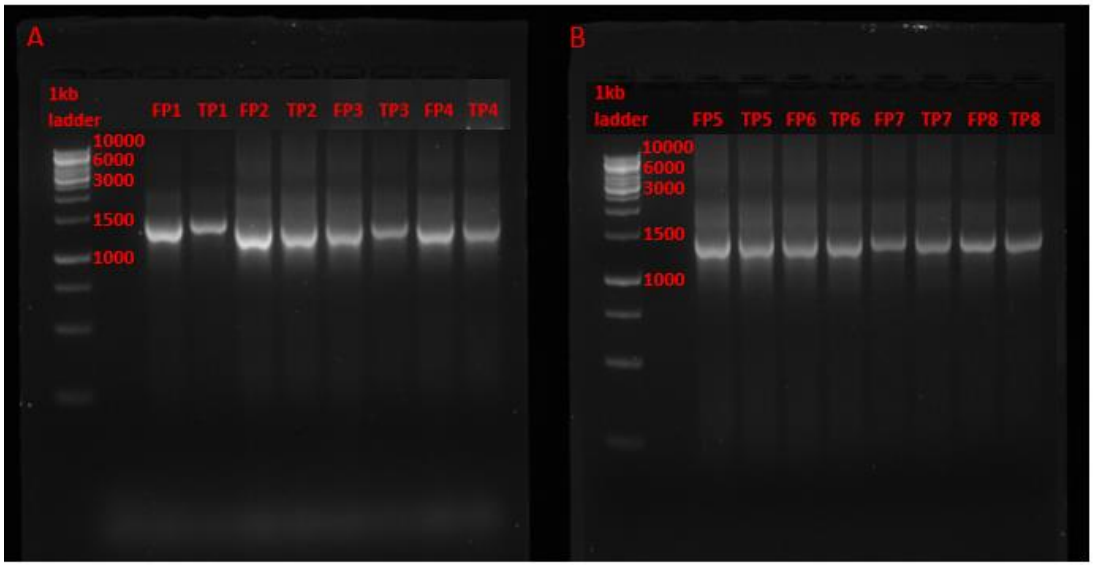

Figure 3.32 Agarose gel of 16S DNA PCR from KWF (**A.** Samples 1-4 and **B.** samples 5-8 of FP & TP).

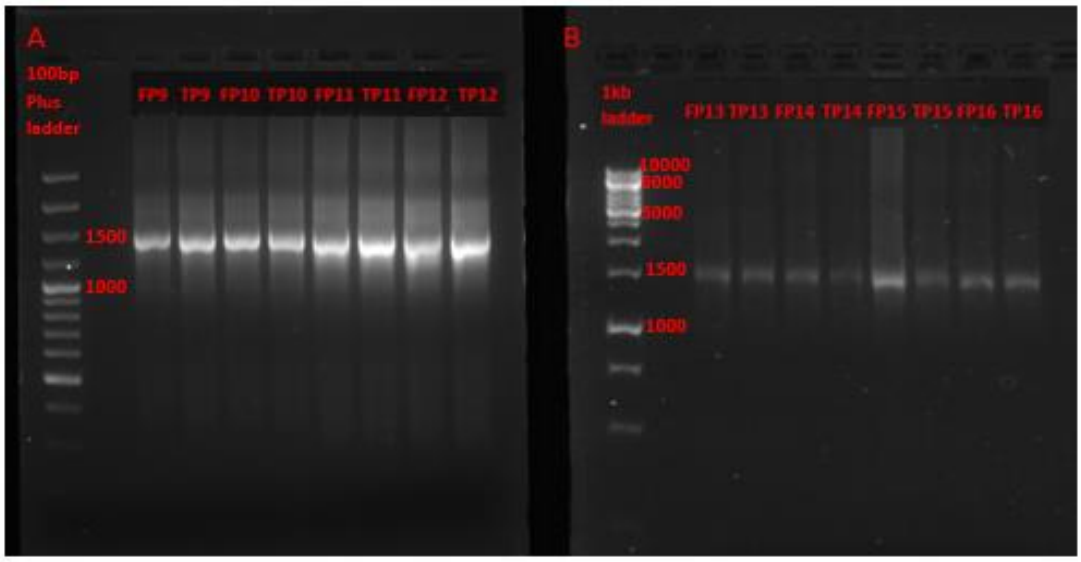

Figure 3.33 Agarose gel of 16S DNA PCR from KWF (**A.** Samples 9-12 and **B.** samples 13-16 of FP & TP).

3.16.3 DGGE 'nested' PCR – Confirmatory gel electrophoresis

The 16S rDNA PCR products were utilised as templates in the nested PCR to generate amplified V3 region PCR products for use in DGGE analysis. **Figures 3.34** and **3.35** display the products of the nested DGGE PCR process. The 100 bp ladder was used and products were approximately 200 bp in size as expected. *A. salmonicida* and *E. coli* were also subjected to DNA extraction and PCR to act as positive controls for PCR reactions. Results of the nested PCR for both bacteria are displayed in **Figures 3.34**

and **3.35**. *E. coli* is a bacterial species found in almost all water sources, and *A. salmonicida* is a problematic pathogen present in KWF, which can lead to disease outbreaks if not monitored.

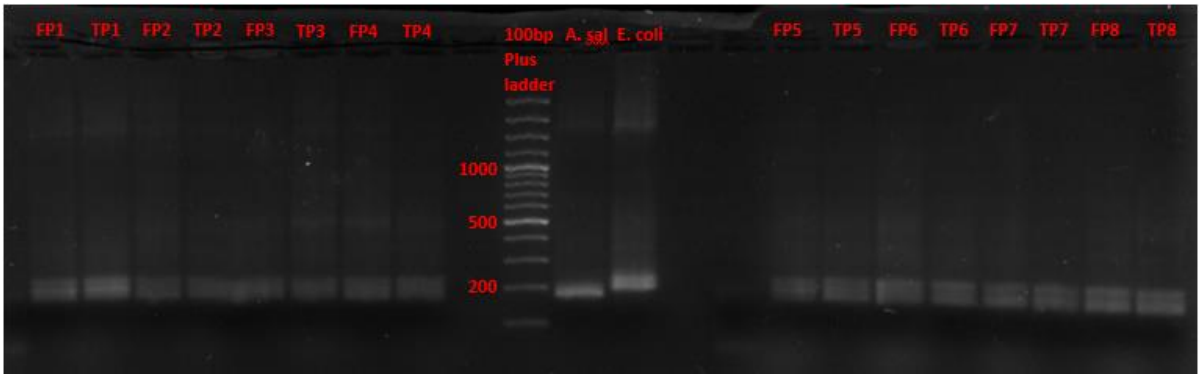

Figure 3.34 Agarose gel of DGGE 'nested' PCR samples 1-8 from FP & TP, *A. salmonicida* (abbr. A. sal) & *E. coli.*

Figure 3.35 Agarose gel of DGGE 'nested' PCR samples 9-16 from FP & TP, *A. salmonicida* (abbr. A. sal) & *E. coli*.

3.16.4 DGGE analysis

For the DGGE analysis, many bands were observed per each sample obtained throughout the study, with greater bacterial diversity being observed from certain samples. **Figures 3.36** and **3.37** illustrate the DGGE gel banding pattern for samples 1 – 16 of the FP. Samples 1 – 16 for the TP are illustrated in **Figures 3.38** and **3.39**. Some bands were common between different samples, however, identical banding between samples does not necessarily confirm the presence of the same species (Muyzer *et al.*, 1993).

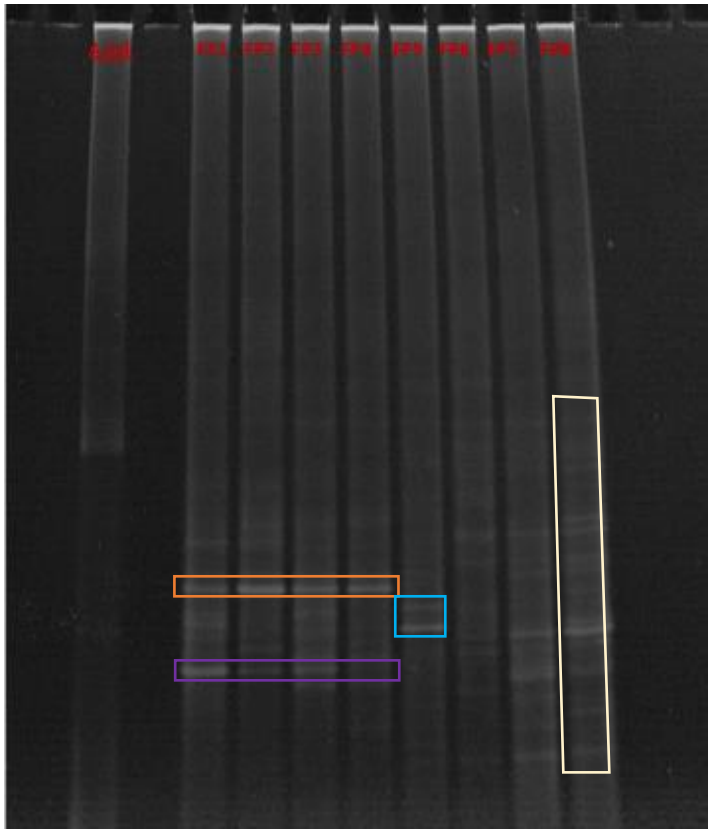

Figure 3.36 DGGE gel banding pattern for samples 1 - 8 of the FP, with *A. salmonicida* as an indicator.

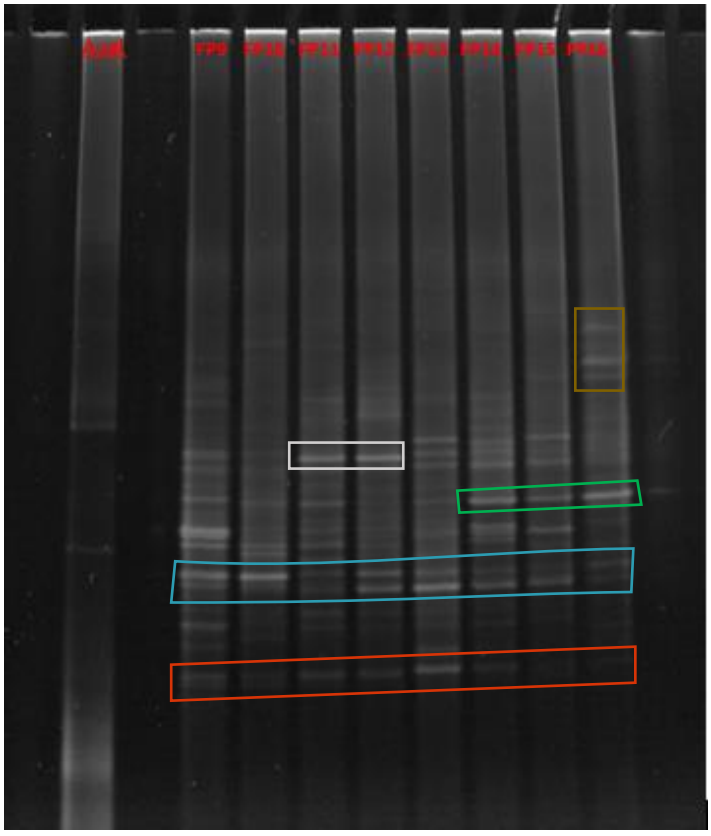

Figure 3.37 DGGE gel banding pattern for samples 9 - 16 of the FP, with *A. salmonicida* as an indicator.

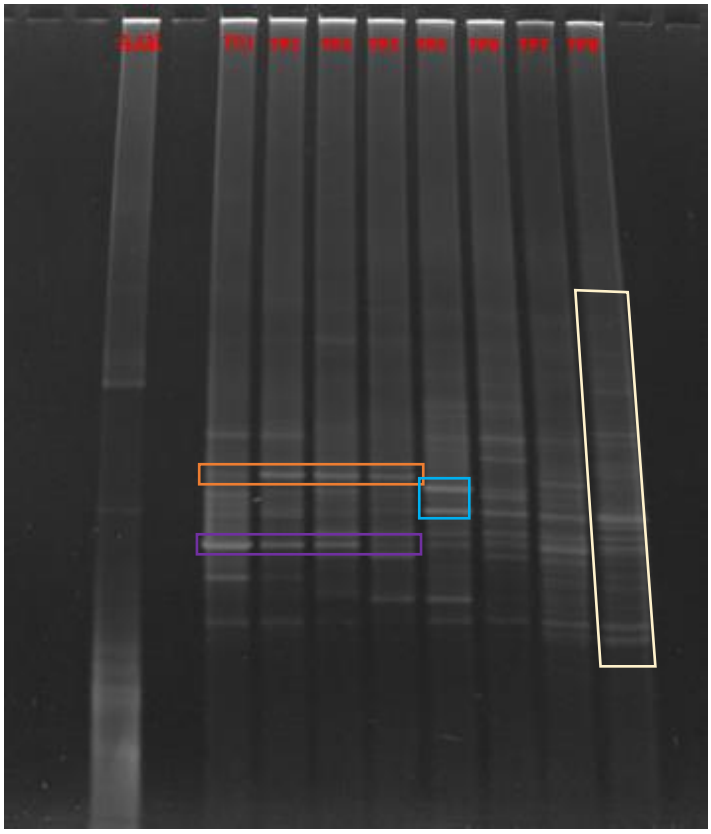

Figure 3.38 DGGE gel banding pattern for samples 1 - 8 of the TP, with *A. salmonicida* as an indicator.

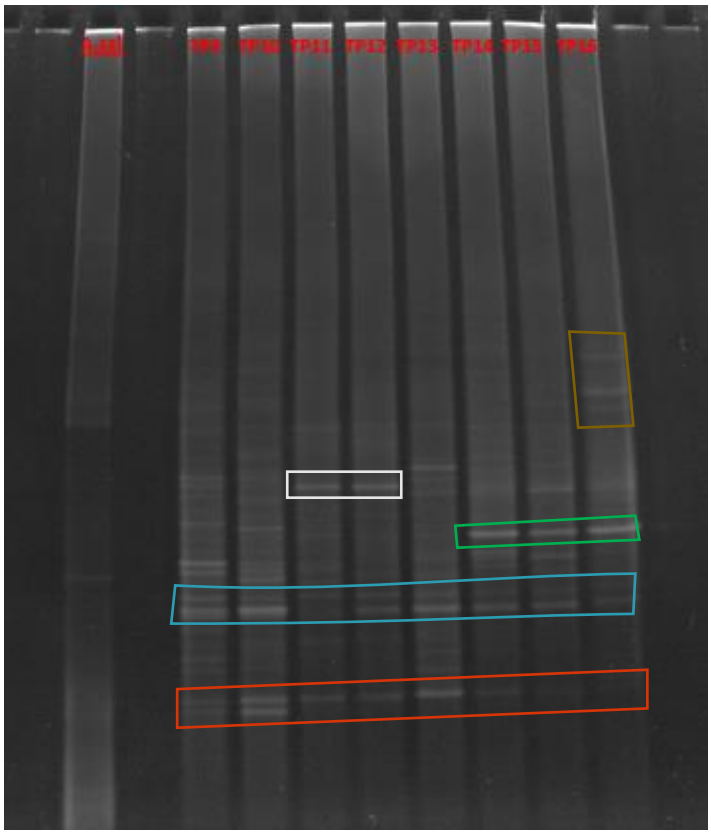

Figure 3.39 DGGE gel banding pattern for samples 9 - 16 of the TP, with *A. salmonicida* as an indicator.

The banding patterns in the DGGE gel images (**Figure 3.36** to **3.39**) of the FP and the TP were very similar for the duration of the study, indicating that a comparable range of bacterial species was present in both ponds, despite one pond containing fish and the other lacking fish. The number of individual bands, of varying size and strength, in the lanes ranged from 11 to 20. The lowest numbers of bands were present in the lanes from March for the FP and the end of April for the TP. The highest number of bands were present in the lanes from mid-July for the FP (19 bands) and the end of June for the TP (20 bands). This indicates that the lowest bacterial diversity, as detected by DGGE analysis, was observed in the rearing water in the first two months of the study, whereas the greatest bacterial diversity occurred between June and July. The temperature peaked during these summer months, which may have had an effect on the overall microbial species dynamics, leading to an increase in the diversity profile. The number of bands in each lane decreased following the summer months, averaging between 12 and 14 bands per lane, but increased to 17 bands for both the FP and TP at the end of October (the last lane in **Figures 3.37** and **3.39**). At this time point, nitrate levels peaked, reaching concentrations of 3.27 mg/l and 3.25 mg/l for the FP and TP, respectively. This sudden influx of organic matter may have provided the opportunity for an increase in numbers of slow growing k selected bacterial species (Vadstein *et al.*, 2018) that had previously been undetected in samples. **Figures 3.36** and **3.38** show the similarity between the banding patterns of the FP and the TP from March to June, with examples of common bands outlined in the images. **Figures 3.37** and **3.39** illustrate the banding pattern for the FP and TP between July and October, with an almost identical banding pattern observed between the two gel images. Examples of similar diversity profiles between the FP and TP are highlighted. There were many bands that were common between samples through the study period. The common bands excised had top sequence matches on BLAST with Proteobacteria, specifically *Brevundimonas* sp., *Rhodobacter* sp. and *Polynucleobacter* sp., Cyanobacteria and phytoplankton chloroplast bacteria. This is explained in more detail in **Section 3.12.6**. Overall, the bacterial population diversity peaked during the summer months, with similar bacterial community profiles observed between the FP and TP. This microbial balance may be partly responsible for the healthy condition of the fish for the duration of the study, as a dysbiosis of

the commensal bacteria by opportunistic bacteria is suggested to be the most likely cause of pathogenic breakouts (Vadstein *et al.*, 2018).

3.16.5 Unweighted Pair Group Method with Arithmetic Mean analysis

In order to determine the seasonal bacterial trends for the duration of the study, the banding patterns on the DGGE gels were compared using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis. The online program 'DendroUPGMA: A dendrogram construction utility' was used in combination with MEGA 7.0 software to carry out this analysis. The total number of unique bands across all lanes was initially determined and a binary matrix recording the presence and absence of bands as 1's or 0's was constructed. This was carried out for individual gels followed by comparison of all gels which resulted in a large binary data file. Using DendroUPGMA and MEGA 7.0 dendrograms were created for all the data analysed. Figures **3.40** and **3.41** illustrate the bacterial patterns in both the FP and TP from March to October, respectively. Figure **3.42** illustrates the similarities/differences of the bacterial trends between the FP and TP for the duration of the study.

The dendrogram created from the banding patterns of the FP revealed a changing bacterial population from the middle to the end of each month in the rearing water ecosystem over the duration of the study. The banding pattern for the end of June was divergent from all other sampling periods, showing the longest branch length in the dendrogram, reflecting greatest differences in the bacterial community at that time point. This may have been in part due to the high temperature peaks in June and the decrease in ammonium levels at the end of June. During the end of June the phytoplankton levels peaked. This may have provided a new source of organic matter for bacteria to thrive on, including bacteria who may have been outcompeted for a source of organic matter at other periods during the study. The dendrogram created for the banding patterns from March to October of the TP also displayed constant changes in the bacterial population in the rearing water ecosystem from the middle to the end of each month. Once again, the bacterial diversity for the end of June was the most distant to all of the other sampling periods.

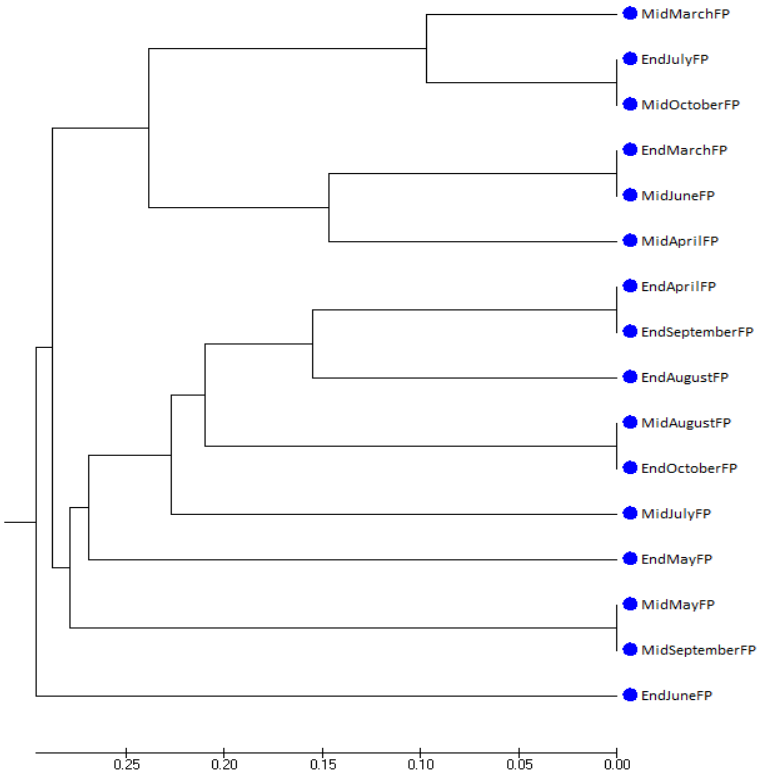

Figure 3.40 Dendrogram following UPGMA analysis of the bacterial trends from March to October for the FP in pond 1 at KWF.

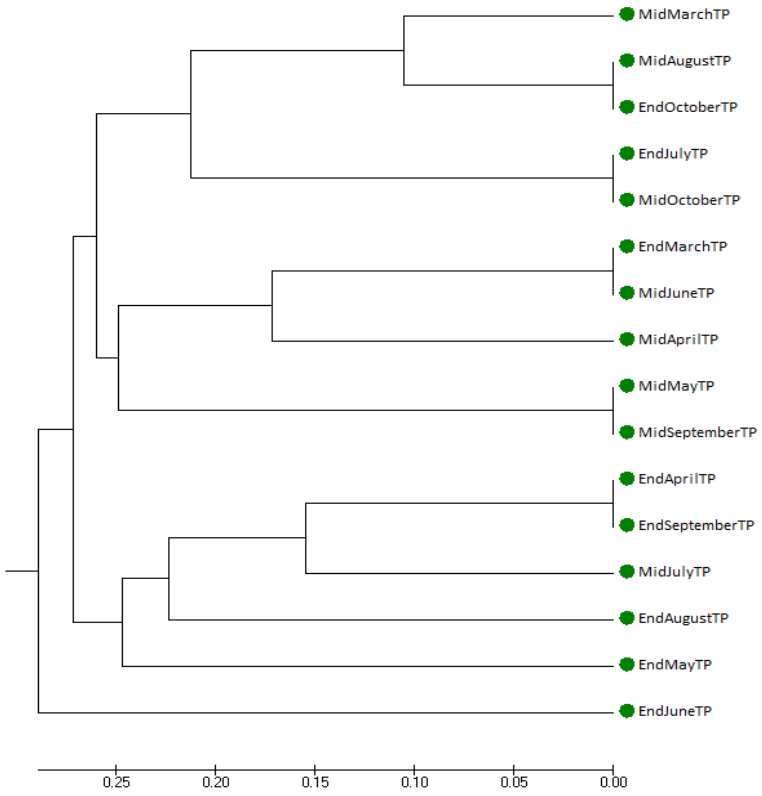

Figure 3.41 Dendrogram following UPGMA analysis of the bacterial trends from March to October for the TP in pond 1 at KWF.

The dendrogram in **Figure 3.42** encompassing all band patterns from both the FP and the TP displays the trends in three clusters essentially. The bottom cluster indicates that band patterns for the FP at the end of May and middle of August shared the least amount of similarity with the rest of the banding patterns. Following that, the band patterns for the end of June for the FP are closest to the band patterns for the middle of June (FP) and the end of April (TP) based on the tree topology. The last two large clusters indicate that the band patterns, in general, share a similar trend.

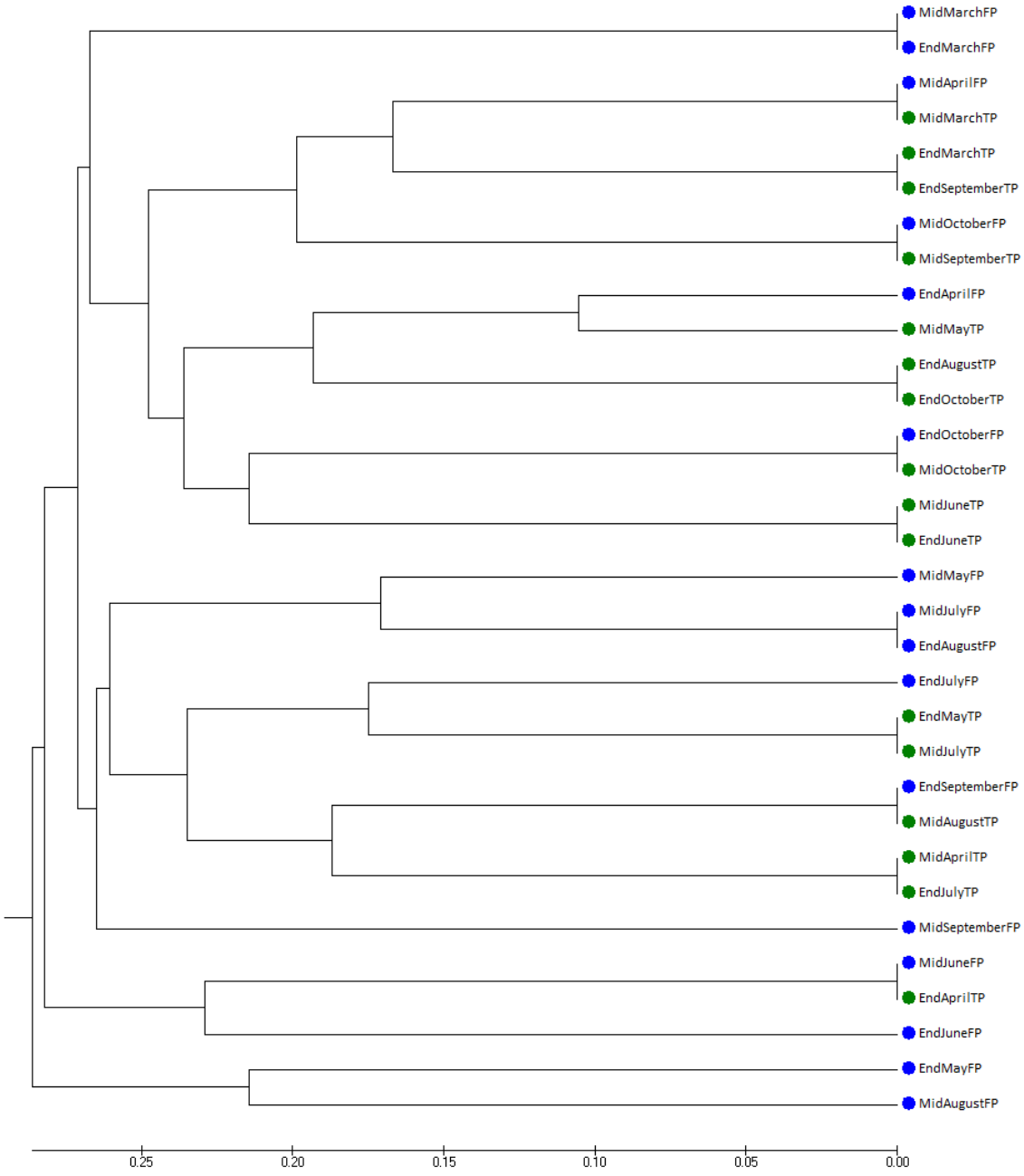

Figure 3.42 Dendrogram following UPGMA analysis of the bacterial trends from March to October for both the FP and TP in pond 1 at KWF.

Although the dendrograms may indicate that the patterns were different between the FP and TP from each month, it gives a closer insight into the band pattern trends. It is clear from the DGGE gel images in **Figures 3.36** to **3.39** that the FP and TP share a great similarity in relation to the bacterial community structure from month to month. This was also investigated further through the sequencing of relevant bands excised from gels which is described in the proceeding pages in **Section 3.12.6.**

3.16.6 Phylogenetic analysis of externally sequenced DGGE DNA band samples

Phylogenetic analysis of all bands excised from the gel is presented in the form of outgroup-rooted phylogenetic trees in **Figures 3.43** to **3.47,** with the evolutionary history inferred by using the ML method based on the Kimura 2-parameter model. The blue dots denote the FP original samples, the green dots denote the TP original samples and the orange dots denote the outgroup *Methanosarcina acetivorans*, a member of the archaea included in the analysis to give perspective on the relationship between the other species. In some cases, the bootstrapping values were quite low. According to Hall (2013), nodes with a bootstrap percentage of < 70% are inaccurate in terms of phylogenetic tree reliability. However, the low bootstrap values, in this case, are most likely due to the short sequence reads (< 161 bp) of the original samples.

The first phylogenetic tree in **Figure 3.43** illustrates the taxa breakdown from the selected excised DGGE bands for the FP from March to June. Sequence FP-3N is more closely related to the Bacteriodetes phylum with more a distant sequence similarity to *Runella* genus based on the tree topology as well as an ancestral relationship to the *Flavobacterium* genus. Sequences FP-8K, FP-4M and FP-2M are more closely related to the cyanobacteria phylum and also possibly phytoplankton in the form of chloroplasts of *Chlorella*, *Cyclotella* and *Streptophyta* species. Sequence FP-7N shares a common ancestor with *Candidatus fonsibacter* ubiquis of the Bacteriodetes phylum but also had a top match on BLAST to a bacterial clone OTU 1230, which was sourced from urban tropical fresh water that ranged in temperature from 23 to 31°C (Nshimyimana *et al.*, 2017). This sequence corresponds to a DGGE gel band only

present for the months of April and June when temperatures were relatively high, which may explain sequence similarity with a bacterium sourced from tropical freshwater. Sequence FP-6B shared a relationship with bacteria sourced from river water and ciliate cells, which are both relevant to the source of the samples analysed in this study, i.e. the rearing water in the fish pond. Sequences FP-5I, FP-5J, FP-8C and FP-7L were all closely related to the phylum Proteobacteria of the *Betaproteobacteria* class. More specifically, these samples shared common ancestral phylogeny with the family *Comomonadaceae* of the order *Burkholderiales* more than any other family. Genus levels included *Delftia* species, which has been isolated from soil and aquatic habitats, freshwater and a freshwater shrimp culture pond; *Rhodoferax* species, isolated from sludge and ditch water, capable of nitrogen fixation using ammonium; and the genus *Limnohabitans* which tend to be abundant in freshwater (Willems, 2014). *Limnohabitans* can use phytoplankton exudate as a carbon source for growth, specifically from *Cryptomonas* sp., *Chlamydomonas noctigama* and *Pediastrum boryanum* phytoplankton species (Šimek *et al.*, 2011; Parulekar *et al.*, 2017). All of the aforementioned phytoplankton species were present for the duration of the study and possibly acted as a source of organic carbon for *Limnohabitans*, which also remained present in the rearing water samples for the duration of the study. This gives an insight into the plausible ecosystem balance ongoing in the rearing water that has proven beneficial in separate research investigations. Sample FP-1M also shared a close relationship to the *Betaproteobacteria* class, but of the *Burkholderiaceae* family with *Polynucleobacter* as the top genus match on BLAST, with *A. salmonicida* a common ancestor to all sequences that were closely related to *Betaproteobacteria* and *Gammaproteobacteria* classes.

All of the remaining bands excised representing the FP between March and June were also of the Proteobacteria phylum but of the *Alphaproteobacteria* class with the exception of the class *Spartobacteria* sp., a bacterium of the Verrucomicrobia phylum, that is associated mainly with soils but has also been isolated from rivers and lakes (Arnds *et al.*, 2010). Of the *Alphaproteobacteria* class, *Rhodobacter* and *Brevundimonas* had the highest number of matches with the excised sequences.

Rhodobacter belongs to a group of bacteria known as purple non-sulfur photosynthetic bacteria with *Rhodobacter* species mainly inhabiting freshwater environments, capable of nitrogen fixation using both ammonium and molecular nitrogen (Pujalte *et al.*, 2014). The *Brevundimonas* genus is the largest of the *Caulobacteracea* family, mainly found in freshwater, with some species capable of reducing nitrates as well as biosorption of heavy metals (Masoudzadeh *et al.*, 2011; Abraham *et al.*, 2014), which would be beneficial in the rearing water to maintain the quality for healthy fish production.

The second phylogenetic tree presented in **Figure 3.44** illustrates the top phylogenetic matches for the bands excised from the FP rearing water samples between July and October. Sequences FP-15A, FP-11Z, FP-9N, FP-9O and FP-12I were more closely related to the Bacteriodetes phylum, predominantly the *Flavobacteriaceae* and *Saprospiraceae* families. As described in **Chapter 2, Section 2.6**, *F. psychrophilium* is the causative agent of RTFS, but there are also other pathogenic species of *Flavobacterium*. These include *F. columnare* and *F. succinicans* (Altinok *et al.*, 2008; Pilarski *et al.*, 2008; McBride, 2014), both of which matched with sequences FP-9N and TP-9K, the bands for which appeared between July and mid-October. Although pathogenic, these bacterial species are commonly isolated from freshwaters without any indication of disease outbreaks, playing a role in nutrient recycling (Rickard *et al.*, 2003; McBride, 2014; Revetta *et al.*, 2015), making them an important genus for aquatic environments once numbers remain below the risk of widespread pathogenesis. The bands that matched with the *Flavobacterium* genus more than any other genus were present when temperatures were below 20°C. This may explain why no disease outbreaks were recorded, as the optimal temperature for rapid growth and transmission of pathogenesis from *Flavobacterium* species tends to be above 20°C (Jean-Francois Bernardet *et al.*, 2002; Declercq *et al.*, 2013; McBride, 2014). Once again, many samples analysed from July to October shared close sequence matches with cyanobacterial clones and phytoplankton chloroplast clones, highlighting the constant presence of cyanobacterial populations throughout the year in the FP. The remainder of the original samples shared a close relationship with the Proteobacteria phylum, including a combination of *Alphaproteobacteria* and

Betaproteobacteria. *Polynucleobacter*, *Brevundimonas* and *Rhodobacter* were the most common genera of Proteobacteria present for samples analysed between July and October based on the bands excised from the gel.

Figures 3.45 and **3.46** illustrate the phylogenetic trees based on the top sequence matches on BLAST with the original DNA samples analysed from the TP from March to June and July to October, respectively. There were no major differences in species diversity compared to the FP based on the bands excised for sequence analysis. Nonetheless, there were some species matches in the TP which had not matched with sequences analysed from the FP. There were two genera from the *Alphaproteobacteria* class that did not match with sequences from the FP samples, the majority of which were *Alphaproteobacteria*. Among these matches was the genus *Novosphingobium*, which has been isolated from a range of different environments including freshwater (Baek *et al.*, 2011; Glaeser *et al.*, 2013; Kumar *et al.*, 2017), and is suggested to play a role in xenobiotic breakdown with one species, namely *N. nitrogenifigens*, involved in nitrogen fixation (Glaeser and Ka, 2014; Kumar *et al.*, 2017). *Actinomyces*, of the Actinobacteria phylum, shared a 96% match with sample TP-4I. Species of Actinobacteria are commonly in the gut and on the skin of fish (Lowrey *et al.*, 2015; Schmidt *et al.*, 2016).

To investigate the applicability of DGGE as a tool for detection of *A. salmonicida*, bands occupying the expected position of the *A. salmonicida* 16S rDNA V3 region (based on the use of an *A. salmonicida* positive control) were also excised and sequenced. No match with *A. salmonicida* was achieved for these samples. Sequencing of bands from gels is challenging, due to closely related sequences in a sample occupying an almost identical position in a gel, resulting in the appearance of thick bands which may be composed of multiple sequences. This is a limitation of the method and often confirmed when high levels of background sequences, that have to be edited out, appear in the sequence data; a phenomenon that was observed with many of the sequences generated for this study. It may be that the *A. salmonicida* V3 region DNA was present in the excised gel bands but was masked by another closely related sequence present at a higher concentration, and therefore *A. salmonicida* was not detected.

A. salmonicida tends to be present in low numbers and can be very difficult to detect (Byers *et al.*, 2002), yet up to 80% of a given population of brown trout tend to be carriers of the bacteria (Gustafson *et al.*, 1992). The bacteria can be transferred to healthy fish from dead infected fish *via* the shedding process of bacterial cells into the water, the rate of which varies depending on the literature. Bullock and Stuckey (1977) recorded total counts of *A. salmonicida* up to 1.2 x 10^5 cells/ml in tank water of infected brown trout. Michel (1980) observed total counts of up to 6.0 x 10^3 cells/ml in the water from a population of infected rainbow trout fingerlings. Rose *et al.* (1989) discovered that infected Atlantic salmon mortalities can shed from 1.7 – 7.0 x 10^4 CFU per hour in freshwater. Traditional detection methods such as plating and biochemical identification can be unreliable and time consuming (Byers *et al.*, 2002). Høie *et al.* (1997) found that PCR methods were not sensitive enough to detect *A. salmonicida* subspecies *salmonicida* from infected kidney and gill samples of Atlantic salmon with the high numbers of fish cells present in the sample, as well as high concentrations of non-target DNA having an inhibitory effect on the PCR reaction (Byers *et al.*, 2002). The 16S rDNA gene sequence similarity for the *Aeromonas* genus is between 98 and 100%, which does not allow for the differentiation of *Aeromonas* species (Fernández-Álvarez *et al.*, 2016). Therefore, alternative nucleic acid based methods for detection of *A. salmonicida* may be more useful for specific detection of the pathogen, such as the use of primers targeting regions associated with *A. salmonicida* virulence factors e.g. the *vapA* surface array protein gene (Gustafson *et al.*, 1992) or the aopO gene (Fernández-Álvarez *et al.*, 2016).

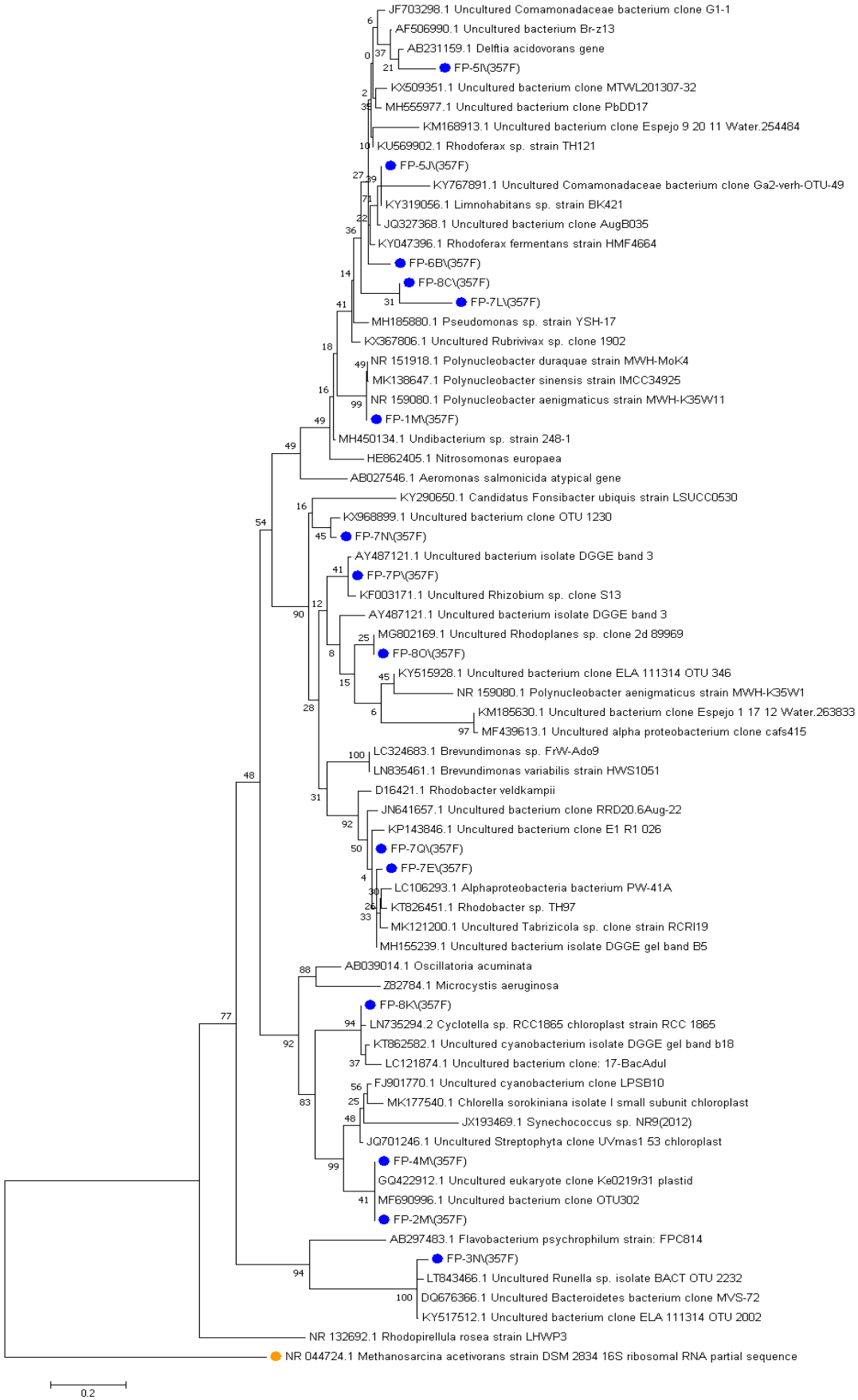

Figure 3.43 Phylogenetic tree of bacterial sequences for the FP from March to June using maximum likelihood analysis.

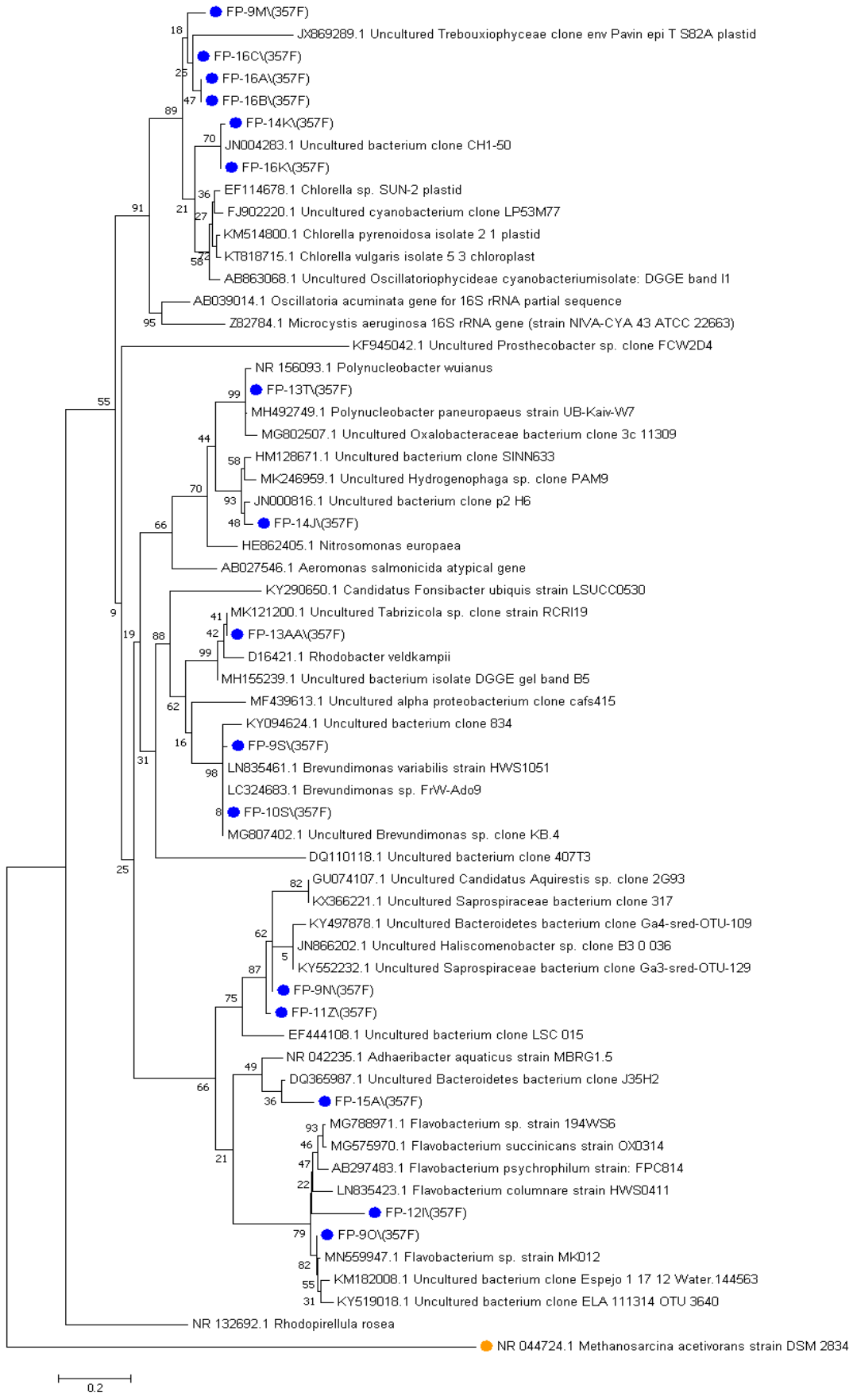

Figure 3.44 Phylogenetic tree of bacterial sequences for the FP from July to October using maximum likelihood analysis.

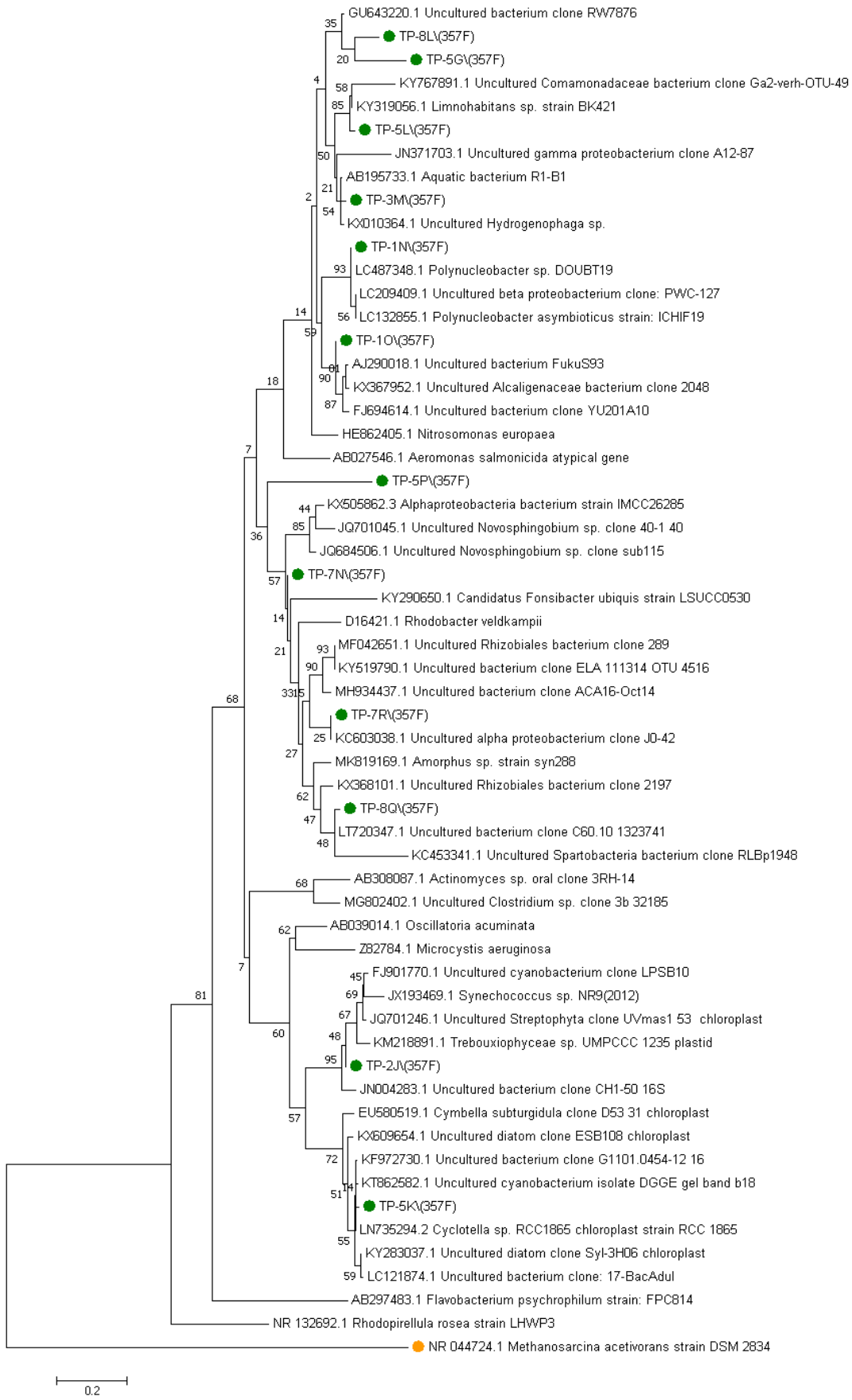

Figure 3.45 Phylogenetic tree of bacterial sequences for the TP from March to June using maximum likelihood analysis.

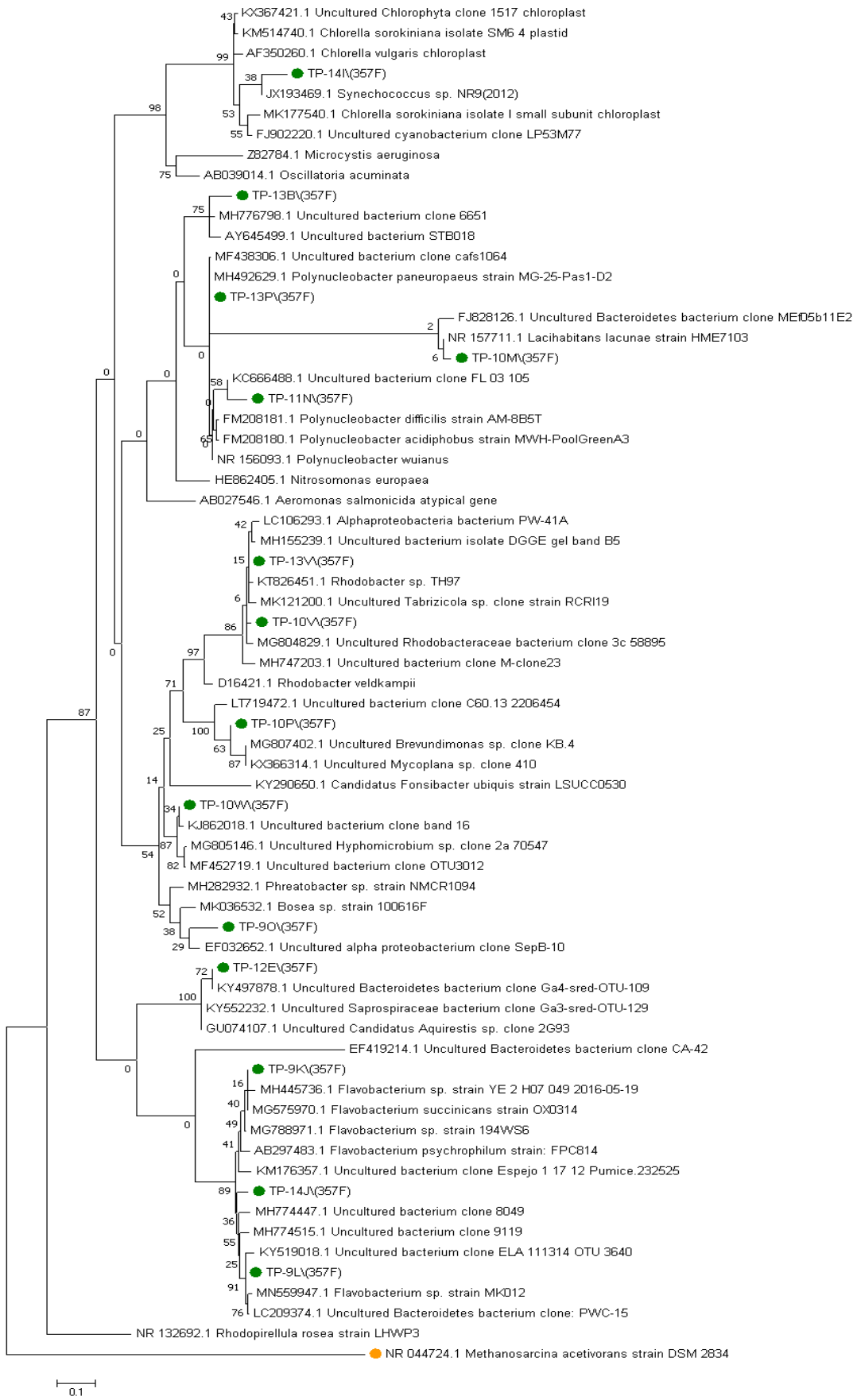

Figure 3.46 Phylogenetic tree of bacterial sequences for the TP from July to October using maximum likelihood analysis.

While the bands excised from the DGGE gels for both the FP and TP DNA samples do give an indication of the bacterial diversity present in the pond, it only represents the diversity from the selected bands excised. Other bands, while present, were extremely weak and therefore too difficult to cut from the gels. **Figures 3.48** and **3.49** indicate the relative abundance of bacterial phyla that had the top sequence matches with the original DNA samples but do not give the relative abundance of the total bacterial diversity in the pond. Of the bands analysed, it is clear from **Figures 3.48** and **3.49** that the most dominant phyla were the Proteobacteria and unclassified bacteria, which is in agreement with other studies (Pope and Patel, 2008; Eiler *et al.*, 2012; Brown *et al.*, 2015; Parulekar *et al.*, 2017; Duarte *et al.*, 2019).

It is also evident that seasonality effected the presence of certain bacterial phyla, particularly in the case of Bacteriodetes, which were more frequent in mid-summer and autumn based on the selected bands excised from the gels. Verrucomicrobia was detected more in the TP samples analysed than the FP samples.

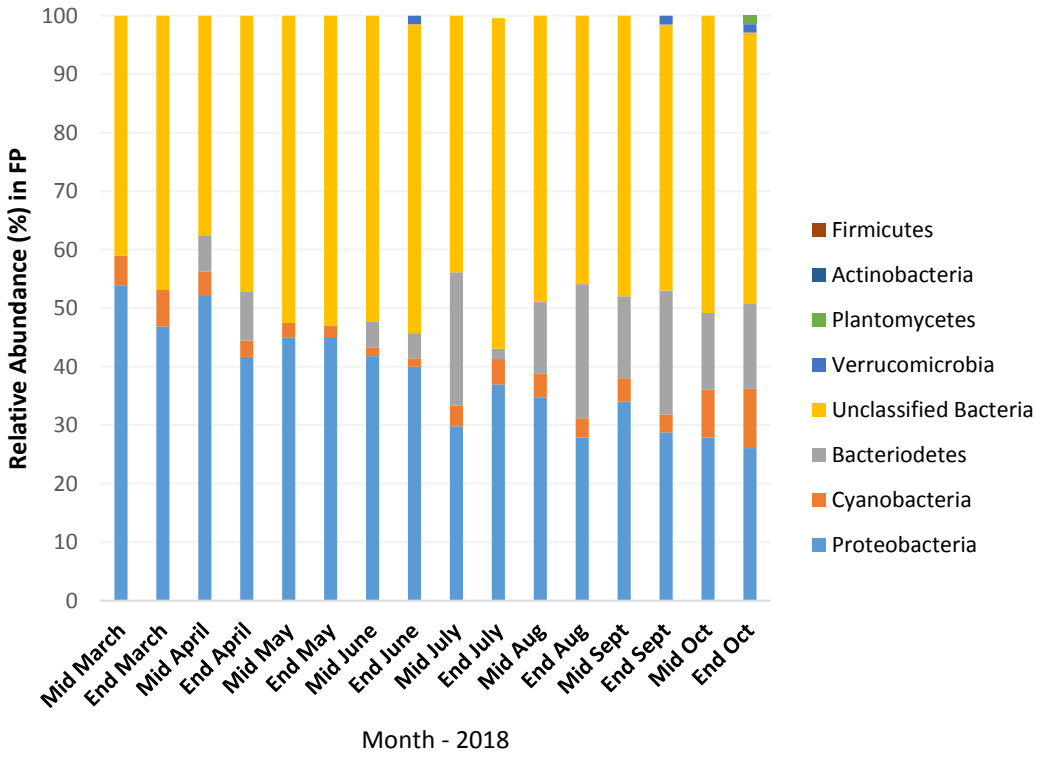

Figure 3.47 Relative abundance of the different phyla as per the DGGE gel bands excised from the FP rearing water samples.

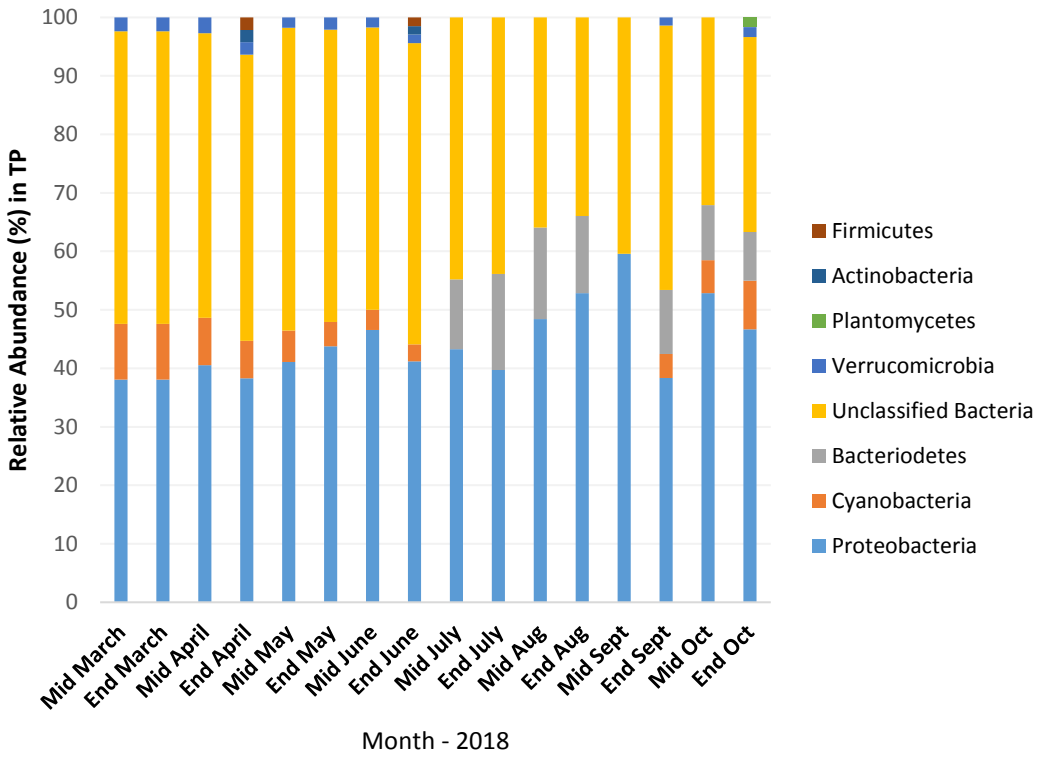

Figure 3.48 Relative abundance of the different phyla as per the DGGE gel bands excised from the FP rearing water samples.

The molecular DGGE-PCR method of taxa identification provides a quick way of sample analysis and has the ability to identify the presence of some bacteria that are pathogenic or toxic such as *Aeromonas* sp. or cyanobacterial species, which cannot be identified using classical microscopic methods. However, there are certain limiting factors to this method. The number of cells/ml cannot be determined and in some cases, analysis *via* DGGE-PCR requires a certain number of cells present for accurate detection. For example, Bukowska *et al.* (2014) indicated that for the efficient detection of *Microcystis* the cell density required was greater than 30 cells/ml. There can also be issues when visualising bands on the DGGE gel; weak bands tend to be difficult to excise from the gel and even once excised can be difficult to reamplify, which was an issue encountered in this study. This does not allow for a full breakdown of all taxa present in the rearing water samples as many bands were either too weak to be visualised under the UV light or there was more than one sequence per band in some cases as encountered during the sequencing process. Therefore, it is important to have a combined approach to profile analysis with cross

validation of results to encompass the entire ecosystem ensemble. A HTS approach may be a more suitable method of including and identifying all taxa present.

3.17 Phytoplankton enumeration

Phytoplankton trends for the study at KWF were firstly determined through enumeration of numbers present using FCM. **Figures 3.49** and **3.50** illustrate the phytoplankton numbers present from March until November of 2018, in phytoplankton cells per ml and in $\log_{10}$ phytoplankton cells per ml, respectively. As the winter months approached, there was a notable decline in phytoplankton numbers as depicted in **Figure 3.49**, therefore, the enumeration for the final months can be visualised more clearly in **Figure 3.50**. As stated in the gating methodology for phytoplankton enumeration, the desired population included the chlorophyll population excluding cells positive for PC, therefore the graphs below do not include the cyanobacteria numbers.

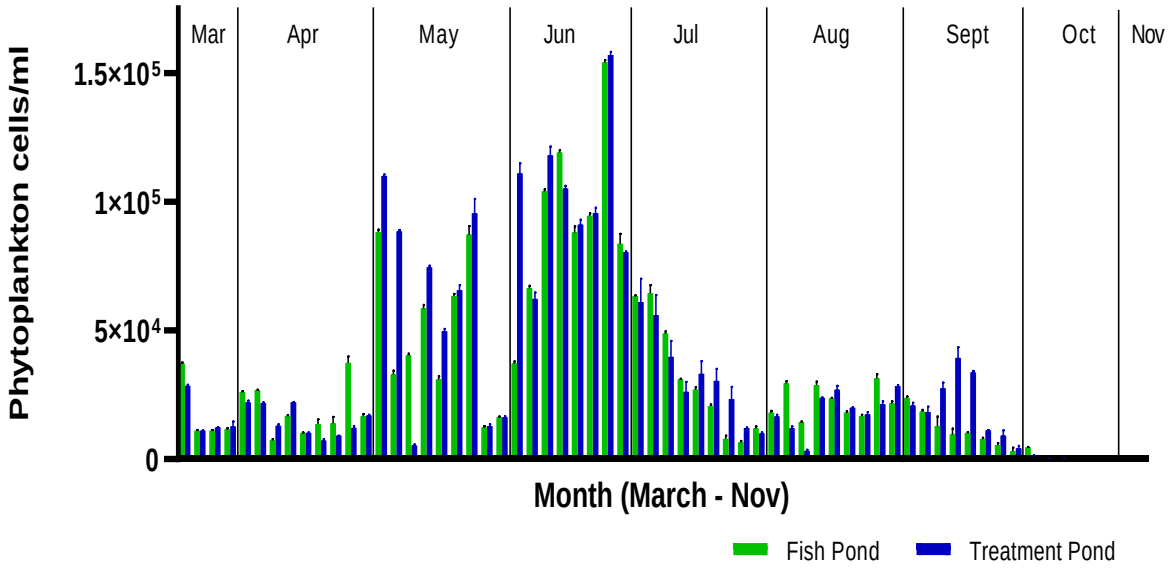

Figure 3.49 Phytoplankton cells/ml in from March to November 2018 in Pond 1 at KWF.

The phytoplankton numbers remained steady for March and April and then increased in May when there was greater irradiance and temperature was at the highest (19°C and 21°C) between May and July. The phytoplankton numbers peaked in late June

for both the FP and TP with a count of 1.54 x 10^5 cells/ml and 1.57 x 10^5 cells/ml, respectively. Lowest numbers were detected in the winter months *via* FCM analysis, of less than 100 cells/ml present. There was a slight decrease in the phytoplankton numbers in the TP in August compared with the FP. This may have been due to the removal of weeds and duckweed from the outer area of the TP, which may have led to a dilution of the phytoplankton population. This trend was reversed in September when a higher phytoplankton count was observed for the TP.

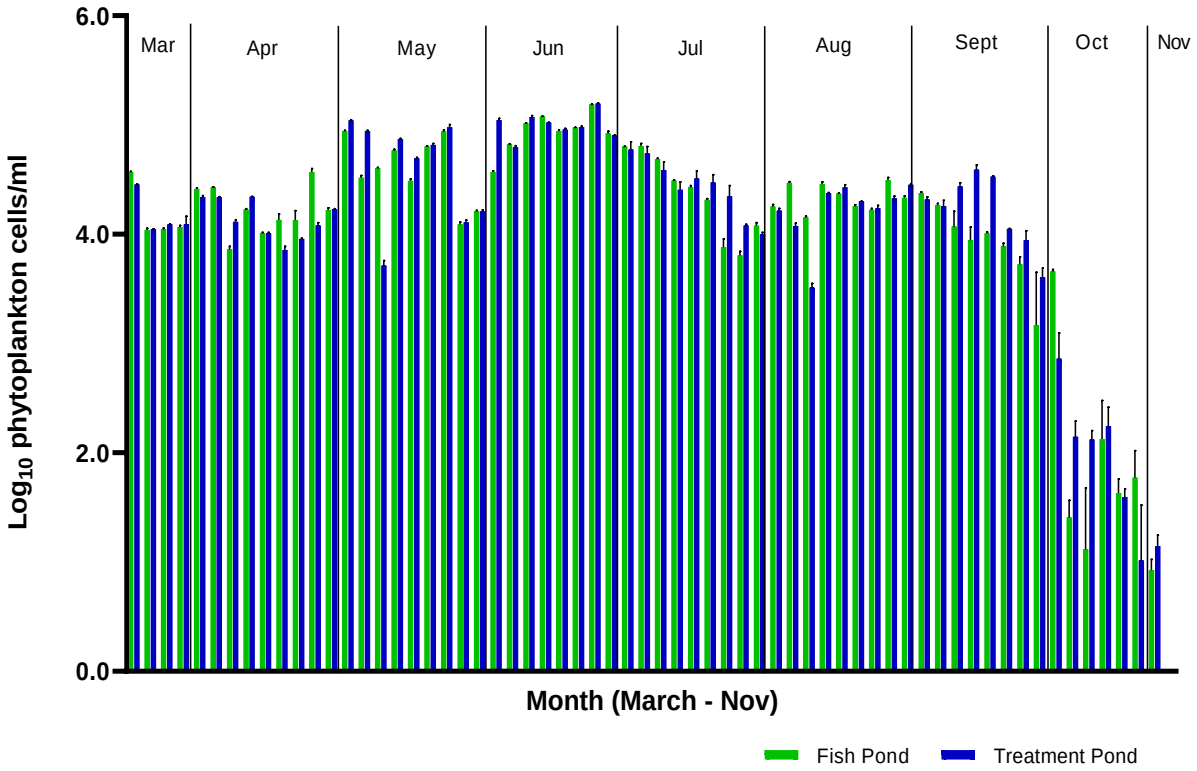

Figure 3.50 Log_{10} phytoplankton cells/ml from March to November 2018 in Pond 1 at KWF.

It is a common trend that phytoplankton cells reach the highest concentrations during the summer months due to increasing irradiance, longer daylight hours and higher temperatures. Li *et al.* (2012) found that phytoplankton numbers were higher in summer than winter as is the general trend of phytoplankton growth in lakes or ponds. However, the decrease from summer to winter was only marginal, from 3.45 x 10^3 to 1.46 x 10^3 cells/l, whereas in this project, cell numbers declined to much lower levels in winter, decreasing to 9 cells/ml in the first week of November. This decease can be visualised in the cytograms displayed in **Appendix A**.

The phytoplankton data was initially analysed for normality and the results of the D'Agostino-Pearson normality test determined that the data for both the FP and the TP was not normally distributed. Therefore, in order to determine if there was a difference between the phytoplankton counts for each pond, the Mann-Whitney non-parametric test was utilised (the equivalent to a t-test). A p-value of 0.821 indicated that there was no significant difference between the values obtained for both the FP and TP in terms of phytoplankton counts, with a significant difference achieved when $p \leq 0.05$ at the 95% level of confidence. This is in agreement with the results obtained for the physiochemical data and the bacterial counts in terms of similarity between both ponds. As previously mentioned, this may suggest that the flow rate between both the FP and the TP was sufficient to ensure an even distribution of the water components, such as phytoplankton, nutrients, etc.

The total number of chlorophyll-containing cells was determined *via* the flow cytometry analysis and therefore the population positive for PC, which was assumed to closely represent the cyanobacterial population in this study. This data was used to establish the cyanobacterial population from the total number of chlorophyll positive cells. **Figures 3.51** and **3.52** illustrate the total chlorophyll positive population and the number of cells in this population that represent phytoplankton cells compared to cyanobacterial cells for the FP and TP, respectively. These figures clearly demonstrate that cyanobacteria accounted for the majority of the chlorophyll-containing cells detected throughout the sampling period in both ponds. This observation is highlighted further with the use of the moving average trend line in both graphs. While certain species of cyanobacteria, for example, *Microcystis* sp., can release toxins into the water and exert detrimental effects on other organisms, cyanobacteria are also beneficial for processes such as nitrification. Throughout this process the nutrients are taken up by the cells and therefore removed from the water increasing the water quality in terms of nutrient pollution. In a study carried out by Liu *et al.* (2018) for the treatment of aquaculture using Chlorophyta, prior to inoculation with the green phytoplankton, Cyanobacteria were responsible for the partial removal of the pollutants from the aquaculture water, highlighting the importance of Cyanobacteria in the rearing water.

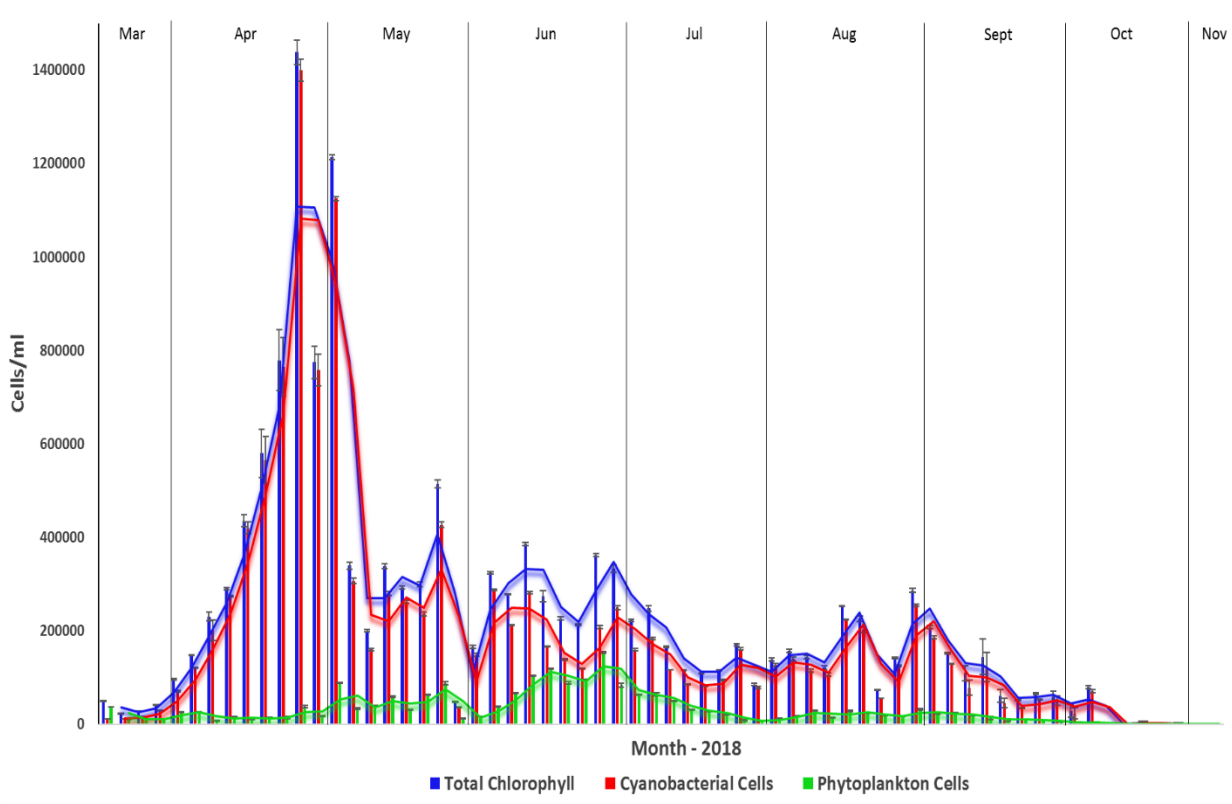

Figure 3.51 Enumeration of total chorophyll-containing cells consisting of cyanobacterial and phytoplankton cells in the FP at KWF.

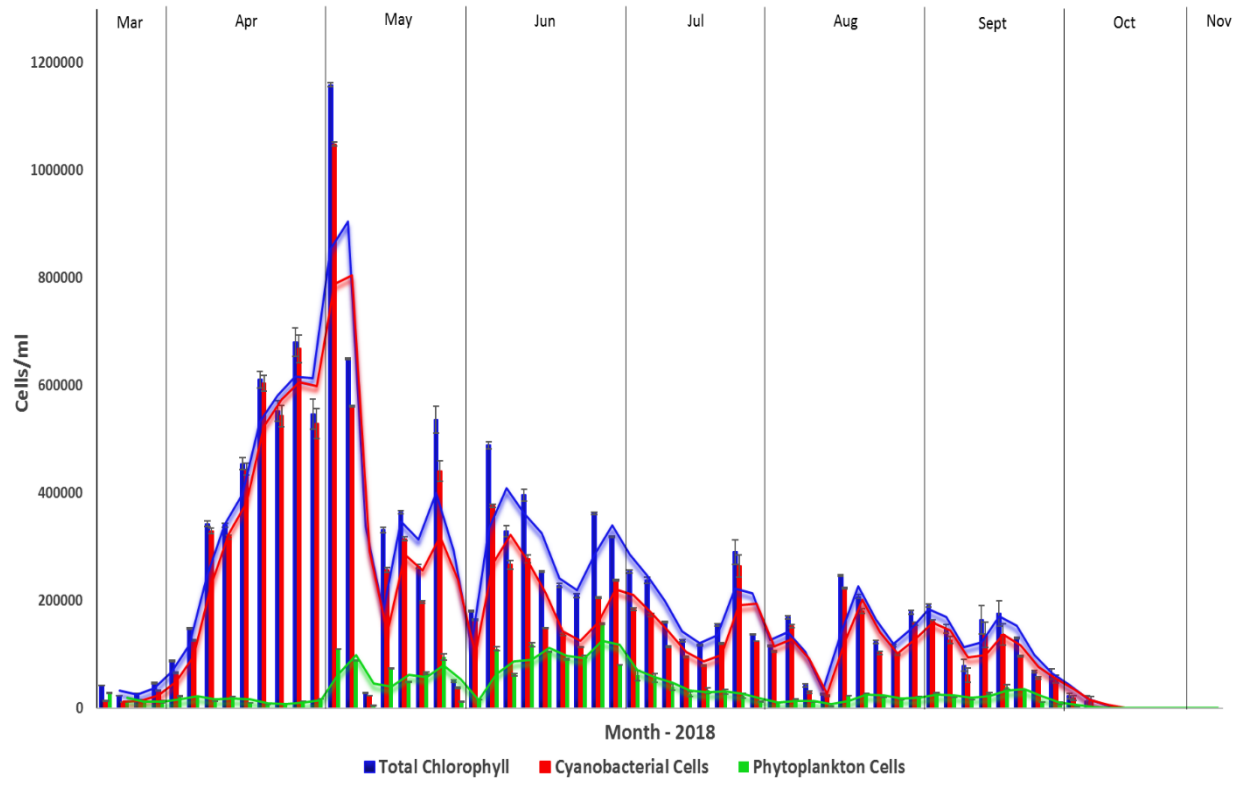

Figure 3.52 Enumeration of total chorophyll-containing cells consisting of cyanobacterial and phytoplankton cells in the TP at KWF.

3.18 Phytoplankton identification

The phytoplankton community profiling was carried out *via* the use of an inverted microscope for imaging followed by the use of photographic identification keys for full and partial speciation. The species dominance varied slightly for some months and drastically for others over the duration of the study. **Figures 3.53 – 3.61** illustrate the different phytoplankton species present in both the FP and TP for each month.

Chlorella and *Monoraphidium* were the most common phytoplankton present in both the FP and TP in March, illustrated in **Figure 3.53**, and both species remained dominant in April. The lack of species diversity for March, as illustrated by the imagery, may be due to the sampling period proceeding a heavy bout of snow. *Chlorella* has been reported as one of the most effective phytoplankton species at nutrient uptake, particularly nitrogen and phosphorus (Wang *et al.*, 2010). In this study, the nitrate, nitrite and ammonium concentrations all decreased in early/mid-June when the *Chlorella* population remained prevalent. The effectiveness of *Chlorella* sp., in particular, *C. vulgaris*, at nutrient removal is also evident from its common use in the treatment of wastewater (Min *et al.*, 2011; Abdel-Raouf *et al.*, 2012; Choi and Lee, 2012; Godos *et al.*, 2012; Delrue *et al.*, 2016). *Chlorella kessleri* synonymous with *Parachlorella kessleri* has also shown great potential for pollutant removal from aquaculture wastewater. Liu *et al.* (2018) inoculated aquaculture wastewater with five Chlorophyta species with *P. kessleri* exhibiting the greatest rate of nutrient uptake in terms of COD, nitrogen and total phosphorus. Based on the findings of the DGGE sequencing results, certain bands had sequence matches with chloroplasts of *C. vulgaris*, *Chlorella pyrenoidosa* and *Chlorella sorokiniana*, which would suggest that one or a combination of these particular *Chlorella* species were present in the rearing water. Chloroplasts are thought to have evolved from ancient cyanobacterial endosymbionts (Ziehe *et al.*, 2017), and as the primers used in the PCR were universal bacterial 16S primers, the chloroplasts of phytoplankton species would have also been detected. *Monoraphidium sp.* have also been reported for successful nutrient uptake. In a biodiesel production study carried out by Holbrook *et al.* (2014) *Monoraphidium* reduced concentrations of nitrates and phosphates to

< 5 mg/l and < 1 mg/l, respectively. Therefore, *Monoraphidium sp.* are potentially useful organisms for phytoremediation of aquaculture water if the cell densities are increased. Sanchis-Perucho *et al.* (2018) discovered that the nutrient removal efficiency of a consortium of *Monoraphidium* and *Scenedesmus* species was more effective than the removal of nitrogen and phosphorus compared to *Chlorella* sp.

There was an increase in phytoplankton diversity for the month of April, with Chlorophyta dominating both in the FP and in the TP. The most numerous Chlorophyta observed, other than *Chlorella* and *Monoraphidium*, included *Pandorina* sp., *Chlamydomonas* sp., *Dictyosphaerium* sp., *Kirchneriella* sp. and *Scenedesmus* sp., with *S. obtusus*, *S. quadricauda*, *S. obliquus*, *S. opaliensis* and *S. acuminatus* all identified. These phytoplankton appear as oval-shaped cells in a row, with spindles protruding from each corner of the overall cell cluster structure in some species as seen in **Figure 3.54**. Similarly to *Chlorella* and *Monoraphidium*, significant research has been carried out into the utilisation of *Scenedesmus* sp. for bioremediation, mainly *S. obliquus* (Chevalier and de la Noüe, 1985; Lavoie and de la Noüe, 1985; Martínez *et al.*, 2000; Ruiz-Marin *et al.*, 2010; Liu *et al.*, 2018; Ramírez *et al.*, 2018). The presence of different clonal populations for some phytoplankton species, such as a four- and eight-colony formation of *Scenedesmus* sp., compared to single-celled organisms may be attributed to the selective pressures in aquatic environments. For example, a study carried out by Zhu *et al*. (2015) demonstrated that upon exposure to *Daphnia* filtrate, the *Scenedesmus* sp. increased the rate of the formation of four- and eight-celled populations. Both four- and eight-celled species were observed in samples obtained from KWF and even two-celled species in some cases, which may just be due to separation. The presence of both the four- and eight-celled *Scenedesmus* sp. may be indicative of the selective pressure that was present in the rearing water for the duration of the study, due to the abundant diversity that was evident from all the samples analysed. There may also have been selective pressures due to the dramatic changes in meteorological and environmental conditions, ranging from snow in March to drought in the summer months. *Dictyosphaerium* sp. was observed for the majority of the months. *Dictyosphaerium* sp. have been associated with the efficient removal of certain pharmaceuticals in urban wastewater

(Gentili and Fick, 2017), which may prove beneficial for improving aquaculture water quality. Cryptophytes and Euglenophyta were also observed in April in the form of *Chroomonas* sp. and *Cryptomonas* sp, and *Trachelomonas* sp., respectively. *Cryptomonas* species are regarded as an important food source in the aquatic environment (Wirth *et al.*, 2019).

The presence of several species continued for the month of May, such as *Chlorella* sp., *Monoraphidium* sp., and *Scenedesmus* sp. The most dominant phytoplankton identified were clonal genera including *Chroococcus, Dictyosphaerium*, *Pandorina* and *Westella*. *Pediastrum* sp. was observed in the sample for May and is denoted by the star-shaped cell depicted in **Figure 3.55**. This species remained present until October, after which it was not observed.

Diatoms, mainly *Cyclotella* sp. and *Stephanodiscus* sp., were the most frequent species observed in the month of June for the FP and the TP. These cells are so similar in morphology that they are both classified as Stephanodiscaeae, which falls under the Thalassiosirales monophyletic group (Stoermer and Julius, 2003). According to Stoermer and Julius (2003) diatoms tend to be specific to certain habitats which allows for their use as indicators of water quality, with *Stephanodiscus* considered to be one of the most common and ubiquitous freshwater diatoms. In July, *Merismopedia* sp. was first observed. This genus is illustrated in **Figure 3.57**, with cells aligned in two box-like shapes. *Synura* sp. were quite dominant in July for both the FP and TP. *Synura* sp. present as up to 50 closely packed pear-shaped cells in a spherical colony.

There was an increase in the presence of *Scenedesmus* sp. in August compared to other months. This agrees with findings from Cellamare *et al.* (2010) who isolated phytoplankton from three freshwater lakes. The diversity was greater in the FP for August, with the TP mainly dominated by *Clamydomonas* sp.

The species diversity for both the FP and the TP was very similar every month with one major exception. In the month of September, the TP was completely dominated by *Cryptomonas* sp. denoted by the numerous oval-shaped cells in **Figure 3.59**. Contrastingly, very large diatoms, possibly *Aulacodiscus*, *Hyalodiscus* and *Cyclotella*

sp. dominated the FP. The presence of these diatoms seemed to cause a decrease in other species present, possibly due to feeding or out-competing with other species for nutrients. In fact, the phytoplankton counts for mid-September were lower for FP compared to the TP, which can be visualised in **Figure 3.50**.

The diversity of species decreased for October, with *Selenastrum*, a.k.a. *Pseudokirchneriella,* and *Craticula* species more dominant than other months. *Selenastrum* is used for testing the toxicity of chemicals, acting as an environmental water quality indicator, due to its sensitivity to a wide range of toxicants (Helfrich and Libey, 1990; Asker, 2011; Perosa *et al.*, 2015). This phytoplankton was used in this project as part of a multi-trophic test battery for the testing of chemical disinfectants commonly used in aquaculture and is described in **Chapter 5**. Although the was a reduction in light intensity and temperature for the month of November, the species diversity increased in comparison to October, with *Chlamydomonas* sp., *Chlorella* sp. and *Dictyosphaerium* sp. among the most dominant.

The species identified from the samples in this study would constitute the biological requirements for the 'green water' concept for sustaining the aquaculture process. This involves the use of naturally present green phytoplankton (microalgae), consisting predominately of *Chlorella* sp. and *Scenedesmus* sp. and other relevant microorganisms i.e. bacteria, protozoa and zooplankton, as a natural food source for fish, with the water also used for treatment (nutrient removal) and a supply of oxygen (Naas *et al.*, 1992; Moriarty, 1997; Neori, 2011; Basri *et al.*, 2015). There are many nutritional benefits to the use of microalgae as a fish feed as outlined in **Chapter 2, Section 2.4**, which enhances the immunity of fish, thereby reducing the financial cost required for the purchase of antibiotics (Han *et al.*, 2019).

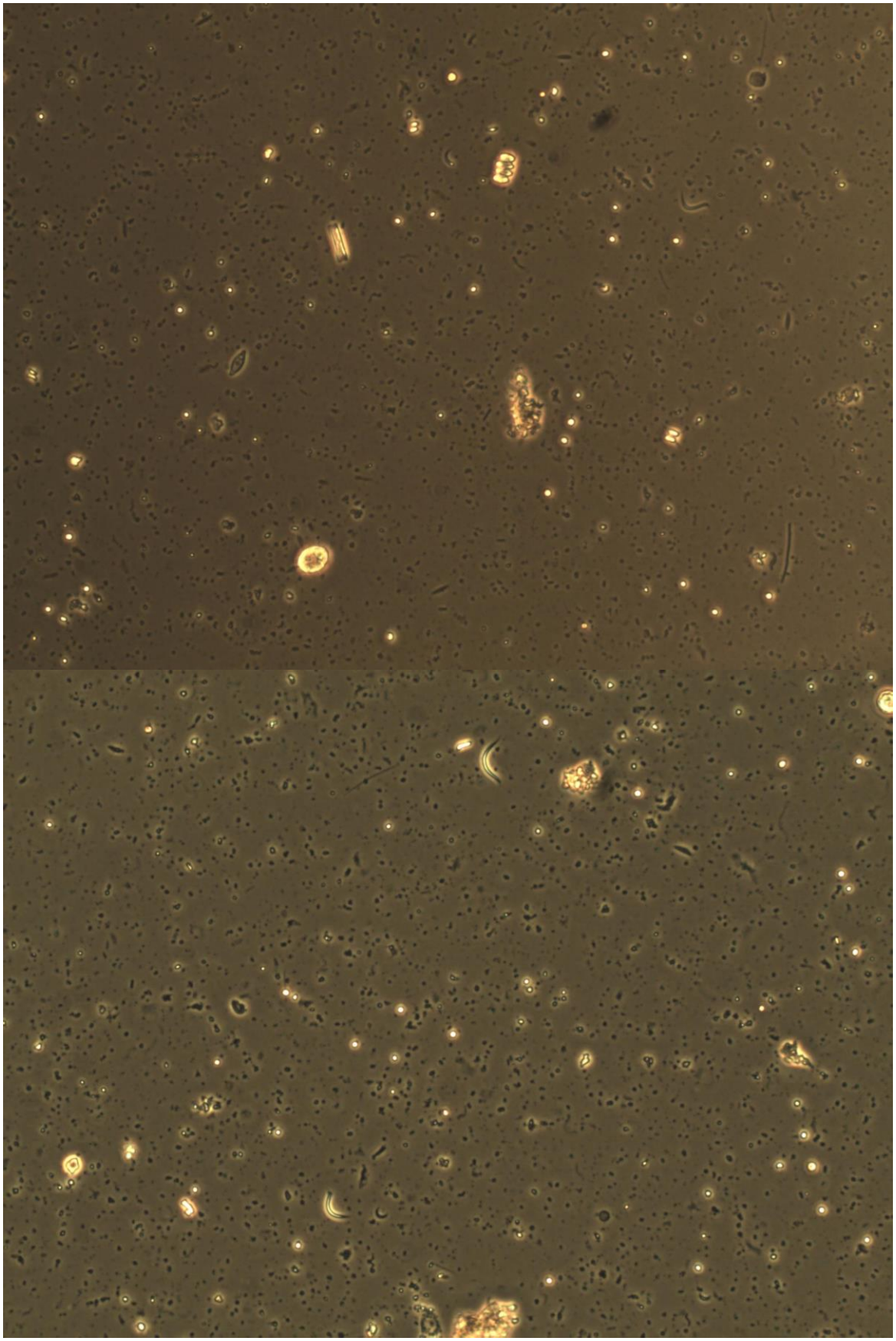

Figure 3.53 Phytoplankton population diversity for the FP and TP in March 2018 including *Chlorella* sp. and *Monoraphidium* sp. (400x magnification).

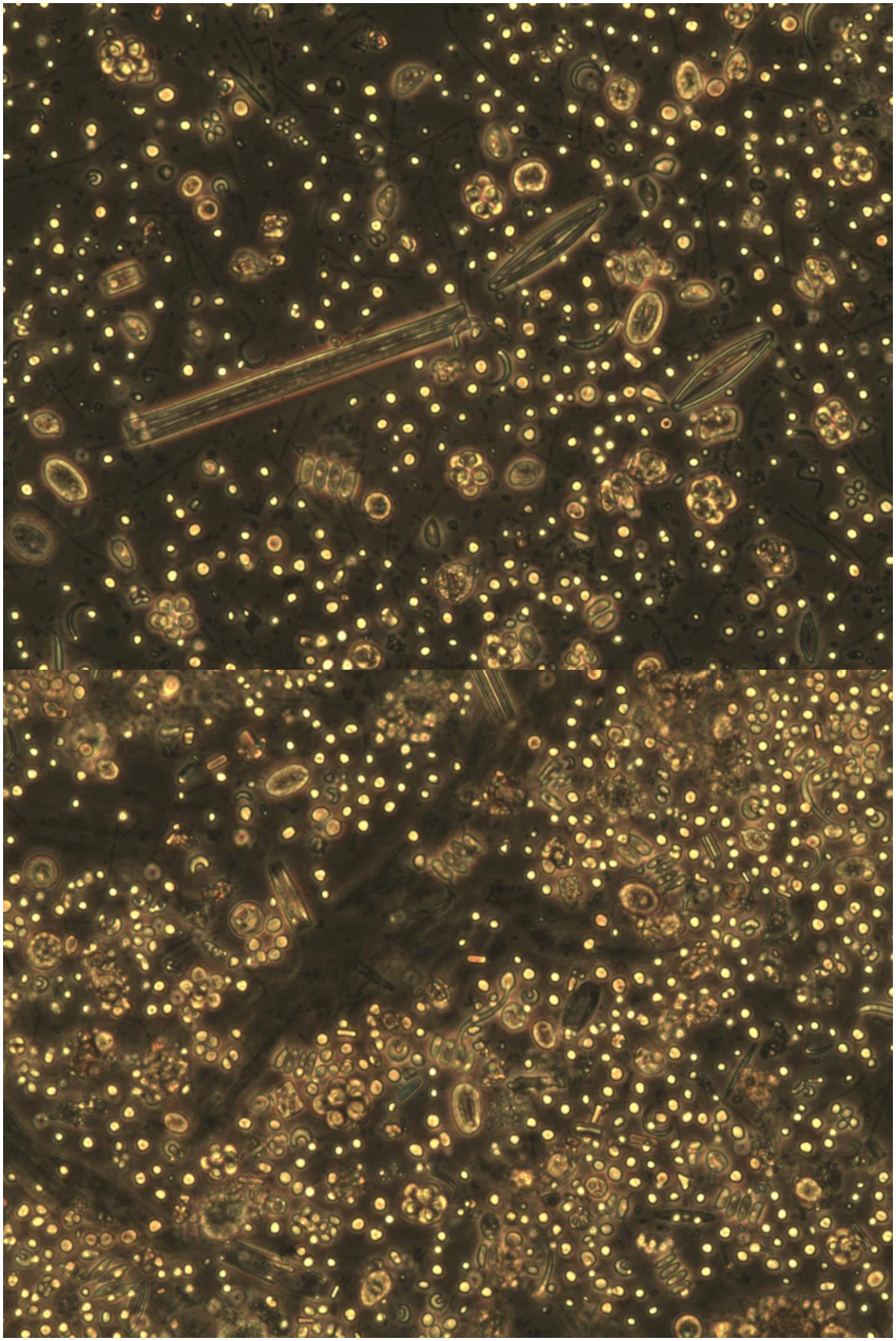

Figure 3.54 Phytoplankton population diversity for the FP and TP in April 2018 including *Chlamydomonas* sp., *Chlorella* sp., *Cryptomonas* sp., *Scenedesmus* sp. and *Monoraphidium* sp. (400x magnification).

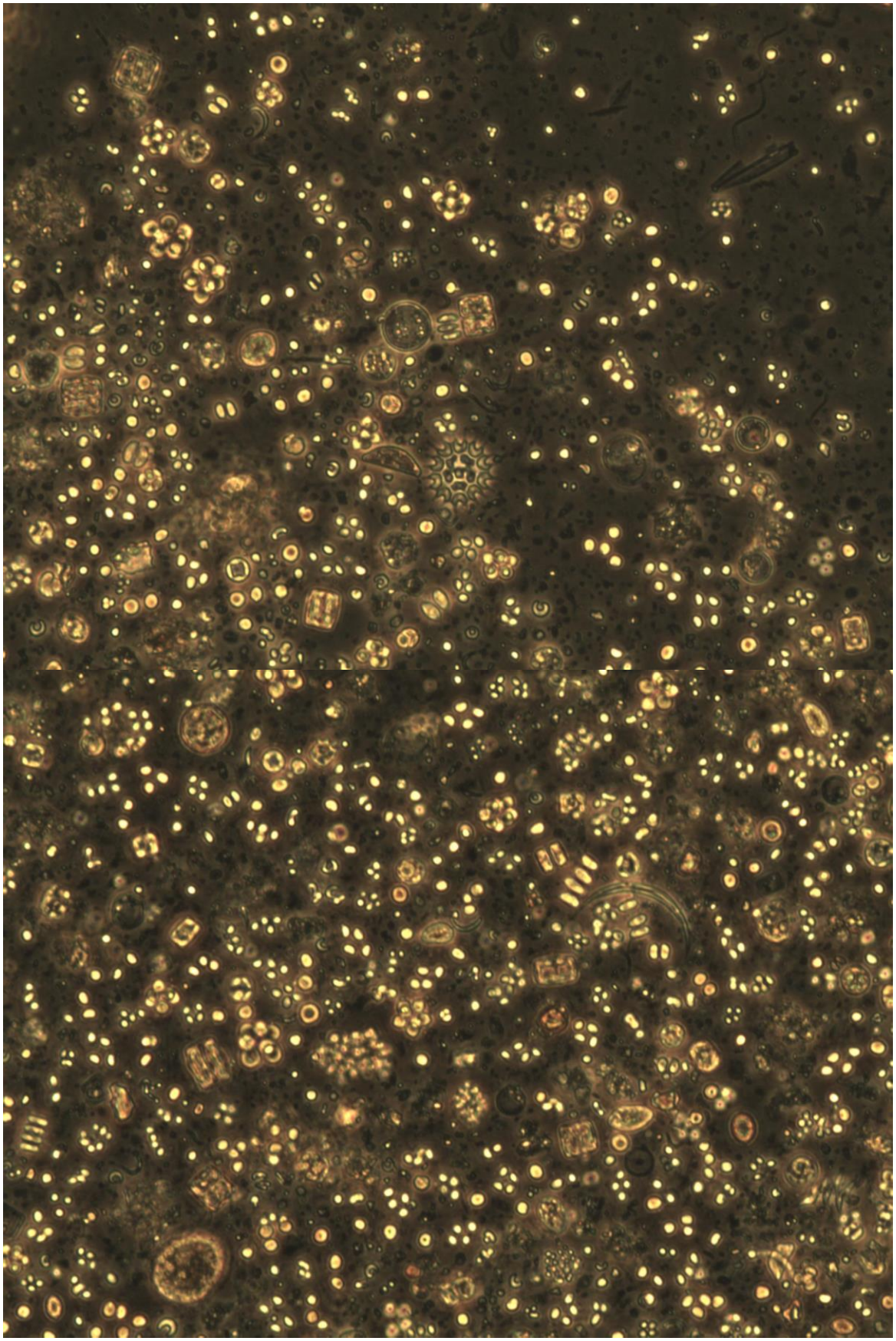

Figure 3.55 Phytoplankton population diversity for the FP and TP in May 2018 including *Chlamydomonas* sp., *Cyclotella* sp., *Dictyosphaerium* sp., *Pandorina* sp., *Stephanodiscus* sp. and *Westella* sp. (400x magnification).

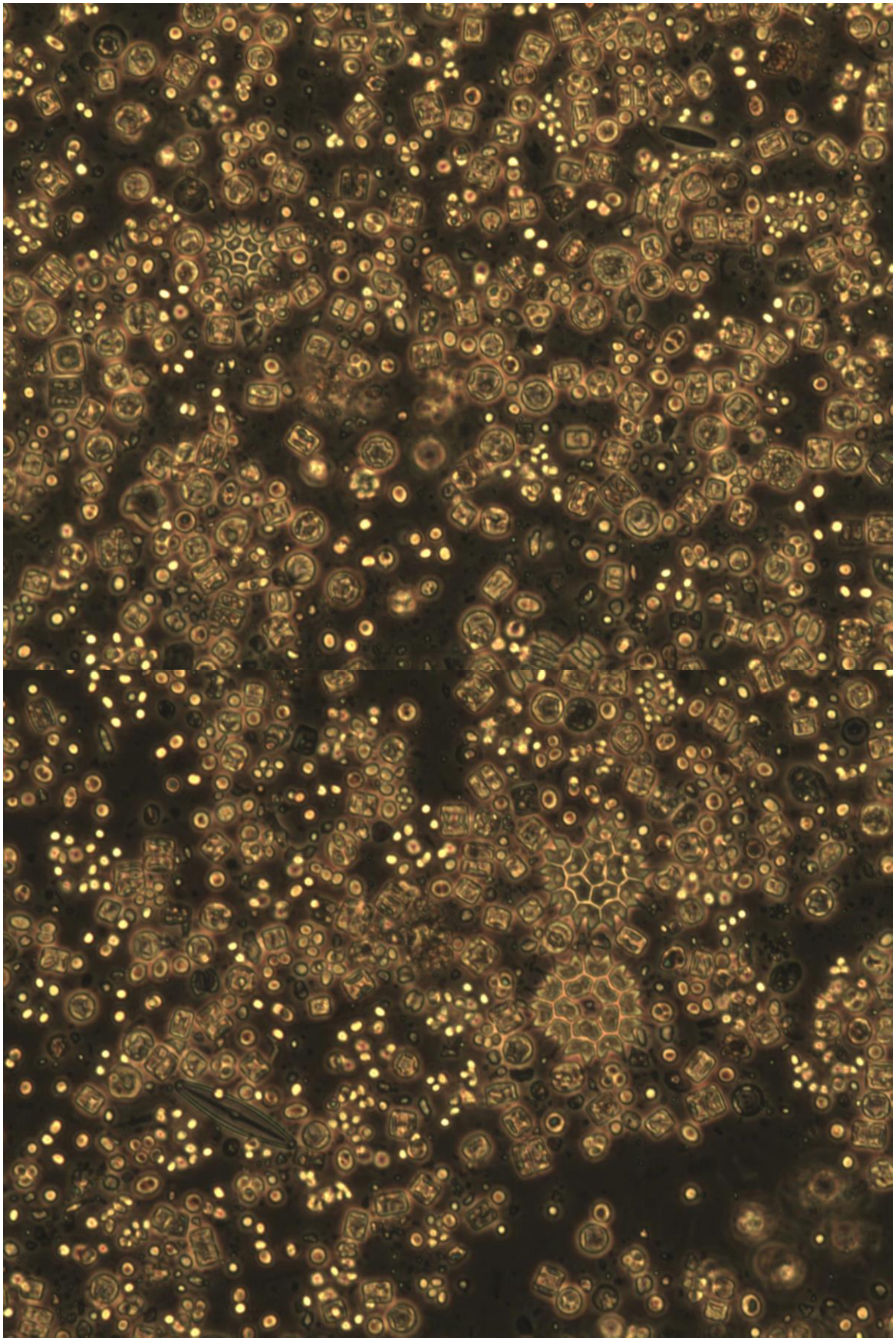

Figure 3.56 Phytoplankton population diversity for the FP and TP in June 2018 including *Chlamydomonas* sp., *Cyclotella* sp., *Dictyosphaerium* sp., *Stephanodiscus* sp. and *Westella* sp. (400x magnification).

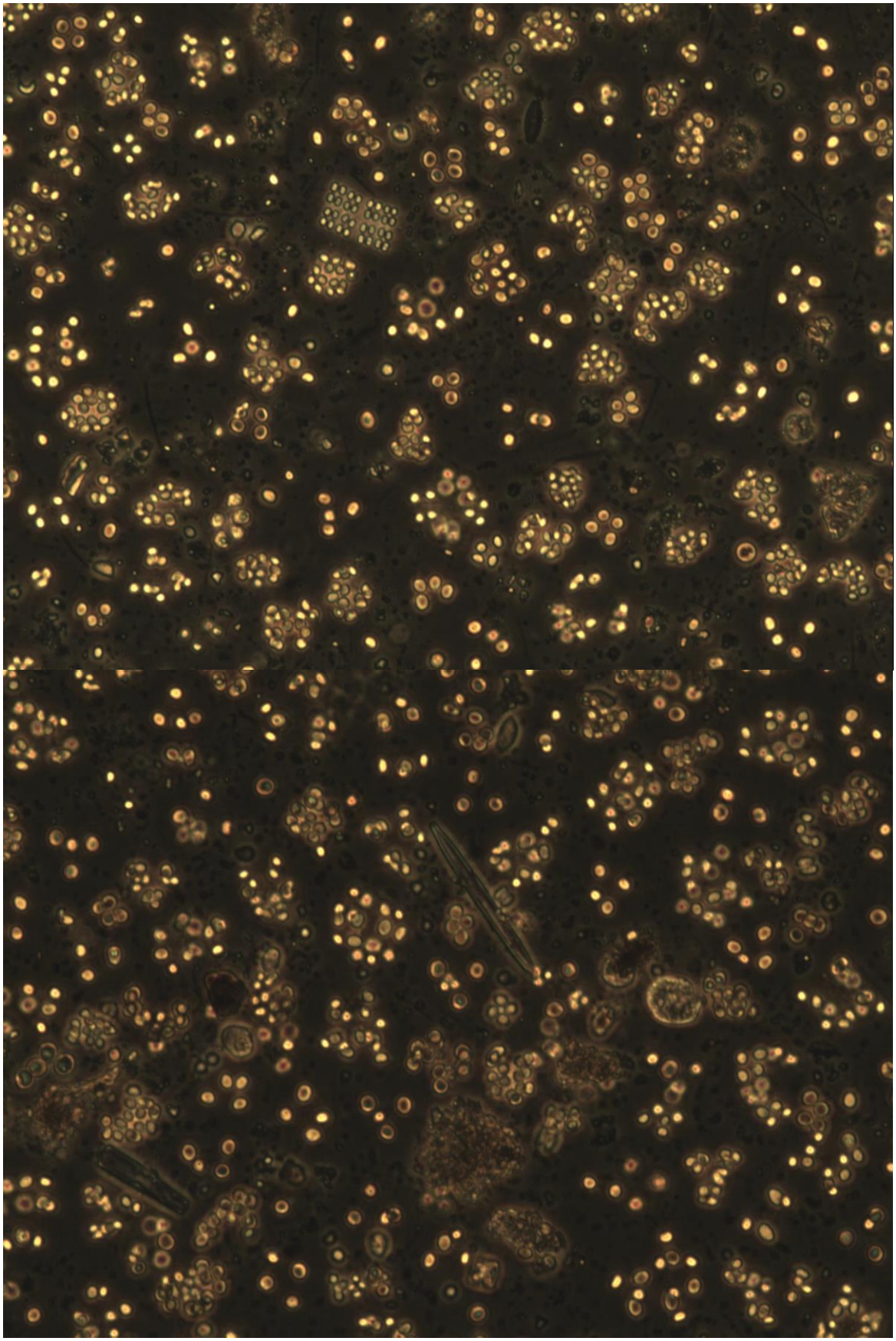

Figure 3.57 Phytoplankton population diversity for the FP and TP in July 2018 including *Chlamydomonas* sp., *Chlorella* sp., *Dictyosphaerium* sp., *Snowella* sp., *Sphaerocystis* sp. and *Westella* sp. (400x magnification).

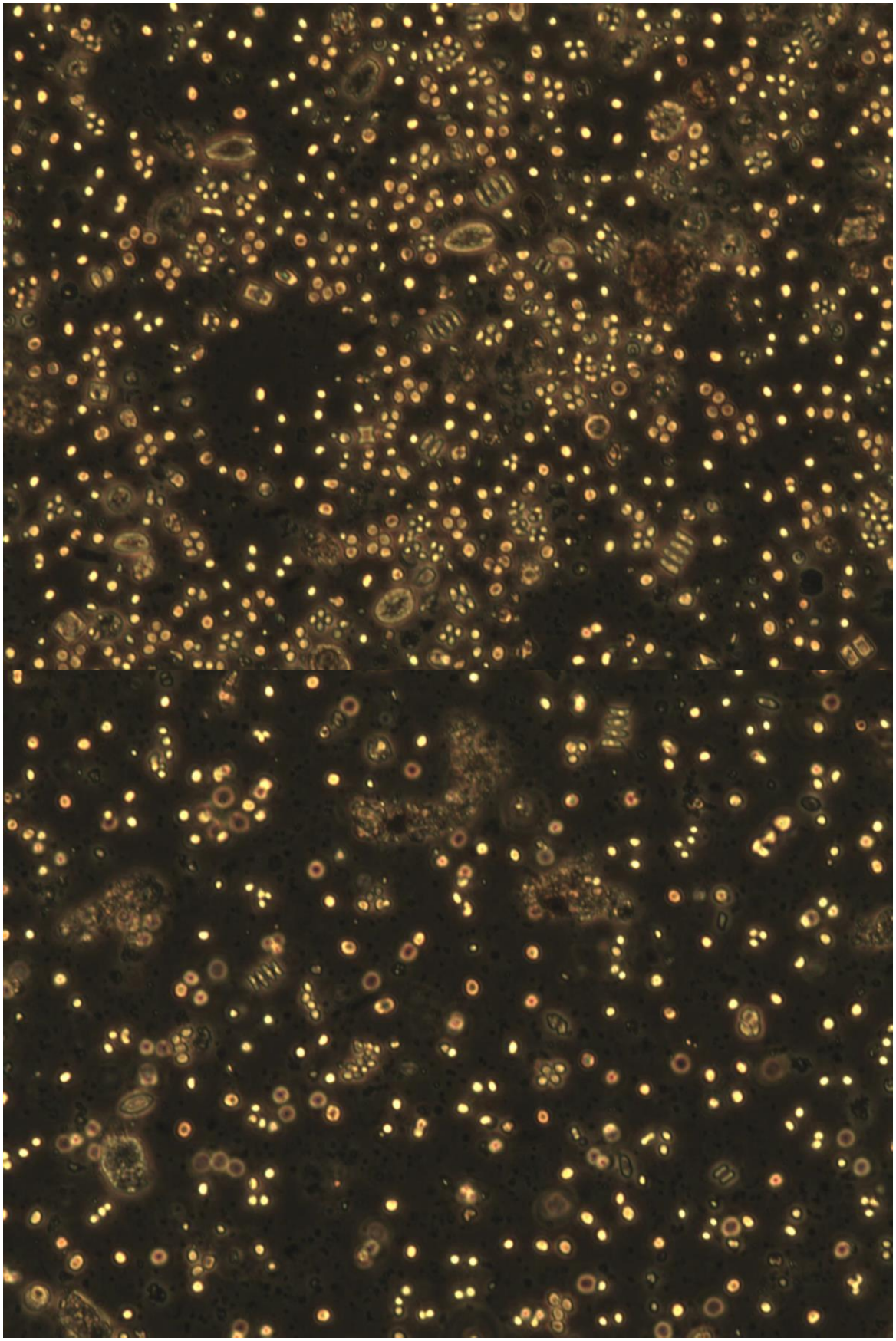

Figure 3.58 Phytoplankton population diversity for the FP and TP in August 2018 including *Chlamydomonas* sp., *Cryptomonas* sp., *Dictyosphaerium* sp., *Scenedesmus* sp., *Tetraspora* sp. and *Westella* sp. (400x magnification).

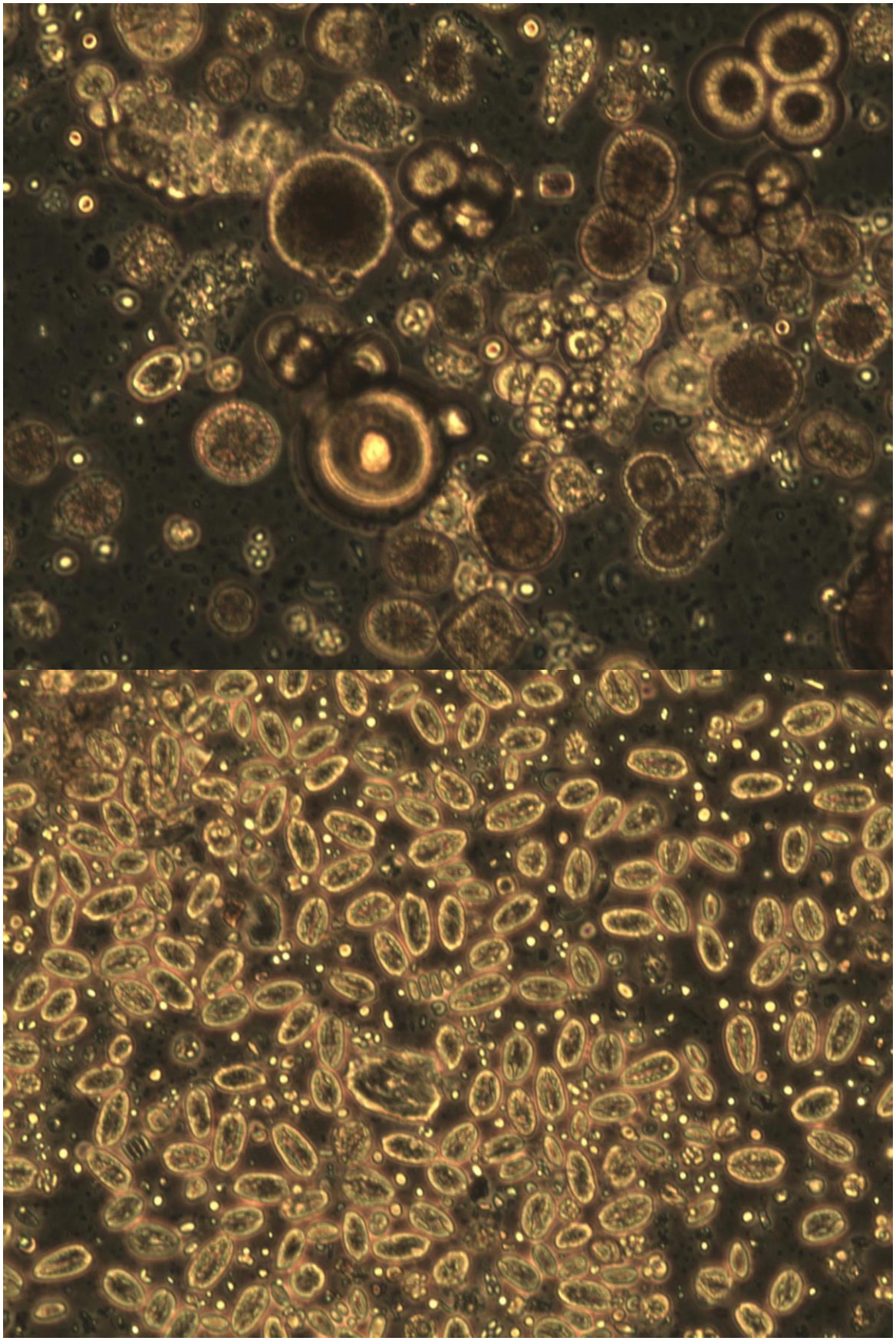

Figure 3.59 Phytoplankton population diversity for the FP and TP in September 2018 including *Aulodiscus* sp., *Cryptomonas* sp., *Cyclotella* sp., *Dictyosphaerium* sp., *Hyalodiscus* sp. and *Selenastrum* sp. (400x magnification).

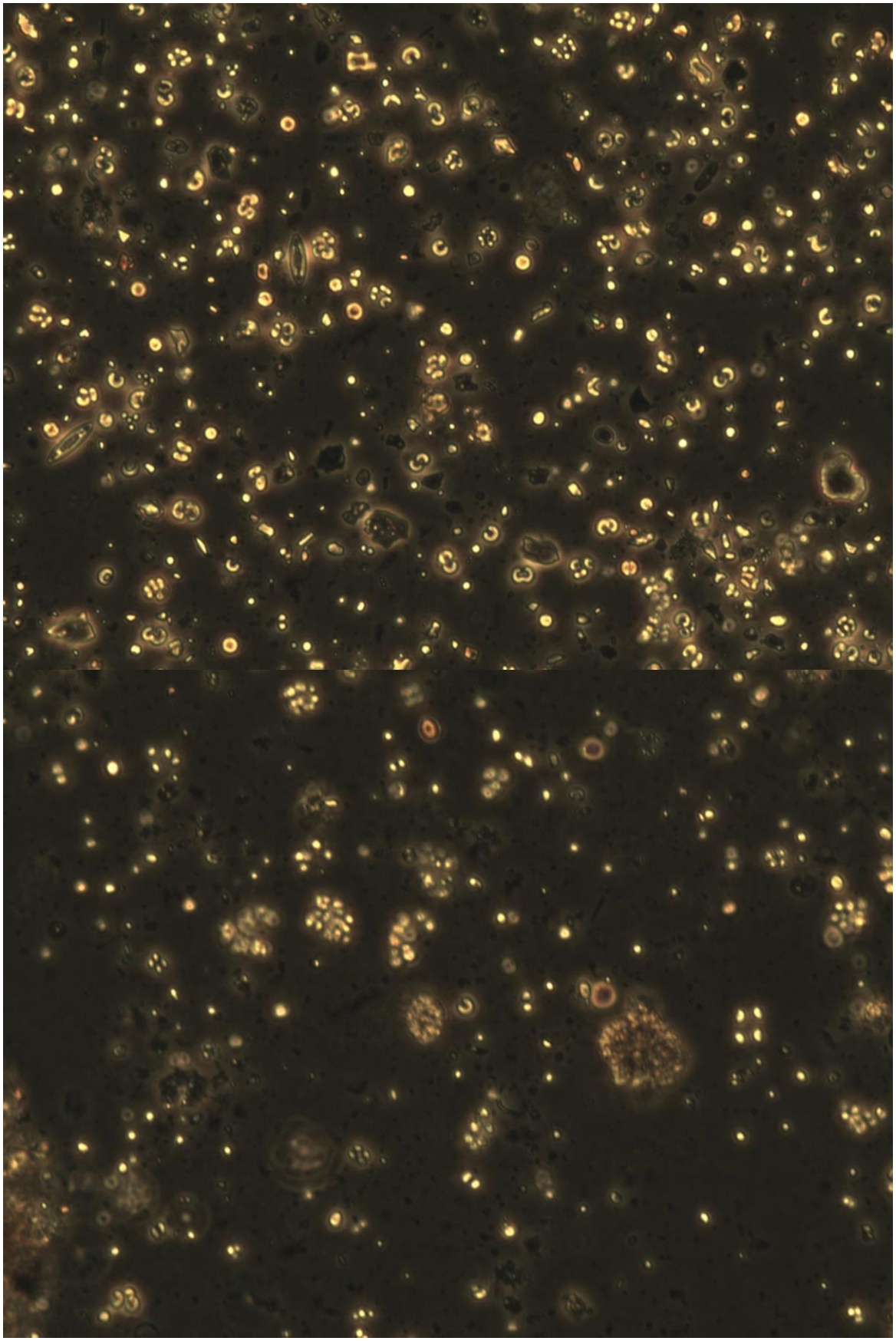

Figure 3.60 Phytoplankton population diversity for the FP and TP in October 2018 including *Aphanocapsa* sp., *Chlorella* sp., *Dictyosphaerium* sp. and *Selenastrum* sp. (400x magnification).

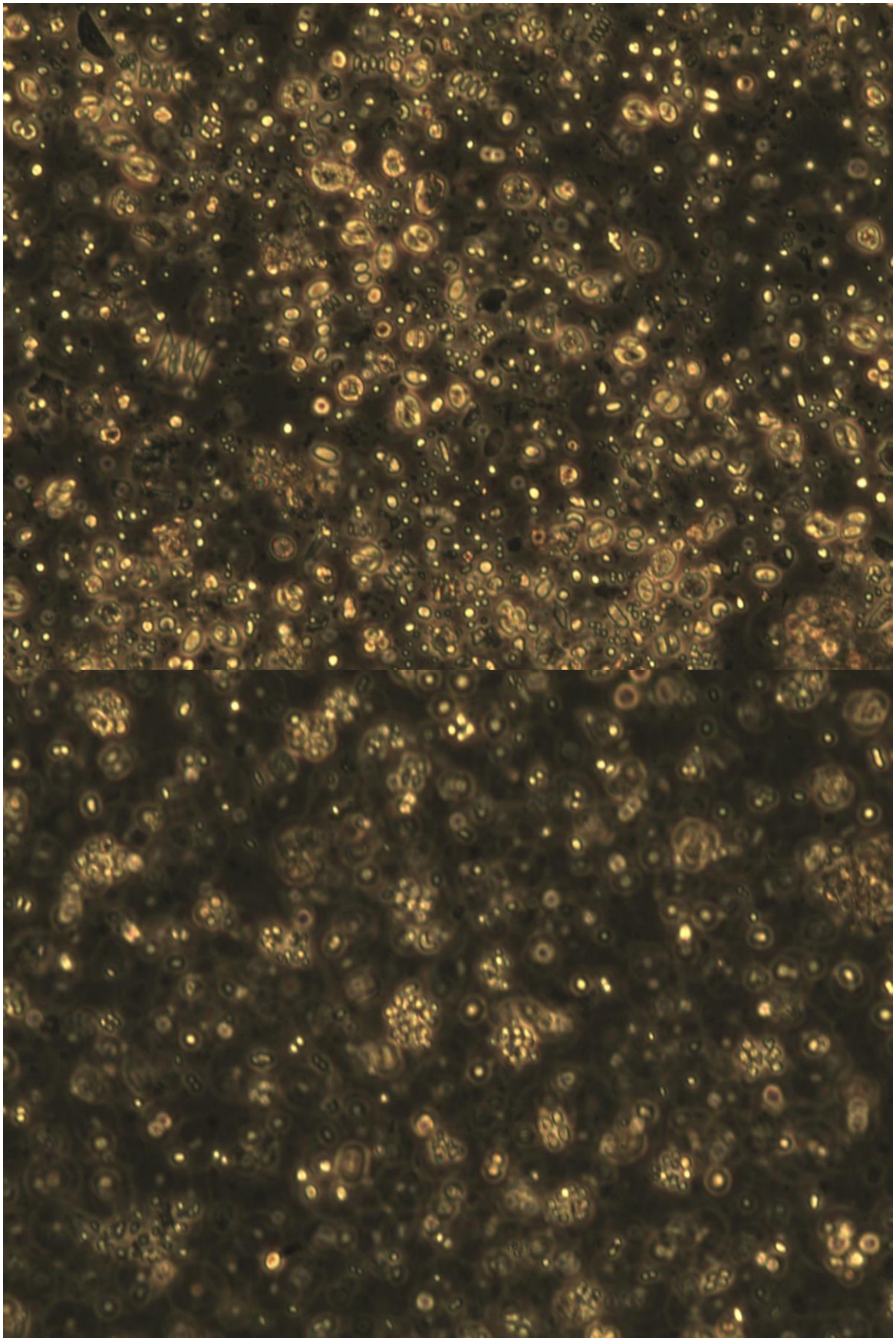

Figure 3.61 Phytoplankton population diversity for the FP and TP in November 2018 including Chlamydomonas sp., *Chlorella* sp., Chryptomonas sp., *Dictyosphaerium* sp. and *Westella* sp. (400x magnification).

It is evident from the images that the species diversity increased from month to month. **Tables 3.7** and **3.8** outline the monthly dominance of each of the aforementioned phytoplankton groups. Chlorophyta was the dominant phylum in both the FP and the TP for the majority of months during the sampling period. Certain Bacillariophyta, a.k.a. diatoms, dominated for June and September in the FP, and for May and June in the TP, with other dominating groups including Cryptophyta and Cyanophyta. While the phyla dominance remained steady, it is clear from the images that the dominant genus varied. For example, in May *Chroococcus*- and *Westella*-like species were the most numerous, whereas in June *Cyclotella* sp. and *Stephanodiscus* sp. dominated the sample. The unusual meteorological conditions that occurred during the months of sampling, from heavy snow to high temperatures and drought, may have led to this constant change in genus dominance.

According to Vuorio *et al.* (2007), when analysing multispecies communities of phytoplankton, enumeration procedures can be complicated and more information regarding water quality can be determined by phytoplankton community analysis compared to basic nutrient or chlorophyll *a* measurements. Therefore, it was important to perform a two-step phytoplankton analysis procedure in the form of FCM and microscopy. Other factors can also be problematic for the phytoplankton identification process. Stoermer and Julius (2003) state that the average size of diatomic cells decreases after each vegetative life cycle, which can lead to variability in cell morphology of the same species. Environmental conditions such as salt levels can also alter diatom morphology (Tuchman *et al.*, 1984; Stoermer and Julius, 2003). This variation in cell morphology may lead to unknown or poorly understood cell functions in terms of nutrient recycling. As well as that, Small *et al.* (2016) stated that the capacity of photoautotrophic systems, such as phytoplankton, to remove nutrient waste from the water depends largely on energy uptake from sunlight, which is very unpredictable in the Irish climate. Also with climate change and increasing temperatures worldwide, certain species may not be able to grow, and as they are a source of oxygen in the ponds, the use of a natural means of production oxygen for biological processes may no longer be an option.

Table 3.7 Dominant phytoplankton species from March to November in the FP of pond 1 at KWF, 2018.

	Most to least dominant in each group	***** Shading indicates dominant phylum	
Month	**Genus**		
March	*Chlorella, Monoraphidium* - **Chlorophyta**		
April	*Chlamydomonas, Chlorella, Scenedesmus, Monoraphidium* - **Chlorophyta**	*Chroomonas, Cryptomonas* - **Cryptophyta**	*Trachelomas* - **Euglenophyta**
May	*Chroococcus, Dictyosphaerium, Pandorina, Chlamydomonas, Tetraspora, Westella*, - **Chlorophyta**		
June	*Dictyospaherium, Westella, Chlamydomonas, Chlorella, Tetraspora* - **Chlorophyta**	*Stephanodiscus, Cyclotella* - **Bacillariophyta**	
July	*Dictyosphaerium, Westella, Chlamydomonas, Chlorella, Sphaerocystis* - **Chlorophyta**		
August	*Westella, Dictyosphaerium, Chlamydomonas, Scenedesmus* - **Chlorophyta**	*Cryptomonas* -**Cryptophyta**	
September	*Dictyosphaerium, Selenastrum* - **Chlorophyta**	*Aulodiscus, Hyalodiscus, Cyclotella* - **Bacillariophyta**	
October	*Selenastrum, Dictyosphaerium, Chlorella* - **Chlorophyta**		
November	*Chlorella, Dictyosphaerium, Chlamydomonas* - **Chlorophyta**	*Cryptomonas* - **Cryptophyta**	*Chroococcus* - **Cyanophyta**

Table 3.8 Dominant phytoplankton species from March to November in the TP of pond 1 at KWF, 2018.

	Most to least dominant in each group	***** Shading indicates the dominant group	
Month	**Species**		
March	*Chlorella, Monoraphidium* - **Chlorophyta**		
April	*Chlorella, Monoraphidium, Dictyosphaerium, Scenedesmus, Chlamydomonas* - **Chlorophyta**	*Trachelomas* - **Euglenophyta**	*Aphanocapsa* - **Cyanophyta**
May	*Chroococcus, Chlamydomonas, Chlorella, Pandorina, Westella, Tetraspora* - **Chlorophyta**	*Stephanodiscus, Cyclotella* - **Bacillariophyta**	*Cryptomonas* - **Cryptophyta**
June	*Dictyosphaerium, Westella, Chlorella, Chlamydomonas, Tetraspora* - **Chlorophyta**	*Cyclotella* - **Bacillariophyta**	
July	*Dictyosphaerium, Westella, Chlamydomonas* - **Chlorophyta**	*Snowella* - **Cyanophyta**	
August	*Westella, Chlamydomonas, Tetraspora* - **Chlorophyta**		
September	*Chlorella* - **Chlorophyta**	*Cryptomonas* - **Cryptophyta**	
October	*Dictyosphaerium, Selenastrum, Chlorella* - **Chlorophyta**	*Aphanocapsa* - **Cyanophyta**	
November	*Dictyosphaerium, Westella, Chlamydomonas* - **Chlorophyta**		

3.19 Ecosystem dynamic analysis of rearing water at KWF

Many parameters were tested and recorded during this study to try to obtain as much information as possible regarding the influencing relations each parameter, be it biological or physicochemical, has on another parameter. Figures **3.62** and **3.63** illustrate nitrogen and phosphate concentrations overlaid with temperature and pH for the FP and TP, respectively. Similar trends were observed for each parameter between both ponds. The temperature ranged from 5.8°C in March to 22.2°C in August, while the pH values remained between 7.2 and 8.4 for the duration of the study. **Figures 3.64** and **3.65** illustrate nitrogen and phosphate concentrations overlaid with the carbonate hardness for the FP and TP, respectively. **Figures 3.66** and **3.67** illustrate the nitrogen and phosphate trends alongside the bacterial and phytoplankton counts from the FP and TP, respectively.

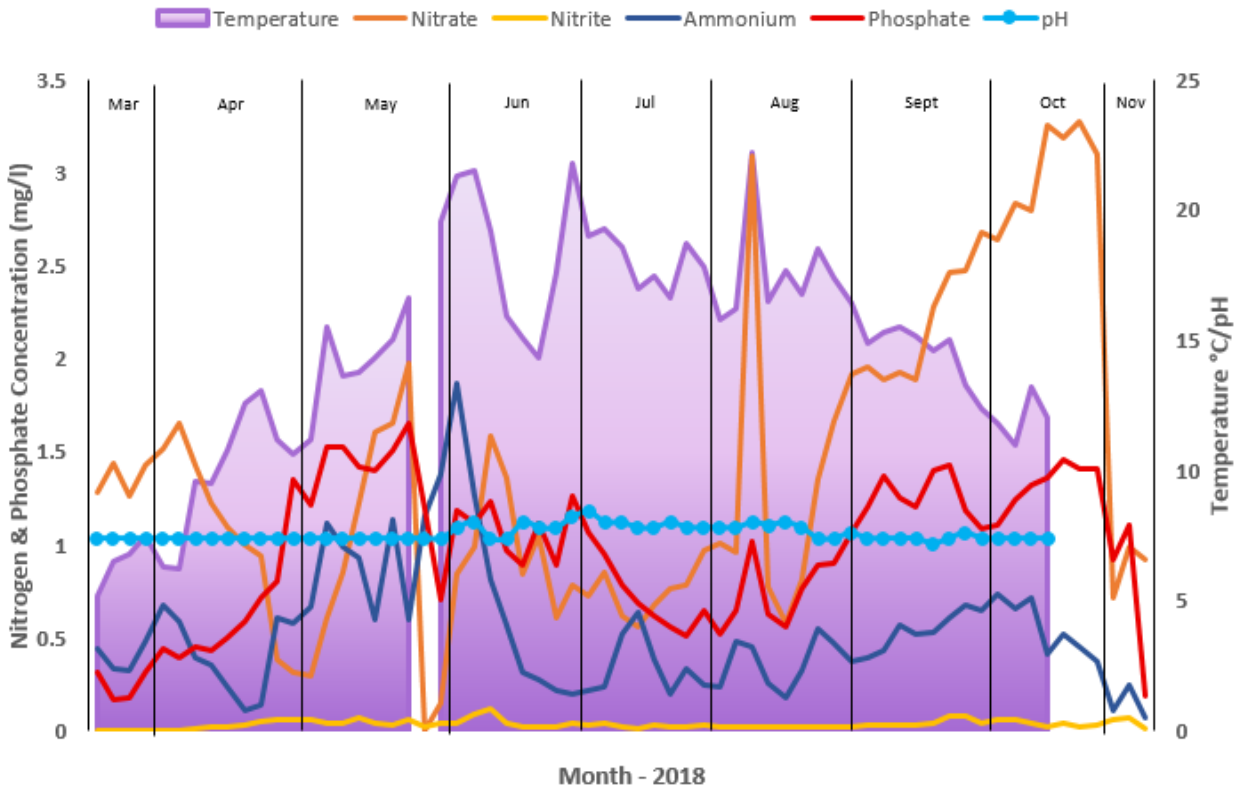

Figure 3.62 Trends for temperature, nitrogen, phosphate and pH for the FP at KWF.

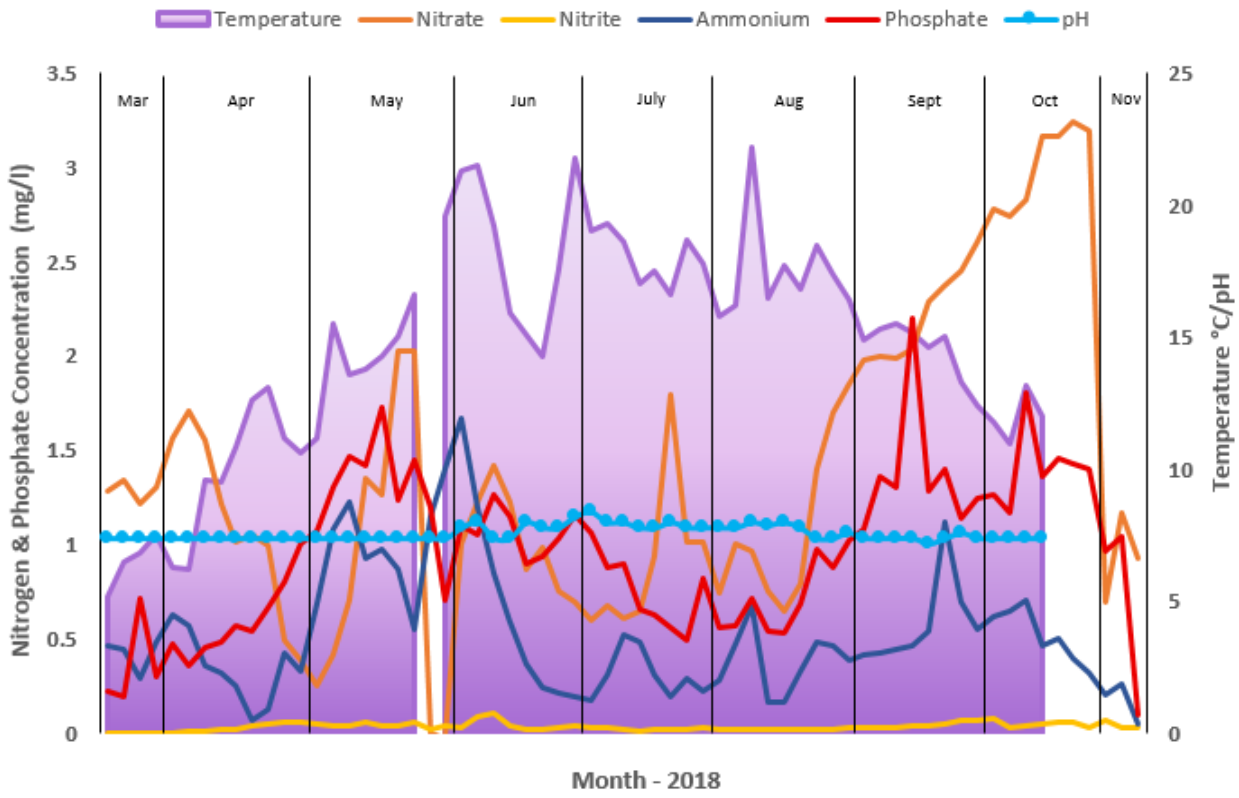

Figure 3.63 Trends for temperature, nitrogen, phosphate and pH for the TP at KWF.

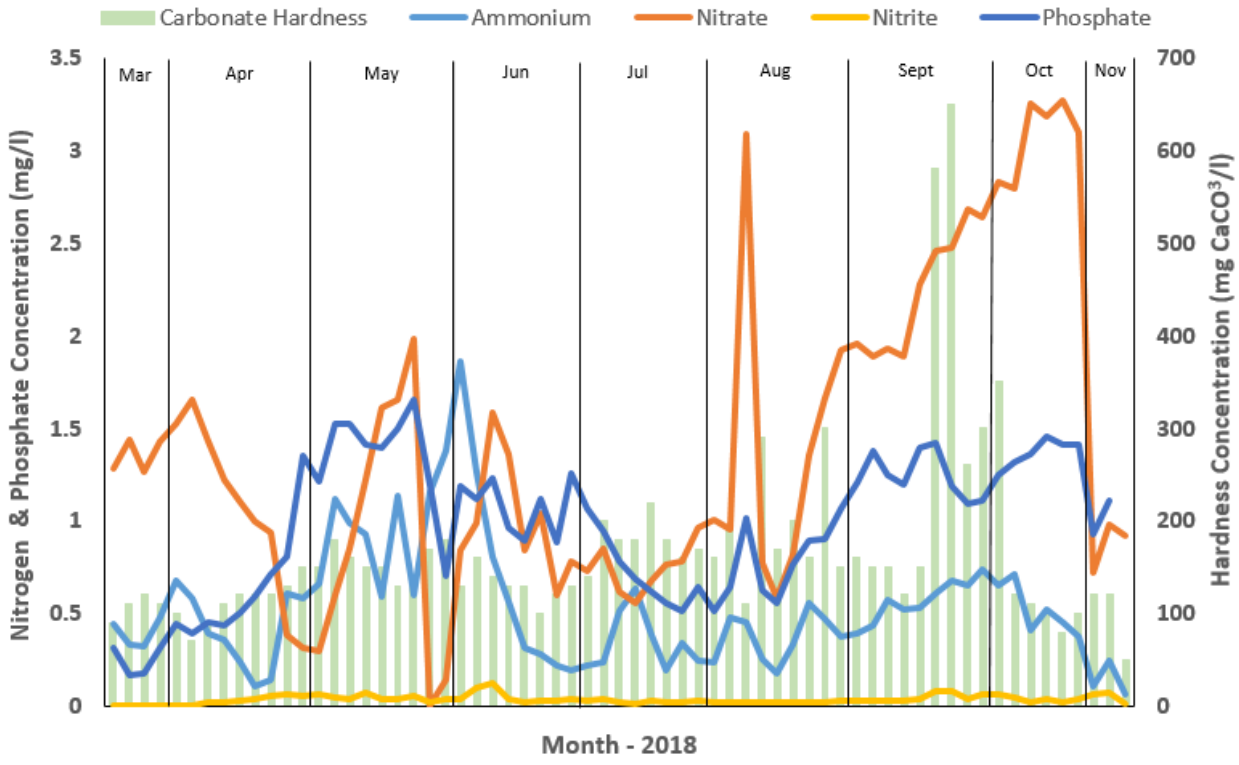

Figure 3.64 Trends for nitrogen, phosphate and carbonate hardness for the FP at KWF.

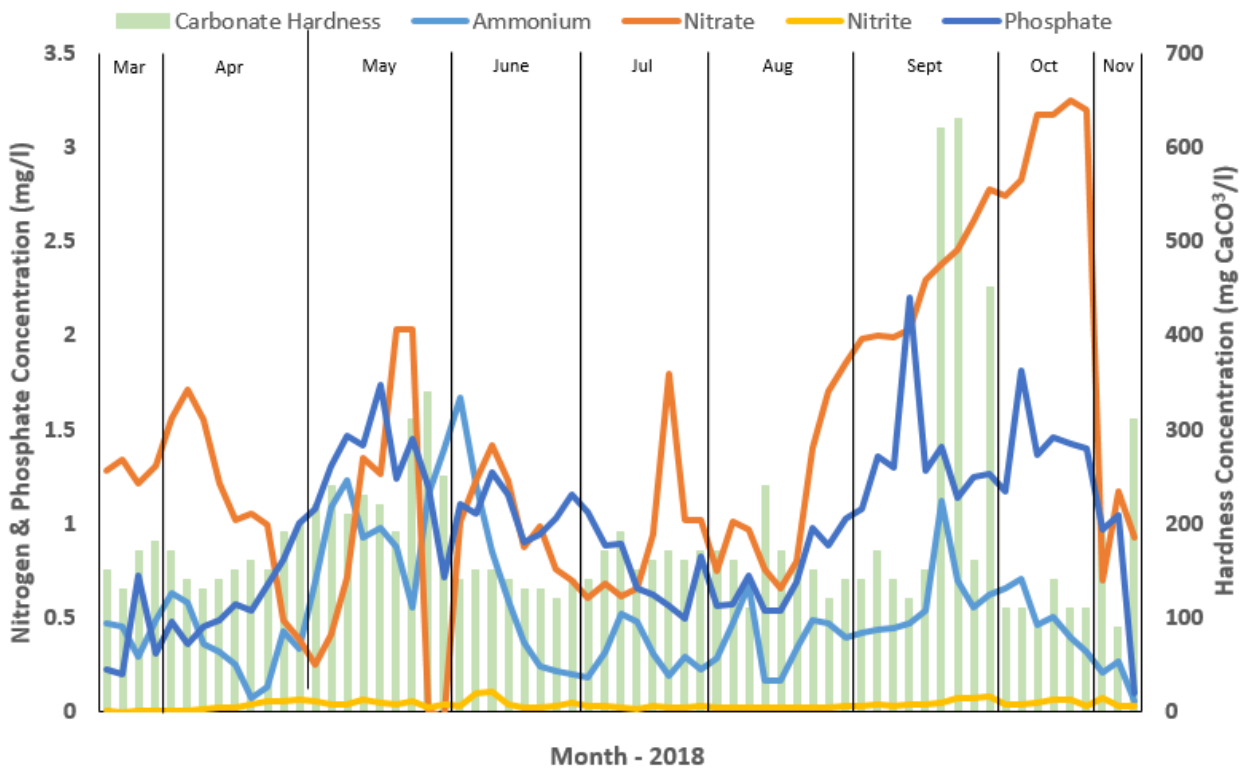

Figure 3.65 Trends for nitrogen, phosphate and carbonate hardness for the TP at KWF.

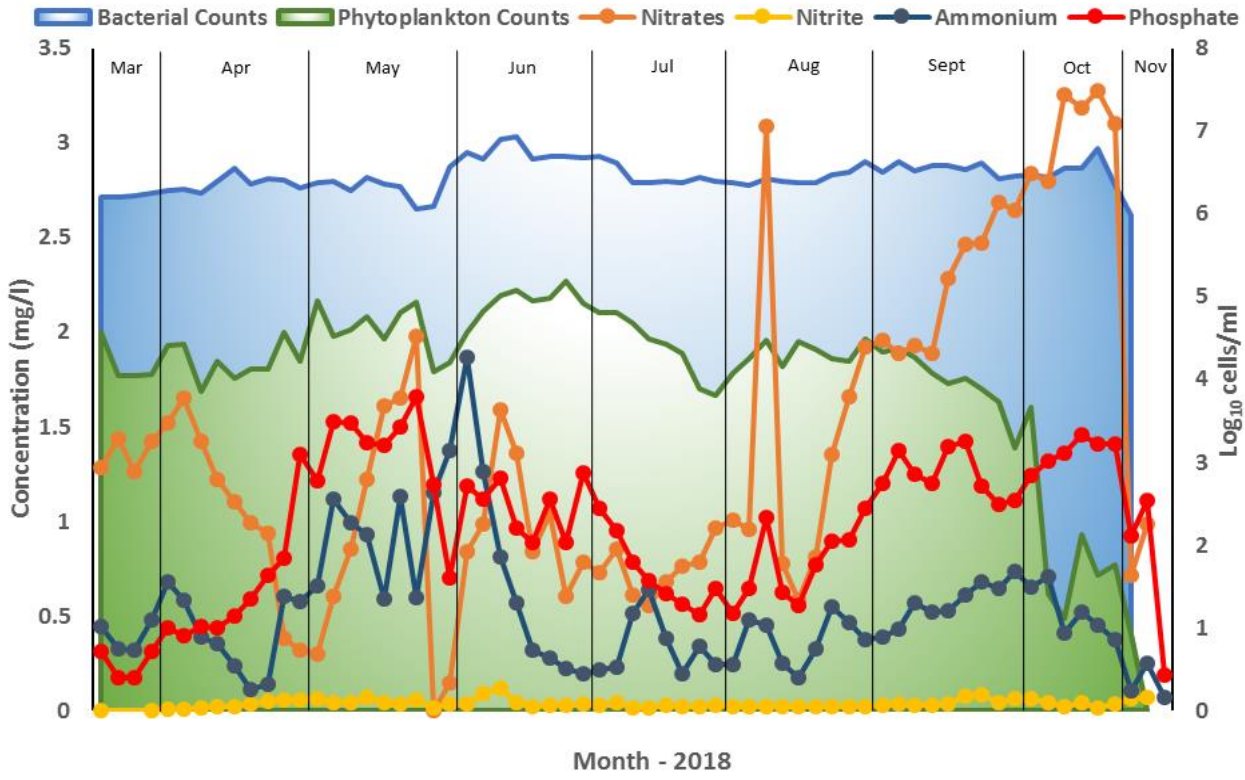

Figure 3.66 Nitrogen and phosphate trends graphed with bacterial and phytoplankton counts in the FP at KWF.

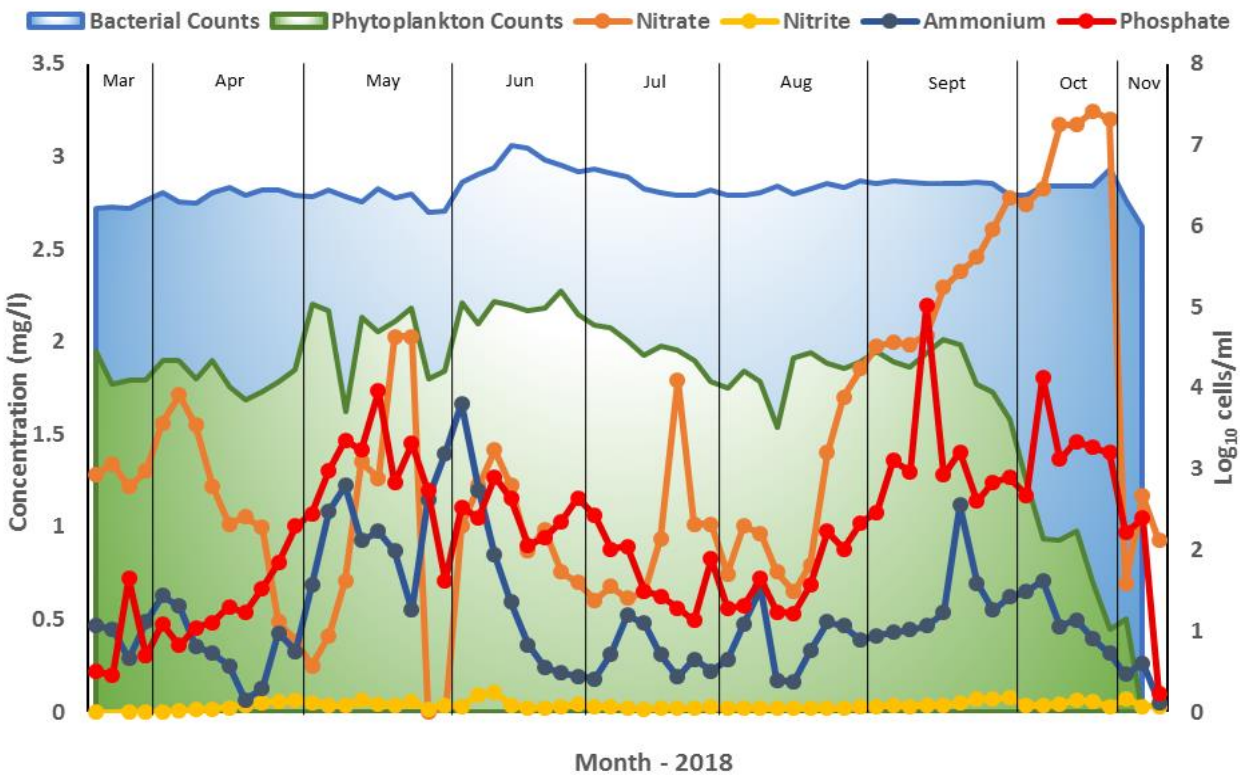

Figure 3.67 Nitrogen and phosphate trends graphed with bacterial and phytoplankton counts in the TP at KWF.

While certain levels of information can be gained from these graphs; similar trends for each parameter between both the FP and the TP, the temperature ranged from 5.8°C in March to 22.2°C in August, while the pH values remained between 7.2 and 8.4 for the duration of the study, it is difficult, however, to make any assumptions about the relationships between variables. In order to analyse this volume of data Principle Component Analysis (PCA) was carried out in order to observe any correlations and/or variability between parameters, which can be difficult to ascertain from raw data. This analysis was carried out in Excel using the XLSTAT software. This type of analysis provides tables and graphs through which observations on the relationships between parameters were made.

The parameters analysed included a combination of variables measured in the laboratory and measured on-site. The lab parameters analysed (mg/l) were nitrate concentration, nitrite concentration, ammonium concentration, phosphate concentration, water hardness (mg $CaCO_3$/l), bacterial numbers, phytoplankton numbers, chlorophyll-containing cells and cyanobacterial cells, with the latter four

parameters measured in cells/ml. The parameters analysed that were recorded on-site included pH, oxygen (mg/l), turbidity (FTU), feeding rate and total chlorophyll and cyanobacteria measured using the AlgaeTorch, with results in μg/l.

The first set of results for determining observations within the data are the correlation matrix (Pearson) scores. The closer the score is to 1 or (-) 1 the greater the positive or negative correlation is between two parameters, respectively. The correlation matrix scores obtained for the parameters analysed in this study are displayed in **Tables 3.9** and **3.10** for the FP and TP, respectively. In the case where correlations existed between parameters, yellow scores denote a moderately strong correlation, red scores denote a strong correlation and blue scores denote a very strong correlation. For this study, the parameters that have a moderately strong to very strong correlation are discussed rather than the parameters that share weak correlations.

The highest correlation for the FP and the TP was the positive one observed between chlorophyll-containing cells and the cyanobacterial cells, with coefficient scores of 0.990 and 0.988 for the FP and TP, respectively. This clearly corresponds to the data displayed in **Figures 3.51** and **3.52** and reiterates the observation that cyanobacteria represented most of the chlorophyll source present in pond 1 at KWF compared to phytoplankton. There was a strong positive correlation between the same two parameters when measured on-site using the AlgaeTorch, which indicates that both on-site and in lab measurements of phytoplankton produced similar trends. There was a moderately strong positive correlation between phytoplankton counts and chlorophyll-containing cells for the FP with a score of 0.441. Whereas a strong positive correlation was established for the same parameters in the TP, with a score of 0.607. This would suggest that the trends for phytoplankton numbers in the TP were more in line with the overall trends for chlorophyll-containing cells compared to the phytoplankton numbers in the FP. This may be due to the presence of fish in the FP, which would have impacted the phytoplankton numbers to a higher extent, by uptake into the diet for example.

Table 3.9 Correlation coefficient scores for PCA analysis carried out on FP parameters at KWF using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano = cyanobacterial; Phyto = Phytoplankton).

Variables	Nitrate	Nitrite	Ammonium	Phosphate	Temp.	pH	Oxygen	Turbidity	Hardness	Feeding Rate	Bacteria No.'s	Phyto No.'s	Chl-Ctn. Cells	Cyano Cells	Chl (Torch)	Cyano (Torch)
Nitrate	1															
Nitrite	0.200	1														
Ammonium	0.118	0.342	1													
Phosphate	0.261	0.550	0.426	1												
Temp.	-0.173	0.249	0.026	0.268	1											
pH	-0.433	-0.203	-0.331	-0.251	0.616	1										
Oxygen	-0.483	-0.234	-0.120	-0.292	-0.338	0.195	1									
Turbidity	-0.167	0.268	0.421	0.493	-0.001	-0.267	0.161	1								
Hardness	0.240	0.512	0.099	0.171	0.108	0.005	-0.220	-0.145	1							
Feeding Rate	-0.093	-0.091	-0.076	0.068	0.420	0.367	-0.292	-0.060	0.030	1						
Bacteria No.'s	-0.030	0.216	-0.080	0.257	0.402	0.304	-0.002	-0.121	0.100	0.235	1					
Phyto No.'s	-0.369	0.230	0.043	0.190	0.329	0.264	0.336	0.319	-0.160	0.108	0.555	1				
Chl-Ctn. Cells	-0.463	0.366	0.065	0.284	0.069	-0.043	0.327	0.484	-0.151	-0.115	0.004	0.441	1			
Cyano Cells	-0.435	0.350	0.063	0.270	0.023	-0.084	0.296	0.463	-0.137	-0.137	-0.082	0.314	0.990	1		
Chl (Torch)	-0.539	0.211	-0.073	0.286	0.731	0.490	0.057	0.357	-0.131	0.308	0.339	0.511	0.555	0.508	1	
Cyano (Torch)	-0.568	0.175	-0.206	0.060	0.445	0.580	0.377	0.100	-0.103	0.151	0.489	0.604	0.527	0.466	0.652	1

0 = No Relationship
< 0.2 =Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.6 – 0.8 = Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

Table 3.10 Correlation coefficient scores for PCA analysis carried out on TP parameters at KWF using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano = cyanobacterial; Phyto = Phytoplankton).

Variables	Nitrate	Nitrite	Ammonium	Phosphate	Temp.	pH	Oxygen	Turbidity	Hardness	Feeding Rate	Bacteria No.'s	Phyto No.'s	Chl-Ctn. Cells	Cyano Cells	Chl (Torch)	Cyano (Torch)
Nitrate	1															
Nitrite	0.282	1														
Ammonium	0.150	0.296	1													
Phosphate	0.398	0.624	0.438	1												
Temp.	-0.261	0.234	0.029	0.276	1											
pH	-0.500	-0.215	-0.338	-0.263	0.616	1										
Oxygen	-0.494	-0.211	-0.150	-0.354	-0.338	0.195	1									
Turbidity	-0.155	0.264	0.391	0.399	-0.001	-0.267	0.161	1								
Hardness	0.246	0.424	0.325	0.209	-0.065	-0.138	-0.079	0.087	1							
Feeding Rate	-0.061	-0.105	-0.081	0.048	0.420	0.367	-0.292	-0.060	-0.052	1						
Bacteria No.'s	-0.110	0.173	-0.068	0.233	0.384	0.282	0.019	-0.100	-0.099	0.276	1					
Phyto No.'s	-0.357	0.281	0.252	0.229	0.370	0.187	0.256	0.377	-0.030	0.140	0.539	1				
Chl-Ctn. Cells	-0.481	0.321	0.119	0.101	0.131	-0.029	0.270	0.496	0.014	0.019	0.067	0.607	1			
Cyano Cells	-0.459	0.303	0.085	0.069	0.069	-0.076	0.247	0.477	0.023	-0.010	-0.032	0.475	0.988	1		
Chl (Torch)	-0.599	0.199	-0.084	0.174	0.731	0.490	0.057	0.357	-0.169	0.308	0.352	0.502	0.567	0.526	1	
Cyano (Torch)	-0.573	0.179	-0.257	0.024	0.445	0.580	0.377	0.100	-0.159	0.151	0.480	0.548	0.481	0.421	0.652	1

0 = No Relationship
< 0.2 =Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.6 – 0.8 = Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

There was a strong correlation between temperature and the chlorophyll content measured using the AlgaeTorch, with a coefficient score of 0.731. This would be expected as temperature and daylight are two of the most important factors in terms of phytoplankton growth, so an increase in temperature will lead to an increase in phytoplankton. This correlation was not, however, present for the chlorophyll analysis carried out in the lab, even though this parameter correlated to the on-site chlorophyll measurements. This result may imply that although certain parameters have been proven to share relationships, this may not always be reflected in the data as other variables may interfere or an increased number of data points may be required. Delgadillo-Mirquez *et al.* (2016) found that temperature was the main parameter that positively affected biomass productivity and the rate of ammonium removal in an investigation of the removal of nitrogen and phosphorus by a microalgae-bacteria consortium in a WWTP. This indicates the influence that one parameter can have on the biological functioning of an ecosystem.

There was a moderately strong positive correlation between bacteria and phytoplankton enumeration data in both the FP and TP, with coefficient scores of 0.555 and 0.539, respectively. This correlation in not unexpected due to the symbiotic relationship shared between these two biological entities. Phytoplankton require nitrogen as an essential element for building structural and functional proteins (Hu, 2004). It is available in the soil organic matter (SOM); however, nitrogen is not in a bioavailable form for phytoplankton to utilise. Nitrogen-fixing bacteria convert the nitrogen (nitrogen fixation) into a form that can then be utilised by phytoplankton (Neospark, 2014; Thajuddin & Subramanian, 2005). The rate of nitrogen fixation largely depends on the bacterial species present in the water and the concentration of ammonia (Hargreaves, 1998). This nitrogen fixation process highlights the important interdependent relationship that exists between phytoplankton and bacteria. Another aspect of this dynamic relation involves organic matter, on which bacteria thrive (Baines and Pace, 1991; Amon and Benner, 1996; Blancheton *et al.*, 2013). One of the principal sources of organic matter in the rearing water is primary production by phytoplankton, followed by excreta and feed pellets (Baines and Pace, 1991; Moriarty, 1997). Aerobic bacteria present in the water body

break down this organic matter into CO_2 and ammonia (Phang, 1991). Phytoplankton then utilise the CO_2 for photosynthesis and release oxygen during the process, which in turn oxygenates the water for the fish (Neospark, 2014). Phytoplankton also uptake the ammonia as well as heavy metals, reducing the availability of toxic substances for fish to consume (Neori *et al.*, 2004).

In terms of the physiochemical parameters, there was a strong positive correlation between pH and temperature which is not unusual as temperature tends to influence pH levels. Li *et al.* (2012) found that temperature positively shaped the biological communities, as the number of organisms increased with increasing temperature in the lake. In this study, for the FP, a moderately strong positive correlation was achieved between the temperature and both bacteria enumeration and cyanobacteria measurement with the AlgaeTorch, whereas a strong positive correlation was achieved between temperature and the chlorophyll content measured used the AlgaeTorch. For the TP, a moderately strong correlation was also achieved between temperature and cyanobacteria measured using the AlgaeTorch.

Phosphorus was a determining factor for plankton richness in the study carried out by Li *et al.* (2012) on an artificial lake, whereas transparency negatively correlated with plankton communities. This negative correlation was related to the negative correlation achieved between transparency and the presence of phytoplankton, indicating that phytoplankton have a major impact on the turbidity of water. This finding agrees with the moderately strong positive correlation achieved between turbidity and chlorophyll-containing cells in both the FP and TP in this study. In the case of nitrates, the data for nitrate concentration in both the FP and the TP was negatively correlated with chlorophyll and cyanobacterial parameters measured. This would indicate that the presence of chlorophyll-containing cells/pigment, the majority of which corresponded to cyanobacteria, had a negative impact on nitrate levels. Phytoplankton are known for the uptake and removal of certain nutrients from the water and the coefficient scores reflect this fact. This finding is comparable to results determined in a study carried out by Choi *et al*. (2010), where the growth of cyanobacteria and phytoplankton inhibited the maximum nitrification rate by a factor of 4 in an autotrophic bioreactor. Hu *et al.* (2000) also established similar

results in an assessment of the removal of nitrate from groundwater by cyanobacteria, with *Synechococcus* sp. displaying the highest rate of nitrate removal.

Positive correlations were displayed for phosphate concentrations when compared with nitrites and ammonium, which tend not to be directly related in terms of concentrations. Jensen and Andersen (1992) investigated the most influential factors of phosphorus release from aerobic sediment surfaces of shallow Danish lakes. Temperature accounted for over 70% of the seasonal variation of phosphate release from sediment in all four lakes investigated in a study by their study. High nitrate concentrations supressed the release of phosphate through oxidation of the surface layer causing iron (Fe) to remain oxidised, hence, less chance to combine with phosphate, therefore decreasing the sediment reactive phosphate (SRP) release. Jensen and Anderson (1992) concluded that mineralisation as a result of steady sedimentation has a major role in the release of SRP from sediment.

This correlation data was also viewed in the form of correlation circles. In order to get the best representation of each parameter data set, the axis which best represented the data required was chosen, based on the squared cosines of the variables. The greater the squared cosine value the greater the link with the corresponding axis. The squared cosines of each of the parameters can be viewed in **Appendix A**. In the case of the FP, axes F1 and F2 represented 47.63% of the variables, which was the highest cumulative percentage for representing the data, and therefore an F1/F2 map was chosen. However, based on the squared cosines of the variables results, an F1/F3 and F1/F4 map were also chosen for the correlation circles, representing 45.53 and 37.01% of the data, respectively. Therefore, three correlation circles were required to determine correct correlations between parameters. For the TP three correlation circles were also used for analysis, with each one representing 49.07, 44.78 and 36.84 % of the data, respectively. This is extremely important as if the correct axes were not used, correlations may be interpreted that did not in fact exist. **Figure 3.68** displays the correlation circles for all parameters analysed for the FP in A, B and C, whereas the parameters analysed for the TP are displayed in D, E and F.

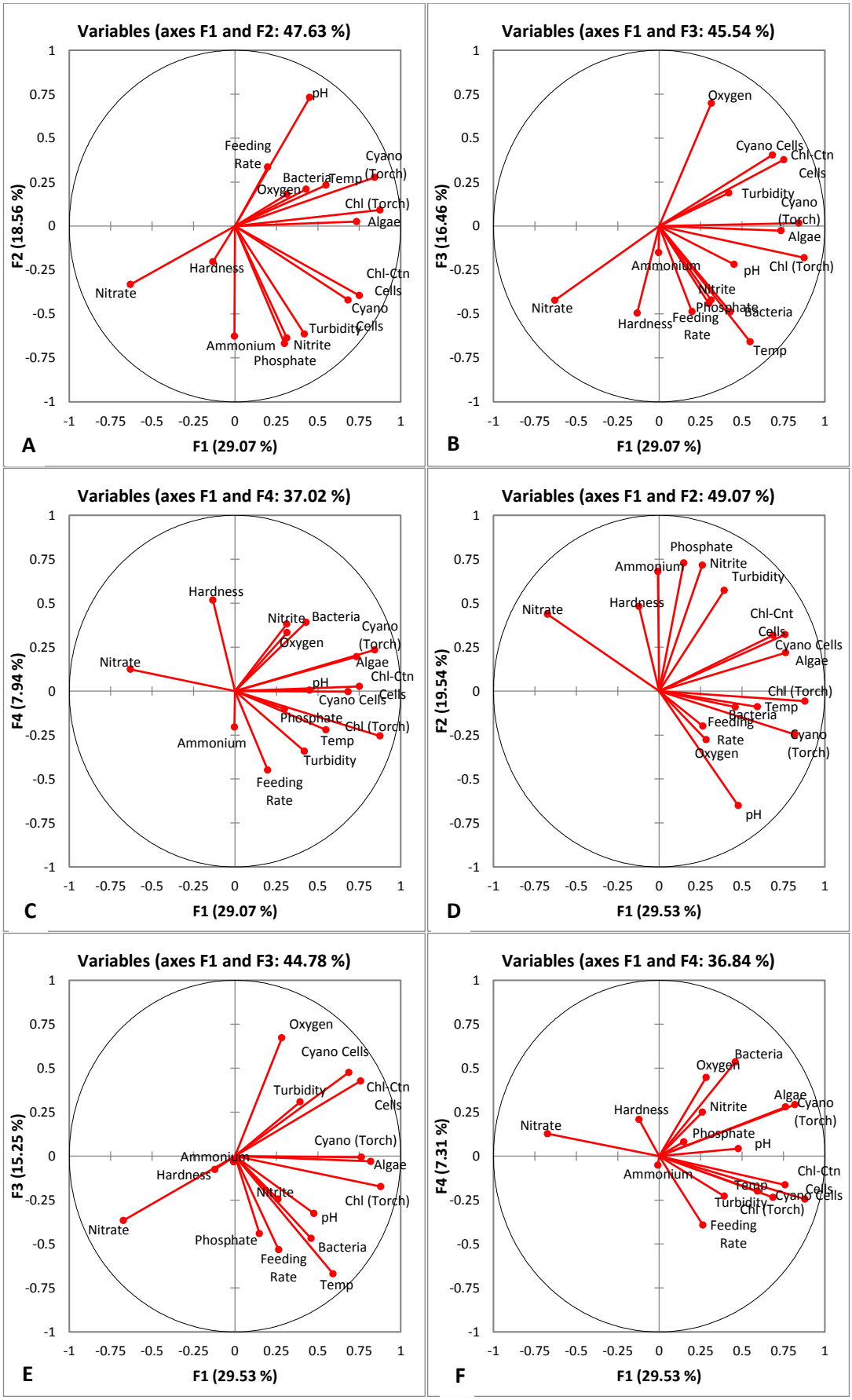

Figure 3.68 Correlation circles for the FP (**A**, **B** and **C**) and the TP (**D**, **E** and **F**) on varying axes for analysis of the relationships between all parameters analysed.

To determine whether correlations exist between variables using a correlation circle, narrow angles between variables indicate a positive correlation, right angles indicate no correlation exists and obtuse angle indicate a negative correlation. Also, the length of the correlation lines indicates how well each parameter is represented by that axis. For example, in correlation circle A, oxygen, bacteria, temperature and hardness have quite short lines, which means the data corresponding to these parameters is not very well represented by the F1 and F2 axes in correlation circle A. The lines are much longer in correlation circle B, indicating that this circle would be the most appropriate to evaluate the aforementioned parameters.

The parameters with a very strong correlation from the correlation coefficient scores for the FP were the chlorophyll-containing cells and the cyanobacterial cells. This relationship was viewed on correlation circle A in **Figure 3.68**, with a very narrow angle between two long lines representing both parameters. The same example can be used for the TP in correlation circle D, where the two parameters also shared a very narrow angle. The negative correlation between nitrates and both chlorophyll containing cells and cyanobacterial cells for the FP and TP was evident by an obtuse angle in correlation circles A and D.

A moderately strong positive correlation that presented in both the FP and TP was between nitrites and hardness levels, which are typically not directly associated either. Many of the results in **Tables 3.9** and **3.10** which displayed correlations between parameters that are normally not directly associated with each other may be simply due to coincidence, however, others may warrant further investigation for future studies. In some cases, results for which correlations have already been reported differed to results obtained in this study. For example, Delgadillo-Mirquez *et al.* (2016) discovered a linear correlation between ammonium concentration and chlorophyll at 15°C and 25°C. In contrast, the results obtained in this project found a correlation coefficient value of -0.073 and -0.084 for the FP and TP, respectively, between ammonium and chlorophyll, indicating a very weak correlation, if any.

Variations in certain water parameters can lead to stress for microorganisms as plankton tend to be sensitive to changes which can affect the community composition (Øvreås, 2000; Li *et al.*, 2009). This may explain some of the variation in

bacterial and phytoplankton numbers when certain physiochemical parameters were unstable. This was evident at the end of May/beginning of June when the nitrate levels dropped and ammonium levels reached their highest, resulting in a temporary decline in bacterial numbers. High temperatures, increased phosphorous concentrations and a decrease in the total nitrogen:total phosphorus (TN:TP) ratio are the common factors associated with phytoplankton blooms, the occurrence of which can lead to decreased oxygen levels, decreased biodiversity and increased turbidity (Bukowska *et al*., 2014). Therefore, it is important to try monitor phytoplankton species diversity in culture water to ascertain the species that are dominating the population, and whether the dominant species are at risk of posing a threat on the biodiversity as well as the fish, in the form of blooms. Common freshwater cyanobacteria that produce toxins in the form of microcystins include *Microcystis* and *Anabaena* (Sivonen & Jones, 1999). Based on the analysis carried out, harmful phytoplankton species were not identified in the rearing water.

Overall, the PCA analysis demonstrated that certain parameters for which correlations were expected were indeed present, for example, correlations between temperature, nitrates and phytoplankton. Whereas other correlations that may not have been expected but showed up and may warrant further investigation such as the positive correlation between nitrite and hardness. The PCA analysis also revealed that the use of on-site measurement methods correlated to the lab analysis in the case of the chlorophyll and cyanobacteria parameters. However, the comparative analysis between the physicochemical parameters measured (**Figures 3.24** to **3.27)** displayed how varied results can be when using less precise methods, emphasising the importance of accuracy when it comes to parameter concentrations. This study resulted in an initial baseline development of pond 1 rearing water at KWF and revealed the fundamental factors encompassed in the ecosystem dynamic, as well as some informative relationships within this pill-pond system.

In summary, the main outcomes of this body of work were:

- The FP and TP shared similar results in terms of physicochemical concentrations, numbers of organisms (both phytoplankton and bacteria)

and biological diversity, with the exception of differing phytoplankton species in September when diatoms dominated the FP and *Cryptomonas* sp. dominated the TP.

- Both bacteria and phytoplankton numbers peaked in June, with the cyanobacterial population peaking between the end of April and the beginning of May.
- Based on the bands excised from the DGGE gels, the bacterial population was dominated by Proteobacteria, Bacteriodetes, Cyanobacteria and unclassified bacteria.
- Chlorophyta, Bacillariophyta and Cryptophyta dominated the phytoplankton community.
- The PCA analysis demonstrated that nitrates, temperature and bacterial numbers had the greatest correlation with phytoplankton numbers, and that there was great correlation between the use of *in situ* technology (AlgaeTorch) and *ex situ* methods (FCM) for the measurement of phytoplankton and cyanobacteria.

The results of the study conducted at KWF provide a baseline for the rearing water ecosystem interactions. In pill-pond aquaculture at KWF, there is less control over environmental conditions than in closed tanks systems, which is why the ecosystem dynamic needs to be understood and possibly manipulated for successful and sustainable production. Identification of the most influential biological species provides valuable knowledge and the possibility of seeding of specific microbial assemblages when required for certain processes, i.e. the addition of a specific bacteria for nitrification or the fertilisation of a specific phytoplankton species for nutrient removal or oxygen supplementation during the daytime.

In addition, more sustainable and effective disease control measures need to be implemented for successful management and eradication of unwanted pathogens and possibly for control of the phytoplankton population, once identified. A novel approach to this is investigated in the proceeding Chapters with the introduction of PUV light for the inactivation of common freshwater pathogens in Ireland.

Chapter 4

Novel Disinfection Technology – Pulsed Ultraviolet Light Studies

Introduction

Several constraints exist in the freshwater aquaculture industry as briefly mentioned in **Chapter 2**. The implementation of an intensification approach of the freshwater aquaculture industry leads to issues regarding water consumption due to the constraints on water abstraction levels (Terjesen *et al.*, 2013). In traditional FT aquaculture systems, where all the incoming water source needed is "new" (i.e. from a river), the water consumption can be up to 100 m^3 per kilogram of fish produced (Roque d'Orbcastel *et al.,* 2009). Intensification of the aquaculture industry by keeping the same FT systems would increase this water consumption, therefore, contributing to water scarcity. Although RAS systems are the way forward, water re-use and recirculation can result in an increased bioload, leading to a huge economic cost to the fish farmer in terms of increased chemical usage, stock mortalities due to widespread disease and decreased income or profit due to reduced productivity. In terms of disinfection, the overuse of chemicals can pose a threat to fish health, wild fish health, the state of the environment and lastly the wellbeing of the consumer, i.e. humans. Aside from the negative impacts that chemicals can have, the possibility of the biological recovery mechanism of bacteria upon exposure to UV light, i.e. photoreactivation, also poses a problem for disease mitigation.

Therefore, there is an urgent need for more effective, sustainable and energy efficient technologies that can aid in the increased production of fish to meet consumer needs, food security needs and health and safety regulations. This section focuses mainly on PUV light as a method that upon execution in aquaculture farms can mitigate diseases, increase water quality, provide more control and, therefore, dramatically enhance production. There is also a focus on the comparison of PUV with CW UV, the effect of PUV on current chemicals used *via* an ecotoxicological assessment (**Chapter 5**) and how this may impact the natural biota in the culture water and the receiving environments.

4.1 PUV light technology

PUV light is emerging as a novel tool for decontamination and sterilisation of water as well as inactivation of microbial organisms that reside in water (Garvey *et al.*, 2015). Pulsed UV flash lamp systems were first patented in 1931 for many different applications (McDonald *et al.*, 2000). The main reason for the emergence of PUV for water treatment in WWTPs and in the aquaculture industry is due to its ability to replace chemical disinfectants without the production of dangerous by-products (Lamont *et al.,* 2004). The high peak power associated with PUV light is capable of eradicating DNA of fungal yeasts, bacteria including spores, molds, and viruses, such as bovine viral diarrhoea virus (BVDV) and poliovirus (Sharifi-Yazdi and Darghahi, 2006; Xenon Corporation, 2006b; Lamont *et al.*, 2007; Azar-Daryany *et al.*, 2009; Farrell *et al.*, 2009; Beck *et al.*, 2015). However, its effectiveness at eliminating freshwater fish pathogens remains to be determined, which was a major objective of this project.

The approach of bacterial inactivation by a pulsed power high intensity UV light yields short bactericidal wavelengths of light with immense intensity, by providing the light source with numerous milliwatts of power (MacGregor *et al.,* 1998). The mechanism behind PUV in its ability to inactivate microorganisms lies in the UV content, which prevents DNA replication of microbes due to alteration of pyrimidine bases of nucleic acids (Lamont *et al.,* 2004). Farrell *et al*. (2009) demonstrated that gram-negative bacteria such as *E. coli* are more susceptible to PUV than gram-positive bacteria, especially during the exponential growth phase, causing irreversible cell membrane damage such as the formation of pyrimidine dimers and single-strand breaks.

PUV light differs from CW UV light exposure as a more intense UV illumination is produced in a shorter period of time. A capacitor stores electrical energy until the system is in operation, after which the energy is released in rapid time into the xenon flash lamp. The result is the production of an intense UV flash with high peak power and high current (Sharifi-Yazdi and Darghahi, 2006). According to Xenon Corporation (2006a), PUV systems consume up to 80% less energy than conventional CW UV systems which consist of a mercury lamp. PUV systems tend to use xenon flash lamps

as the source of the UV light which are much more environmentally safe than the hazards that may be associated with the use of mercury lamps (Xenon Corporation, 2006a). The intrinsic difference in peak energy between PUV and CW UV light illustrated in **Figure 4.1**, demonstrates that the intensity of PUV light delivers a much higher peak power than CW UV, reaching up to 100,000 times the intensity of the sun on earth's surface.

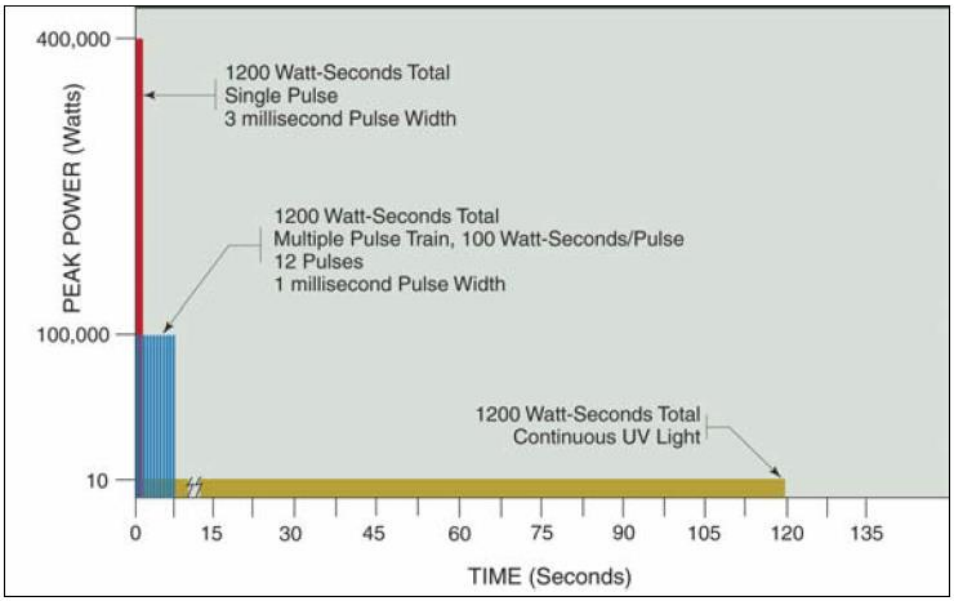

Figure 4.1 Comparison of pulsed vs. continuous UV light; pulsed light illustrated in single pulse and burst modes (Xenon Corporation, 2006b).

The high-peak power property of the PUV attributes a significant advantage over conventional UV lamps (Farrell *et al.*, 2009). If high-peak pulse power is employed, along with the greater penetration depth, irreversible DNA alteration can be achieved, damaging the genome resulting in rapid inactivation and lethalities (Hosseini *et al*., 2011). Bohrerova *et al.* (2008) discovered that the PUV system was 2.4 times more effective at inactivating *E. coli* than MP CW UV irradiation. A study which observed the outperformance of PUV over CW UV light is illustrated in **Figure 4.2**, where McDonald *et al.* (2000) compared the two systems for the inactivation of *B. subtilis* spores. In order to reduce the percent survival of the *B. subtilis* spores below 0.1 percent, 16 mJ/cm^2 of CW UV light were required, whereas only 8 mJ/cm^2 of UV light was required to reduce the spores to the same percent (McDonald *et al.*, 2000).

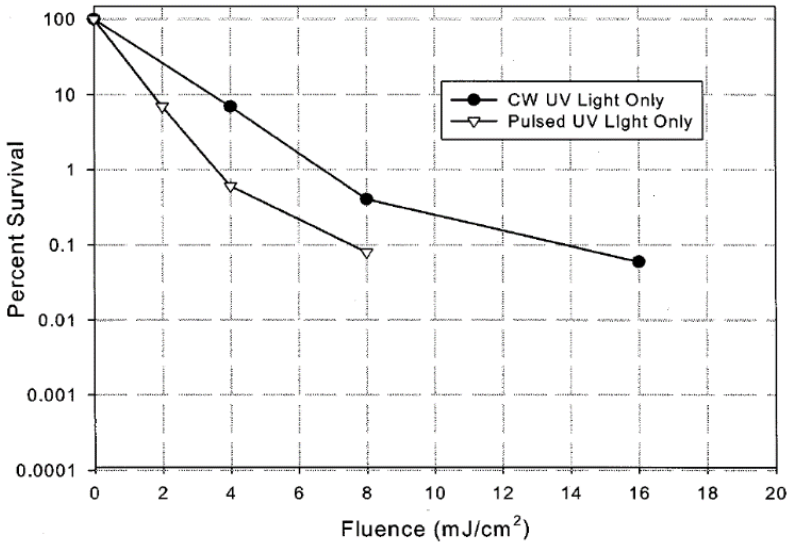

Figure 4.2 Inactivation of *Bacillus* spores on surfaces by continuous and pulsed UV light (McDonald *et al.*, 2000).

For conventional CW UV disinfection, the system is continuously in operation while the sample is exposed to UV treatment. In contrast, the PUV system operates on the basis of pulses emitted and a cooling period is achieved in between pulses, which does not occur with conventional UV systems as illustrated by Xenon Corporation (2006a) in **Figure 4.3**. In addition, no warm-up period is required for PUV operation, unlike CW UV setup (Bohrerova *et al.,* 2008). PUV system parameters can also be changed in terms of voltage and pulse rate, therefore allowing the system to be optimised for specific applications (McDonald *et al.*, 2000).

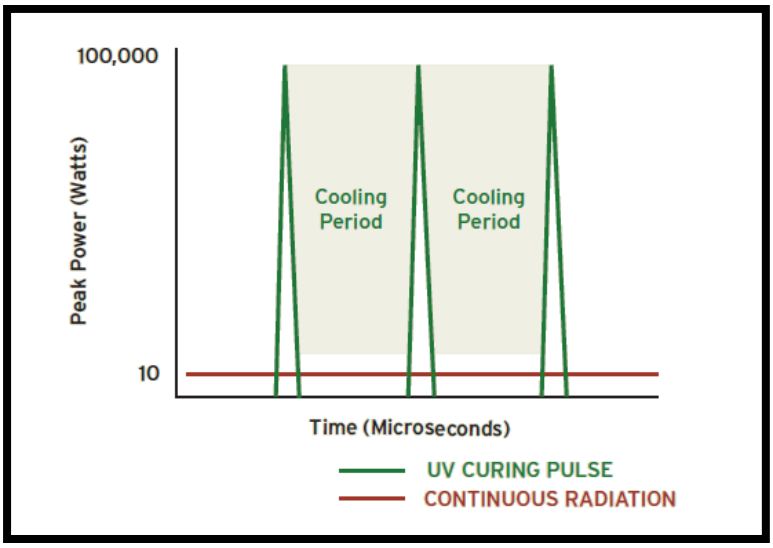

Figure 4.3 Pulsed UV light provides cooling between pulses whereas CW UV continuously warms the substrate (Xenon Corporation, 2006a).

4.1.1 PUV light & photoreactivation

Photoreactivation responses can occur for some pathogens, following exposure to CW UV light (Maclean *et al.*, 2008). Exposure to PUV light unlike exposure to UV light doesn't just target DNA but also has the ability to destabilise the integrity and functionality of plasma cell membranes as discovered by Farrell *et al.* (2011) in the disinfection of *Candida albicans* using PUV light. A study carried out by Lamont *et al*. (2004) investigated photo-repair mechanisms associated with PUV. It was discovered that whilst photoreactivation can indeed occur following exposure to PUV light, in order to photo-repair they needed to be exposed to high amounts of visible light and UVA light post treatment with PUV (Maclean *et al.*, 2008). Another factor involved in photoreactivation is time; the most common time required in order for photoreactivation to occur is 1 to 3 hours of exposure to UVA light (Oguma *et al.,* 2002) and/or high amounts of visible light. So in order for pathogens to have the ability to photoreactivate following PUV treatment, they must be exposed to UVA light for a long period of time.

4.1.2 Potential applications of PUV technology in aquaculture

There are three main areas of the fish farm system in which PUV technology may be implemented: (i) treatment of the incoming water, (ii) decreasing the pathogenic load throughout the system and (iii) treatment of effluent before it is discharged into the receiving water body. When therapeutic agents are administered in RAS systems, the potential of that substance being circulated through the rearing water is high, however, it will be much more dilute than the initial dose administered (Verner-Jeffreys and Taylor, 2015). Therefore, if PUV disinfection is implemented downstream in the fish farm there is potential for therapeutic or chemical substances to encounter this technology, albeit at a diluted dose. Thus, it is important to investigate the impacts of PUV on such substances in terms of toxicity. Discharge from aquaculture farms can lead to the introduction of pathogens into receiving waters impacting the natural ecosystem if there is no treatment method in place (Hopkins *et al*., 1995), and so disinfection methods are required to decrease the

amount of waste and microbes released into these waters. PUV technology may be used as a tool to help with remediation of the environmental impacts of aquaculture wastewater effluent as it has already proven effective as a method of disinfection for WWTPs (Garvey and Rowan, 2011). PUV light represents an exciting and novel opportunity to treat fish pathogens and their concomitant disinfection chemical before they are discharged into the freshwater ecosystem.

Methodologies

A range of inactivation studies were carried out on common problematic freshwater bacteria. This involved the correct identification of selected test microbial strains to ensure the organisms sourced were relevant to the freshwater industry in Ireland. Once the identity was confirmed, the bacteria in question were subjected to PUV light exposure at varying voltages and for varying time periods. Due to the fact that CW UV light is already a common disinfection procedure in the aquaculture industry (**Chapter 2, Section 2.10**), the bacteria were then exposed to this method of disinfection for comparison studies in term of inactivation efficiency.

4.2 PUV inactivation studies

The PUV light system was used in this project in order to determine its applicability as a prototype for use in the freshwater aquaculture industry. A validation procedure was first employed using the bacterial strain *E. coli* before exposing a range of other bacterial species, more relevant to the freshwater aquaculture industry presented in **Chapter 2 - Aquaculture**, to the PUV light system. This validation procedure was carried out as per the method employed by Garvey *et al.* (2009) with any deviations from the method outlined. Following validation procedures, other problematic freshwater pathogens were subjected to the PUV technology to determine their susceptibility to this disinfection method. The two selected pathogens investigated were the bacteria *A. salmonicida* and *F. psychrophilum.* Both bacteria were subjected to a number of pulses of UV light over a range of voltages in order to determine the optimal kill zone, therefore employing the desired disinfection level at the most

energy efficient power of PUV. Comparative studies using conventional CW UV were carried out for both *E. coli* and *A. salmonicida*.

4.3 Test microbial strains

All microbial test strains used in this study were obtained in a dehydrated form from either the American Type Culture Collection (ATCC) or the National Collection of Industrial Food and Marine Bacteria (NCIMB), which is outlined in **Table 4.1**. *E. coli* was chosen as the bacterial surrogate to optimise the use of PUV technology *via* validation procedures as per Garvey *et al.* (2009). The basis for use of *A. salmonicida* and *F. psychrophilum* lies in the fact that they are well known salmonid pathogens in Ireland and worldwide (Morgan *et al.*, 1993; Austin, 1997) and *A. salmonicida* has also been isolated from non-salmonid marine and freshwater fish (Beaz-Hidalgo *et al.,* 2008). All strains were streaked to purity *via* colony isolation on appropriate agar for each microorganism followed by incubation at the relevant incubation periods and temperatures (see **Table 4.2**). The identity of each strain was confirmed *via* a range of biochemical, morphological and physiological tests including gram stain retention, growth on selective agar, API biochemical kits and IMViC tests, comprised of indole, methyl red (MR), Vogues-Proskauer (VP) and citrate characteristics. To maintain the stability of each culture, they were stored at -80°C in a microbank preparation and new cultures were plated every two weeks on appropriate selective agars.

Table 4.1 Source of test microorganisms used in this study.

Test Microorganism	Code	Origin	Host Source
Bacterium			
Escherichia coli	25922	ATCC	FDA Strain Seattle 1946
Aeromonas salmonicida, **subspecies *salmonicida***	1102	NCIMB	Salmon kill, River Cletter
Flavobacterium psychrophilum	1947	NCIMB	Kidney of Coho salmon (*Oncorhynchus kitsuch)*

Table 4.2 Growth requirements of the bacterial strains used in this study.

Bacterium	Growth Media		Incubation Conditions*
	Non-selective	**Selective**	
Escherichia coli	Nutrient Broth/Agar (LabM)	MacConkey Agar (LabM)	37°C aerobic 12 - 24hrs
Aeromonas salmonicida	Nutrient Broth/Agar (LabM)	Furunculosis Agar (HiMedia Laboratories)	20°C aerobic 24 - 48hrs
Flavobacterium psychrophilum	R2A Broth/Agar (LabM)	Selective Cytophaga Agar (Cosmos Biomedical Ltd.)	20°C aerobic 48 - 72 hrs
***To ensure homogenous distribution of samples incubation of *E. coli* required the used of agitation in an orbital shaker at 125rpm. *A. salmonicida* and *F. psychrophilum* were mixed manually at frequent intervals as no orbital shaking incubator was available at the appropriate temperatures required for the culturing.**			

4.4 Identification of microbial strains

Initial confirmatory tests of each strain involved visual examination of colonies on selective and non-selective agar to examine the purity of the strain. More in-depth morphological examination of each microorganism was performed *via* the gram stain retention procedure, during which a gram-positive bacterial species retains the crystal violet dye, whereas a gram-negative bacterial strain does not. Microorganism morphology was identified by this method microscopically under oil immersion (1000x magnification). *E. coli* colonies were grown on MacConkey agar (LabM) and their identity confirmed *via* determination of biochemical characteristics by API 20E profiles as depicted in **Figure 4.4**. *Aeromonas salmonicida* was cultured on furunculosis agar followed by identity confirmation *via* the preparation of API 20NE biochemical profiles. IMViC reactions were employed for both *E. coli* and *A. salmonicida* as preliminary biochemical confirmatory tests for Enterobacteriaceae; although *A. salmonicida* belongs to the Aeromonodaceae family, it morphologically resembles the family of Enterobacteriaceae and so IMViC tests were reliable. This involved the determination of indole production, MR and VP for glucose use and finally citrate utilisation. *F. psychrophilum* was grown on Cytophaga agar and identity was confirmed *via* the preparation of API ZYM profiles. IMViC tests were not performed for *F. psychrophilum*, however, as it is not an Enterobacteria.

Figure 4.4 API 20E biochemical profile set up for E. coli.

All of the API biochemical profiles were prepared and results determined as per the standard methods described in the manufacturers manual. In order to accurately differentiate *F. psychrophilum* from other *Flavobacterium, Flexibacter* and *Cytophaga* species, Bernardet *et al.*, (1990) have described the following characteristics as sufficient for investigation: cell morphology, colony morphology, ability of continuous growth on blood agar, flexirubin and cytochrome oxidase production, Congo red absorption ability, hydrolysing ability of starch and tyrosine, and API ZYM gallery reactivity.

4.5 Enumeration of bacteria

In order to determine the initial density of each microorganism, a standard curve was prepared for each bacterial strain. Bacterial cultures were prepared in the appropriate broth until the exponential growth was reached. A 1:2, 1:4, 1:6, 1:8 and 1:10 dilution was prepared and the optical density recorded for each dilution at 600 nm for *E. coli* and 610 nm for *A. salmonicida.* The optimal parameters for readings on a spectrophotometer are between 0.1 and 1 and each reading fell in between these parameters as measured on the Jenway UV-Vis Spectrophotometer. Each dilution was inoculated onto agar using the conventional spread plate method where 100 µl aliquots of each sample and the appropriate dilutions were transferred onto agar using sterile spreaders and plated in triplicate. Each plate was counted terms of colonies (**Figure 4.5**) with the limit of detection set at 1 CFU, using the following formula which accounts for the dilutions made:

$$\mathbf{CFU/ml} = \mathbf{Number\ of\ colonies} \times \frac{\mathbf{1}}{\mathbf{SD}} \times \mathbf{DF}$$

where **CFU** = colony forming units, **SD** = serial dilution, and **DF** = dilution factor.

This formula allows the results on plated counts to be expressed in CFU/ml, which is the standard method of expressing microorganism growth on agar.

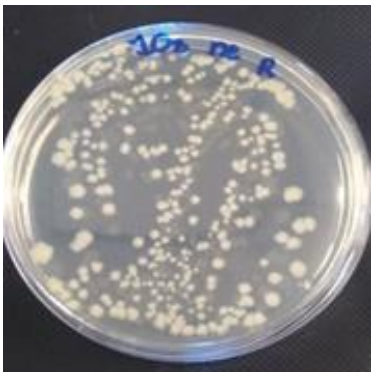

Figure 4.5 *A. salmonicida* colony growth on nutrient agar.

4.6 PUV system used in this study

The PUV light system used throughout this study was the Samtech PUV-01 system manufactured and supplied by Samtech Ltd, Scotland. This bench-top, portable system was used as a prototype to determine applicability in disinfection of problematic finfish pathogens. It was important to employ the use of all personal protective equipment (PPE) whilst operating the PUV system as this technology emits potentially lethal high voltages of UV light. All precautionary measures were also employed during PUV operations which are outlined in the 'Pulsed UV System, PUV-01 Operating Instructions' manual supplied by the manufacturer. This equipment was designed to cover UVA, UVB and UVC regions of UV light with continuously adjustable discharge voltages ranging from 400 V to 1000 V. The PUV system is comprised of two main components: the driver unit and the flash lamp chamber as illustrated in **Figures 4.6** and **4.7**.

The driver unit consists of the control panel for operating the system, where the voltage, pulse frequency, and trigger (automatic or manual) are set depending on parameters required. The pulse frequency, which determines the frequency output of the flash of UV light, can be set to between 0.1 and 10 pulses per second (pps).

Figure 4.6 The pulsed UV system used in this study.

The flash lamp chamber was designed to house a xenon-flash lamp with the capacity to produce high intensity UV light for microbial disinfection. The chamber also consists of an adjustable metal stand for the mounting of an 8.5 cm petri dish which contained each sample for treatment. The petri dish was placed at an 8 cm length distance from the flash lamp for all of the disinfection treatments carried out in this study.

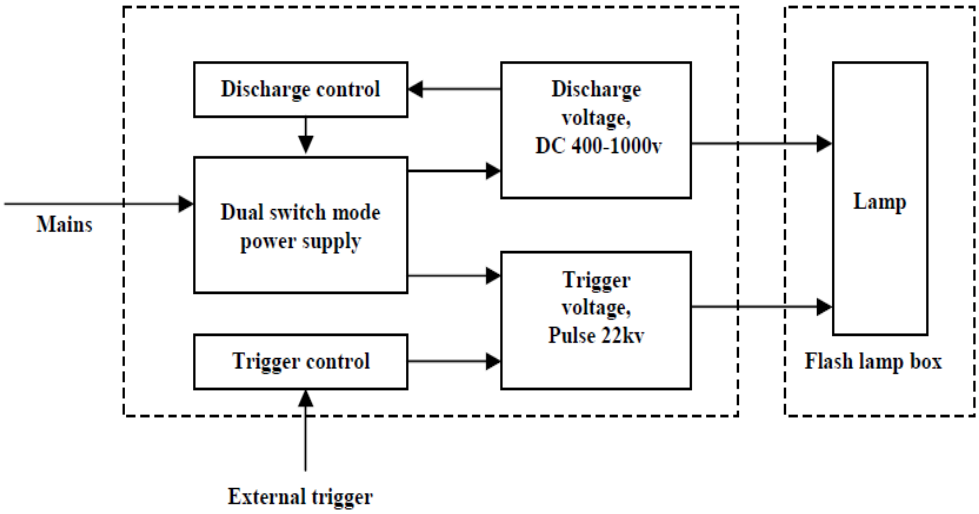

Figure 4.7 Block diagram of the pulsed UV system layout (Sharifi-Yazdi & Darghahi, 2006).

The concept behind the PUV system lies in the combination of pulsed power with flash lamp technology; therefore, a high intensity of UV illumination is emitted within a much shorter timeframe compared to conventional CW UV systems. Electrical energy is stored in a capacitor, which is rapidly released into the flash lamp, ionizing the xenon gas. The electrical energy produced is then converted to pulsed radiant

energy by the flash lamp resulting in the production of an intense UV flash due to high peak power and the high current generated. The UV dose emitted at a distance from the xenon lamp, also known as the UV irradiance, depends on the discharge voltage applied (400 V to 1000 V) which corresponds to the amount of energy released in joules (J) per pulse of UV light, outlined in **Table 4.3**. Depending on the pulse frequency and the voltage applied, one joule of energy may contain up to 10^{18} photons of UV light (Xenon Corporation, 2006b; Samtech Ltd, 2015)

Table 4.3 Discharge voltage and energy per pulse delivered by the PUV system (Samtech Ltd., 2015).

Voltage (V)	400	500	600	700	800	900	1000
Energy (J)	3.2	5.0	7.2	9.8	12.8	16.2	20

Due to the directly proportional relationship between discharge voltage and energy per pulse, an increase in the level of voltage applied will result in an increase in energy. This relationship is explained by the following equation:

$$\mathbf{E} = \frac{\mathbf{1}}{\mathbf{2}}\,\mathbf{CV^2}$$

where **C** = energy-storage capacitance (40 µF) and **V** = the discharge voltage.

The dose released by the lamp onto the specimen for treatment, measured in millijoules per centimetre squared (mJ/cm^2), is the product of irradiance in milliwatts per centimetre squared (mW/cm^2) and time in seconds (sec), therefore adjustment of discharge voltage or pulses can be applied to employ the required dose. Effective inactivation of microbes with UV light occurs within the UVC spectrum of UV light from 200 to 280 nm, known as the germicidal wavelength (see **Section 2.9**). **Figure 4.8** illustrates the emitted spectrum from the flash lamp of the PUV system recorded at discharge voltages of 600 V, 800 V and 1000 V. This demonstrates a rich output in the UVC region with three observed peaks at 229 nm, 247 nm and 260 nm (Samtech Ltd, 2015).

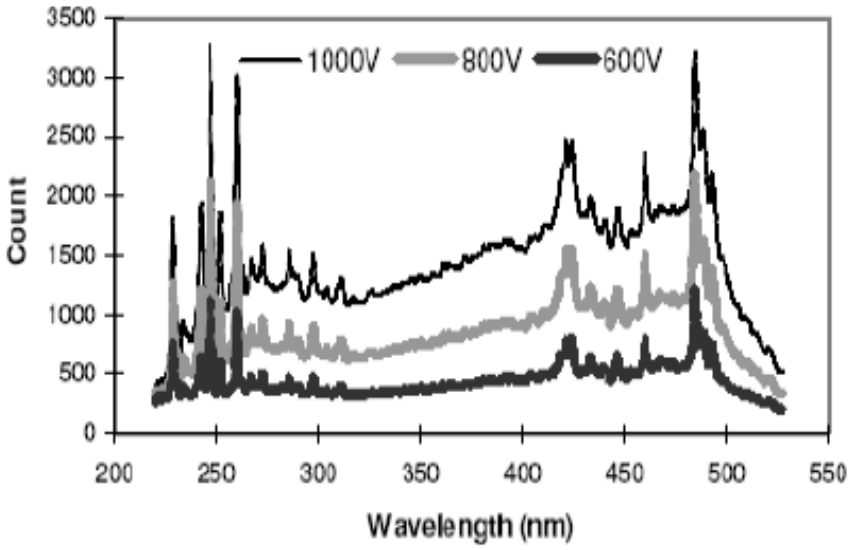

Figure 4.8 Emitted spectrum of the PUV system at different discharge voltages (Samtech Ltd., 2015).

4.7 Pulsing of microbial samples

The microbial test strains subjected to PUV treatment in this study were *E. coli, A. salmonicida* and *F. psychrophilum*. All species were cultured in the respective broths under the required conditions (see **Table 4.2**) to a cell density of $1.0x10^9$ CFU/ml. The cultures were subsequently adjusted to a cell density of $1.0x10^6$ CFU/ml based on standard curve results using quarter strength ringer's (QSR) solution as a diluent *via* the following equation:

$$\mathbf{C_1V_1 = C_2V_2}$$

where $\mathbf{C_1}$ = concentration of sample 1, $\mathbf{V_1}$ = volume of sample 1, $\mathbf{C_2}$ = concentration of sample 2, and $\mathbf{V_2}$ = volume of sample 2.

A 10 ml aliquot of each liquid sample was transferred to a sterile petri dish which was placed in the flash lamp chamber of the PUV instrumentation. The lid was removed from the petri dish before the commencement of pulsing at desired electrical parameters. For initial inactivation studies, high-range voltages of 700 V and 900 V were applied to samples with the number of pulses deployed ranging from 20 to 220 pulses at a frequency of 1 pps. Range finding procedures led to the establishment of optimal inactivation parameters at 400 V in order to provide a kill curve for each microorganism using the lowest possible energy in terms of voltage. This range

finding process also ensured that the most energy efficient mechanism of PUV-associated inactivation was applied. The enumeration process was carried out as previously described in **Section 4.5**. The agar plates were incubated at 37°C for *E. coli* and 20°C for both *A. salmonicida* and *F. psychrophilum* with results determined *via* colony counts. Every aspect of the experiment was carried out with the highest degree of microbial practice and aseptic technique ensured. For sterilisation of all solutions, glassware and apparatus required in this study the autoclave was utilised at the parameters of 121°C for 15 minutes to ensure adequate sterility and purity of components. All experiments were carried out as at least two independent tests, with sample plating on agar for colony enumeration carried out in triplicate.

4.8 CW UV inactivation studies

In order to perform comparative studies regarding the efficiency of PUV light as a superior non-chemical disinfection method, inactivation studies were carried out using the conventional fixed wavelength CW UV lamp, **Figure 4.9**. This source of UV light emits energy at 254 nm and is therefore referred to as monochromatic or CW UV. The inactivation studies were carried out using a benchtop LP mercury UV lamp, which was attached to a stand. The area for sample exposure was set up so that the petri dish containing the test microorganism was at a distance of 8 cm away from the light source to correspond with the PUV system set up. A warm-up period of 30 minutes was required for the UV lamp to ensure optimal performance. It was essential to have a bunsen burner in the surrounding area of the apparatus to ensure no contamination, as the handheld UV system does not consist of an enclosed chamber, unlike the PUV system.

Figure 4.9 UVGL-55 handheld UV lamp (Mineralogical Research Co., 2009).

The bacterial cultures were prepared as previously described and 10 ml aliquots of each sample were exposed to the UV light with the lid removed. Initial experimental analysis involved range finding to determine what time period was required for complete inactivation of the test microorganisms. Following this, both *E. coli* and *A. salmonicida* were exposed to the UV light for a timeframe ranging from 0 to 40 secs. Sample preparation, incubation and enumeration were carried out using the same methodologies as described for the PUV inactivation studies.

4.9 Statistical Analysis

All bacterial counts were expressed in terms of $\log_{10}$ CFU/ml with results illustrated in the form of 'Kill Curves' for each bacterial sample treated. Data are expressed in graphs as the arithmetic mean ± standard error of the mean (SEM). Death (D) values were calculated as a representation of the number of pulses (PUV) or length of time in seconds (CW UV) required to reduce a bacterial load by one $\log_{10}$ order. This is achieved by utilising the 'What if Analysis' tool on Excel, where the X values (secs/pulses) for two selected Y values ($\log_{10}$ CFU of bacteria) are determined and the difference between the two X values is considered the D-value. The smaller the D-value, the lower the number of pulses or the amount of time required for inactivation, which is reflected in the size of the slope, indicating a greater inactivation efficacy. Statistical analysis was also performed using the GraphPad Prism work package. Differences were considered significant at $p < 0.05$.

4.10 Confirmation of microbial identity for test strains

To ensure the purity of the test organisms utilised in this study, several identification test measures were performed. All microbial test strains exhibited characteristic morphological, physiological and biochemical properties associated with each microorganism which are summarised in **Table 4.4** and **Figure 4.10**. It was essential to ensure the purity of each selected organism not only to confirm the identity but also to eliminate contamination, which may render false results.

Table 4.4 Confirmation results of microbial test strain identity.

Microorganism	Morphological Characteristics	Biochemical Characteristics
Escherichia coli **(ATCC 25922)**	Gram negative; Rods; Motile; Circular, whitish colonies	Growth on MacConkey agar; **IMViC:** Indole positive, MR positive, VP negative, Citrate negative; Oxidase negative, Catalase positive; API 20E profile (99.5% ID)
Aeromonas salmonicida **(NCIMB 1102)**	Gram negative; Rods; Non-motile; Circular, whitish colonies	Growth on Furunculosis agar; **IMViC:** Indole negative, MR positive, VP negative, Citrate negative; Oxidase positive, Catalase positive; API 20NE profile (89% ID)
Flavobacterium psychrophilum **(NCIMB 1947)**	Gram negative; Rods; Motile; Circular, pale yellow colonies	Growth on Cytophaga agar; Oxidase positive, Catalase positive; API ZYM profile (72% ID)

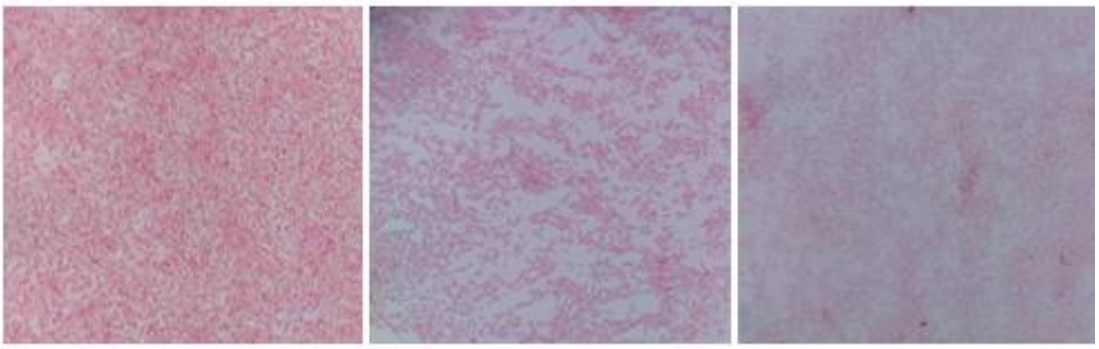

Figure 4.10 (A) *E. coli;* **(B)** *A. salmonicida;* **(C)** *F. psychrophilum,* (1000x magnification).

4.11 Standard curve preparation of test species

In order to adjust the cell density of each bacterial population before subjecting them to UV light disinfection, standard curves were prepared for the microorganisms. Initially, the aim was to use optical density readings at 600 nm and 610 nm to determine the decrease in bacterial load following treatment with PUV light.

However, a bacterial density of $1.0x10^6$ CFU/ml did not fall in between the normal spectrophotometer reading parameters of 0.1 to 1.0, as a much lower reading was observed. Therefore, this method of enumeration could not be used for the test microorganisms. The purpose of the standard curve was then utilised to make correct dilution calculations based on the bacterial density reached following the specific growth times for each species. For *E. coli,* the density was approximately $1.0x10^9$ CFU/ml following inoculation in nutrient broth for 15 hours at 37°C. A cell density of approximately $1.0x10^8$ CFU/ml following cultivation of *A. salmonicida* based on the appropriate culture conditions at 20°C was achieved. In order to adjust the cell density of both cultures to $1.0x10^6$ CFU/ml, a dilution calculation was performed using the C_1V_1 formula (**Section 4.5**). Thus, the initial cell density for both test microorganisms was $1.0x10^6$ CFU/ml pre-inactivation studies. It was important to know the starting density for comparison of results and to rule out the starting cell density as a contributing factor if inconsistencies occurred between replicate tests.

Standard curves prepared for *E. coli* and *A. salmonicida* illustrating the optical densities (OD) plotted against the $\log_{10}$ CFU/ml are presented in **Figures 4.11** and **4.12,** respectively. The OD readings at 600 nm for *E. coli* and 610 nm for *A. salmonicida* were employed. The correlation coefficient (R^2) values were 0.9381 and 0.9154 for the *E. coli* and *A. salmonicida* standard curves, respectively. This explains how well the data generated fitted the standard curve established. The closer the R^2 value to 1 the greater the predictability of the graph to indicate the $\log_{10}$ CFU/ml at a certain optical density. When working with biological specimens it is very difficult to obtain a perfect linear regression curve due to the natural biological variation that exists. **Figures 4.11** and **4.12** indicate that the R^2 values were close to 1 which implies that the adjustments made to the cell density of the cultures to establish a cell

density of 1.0x10^6 CFU/ml were accurate enough in terms of bacteria based on the graphs generated. It was not possible to establish a standard curve for *F. psychrophilum* as the growth rate fluctuated each time the assay was performed, therefore the control plate was counted following the experimental process to establish the initial cell density.

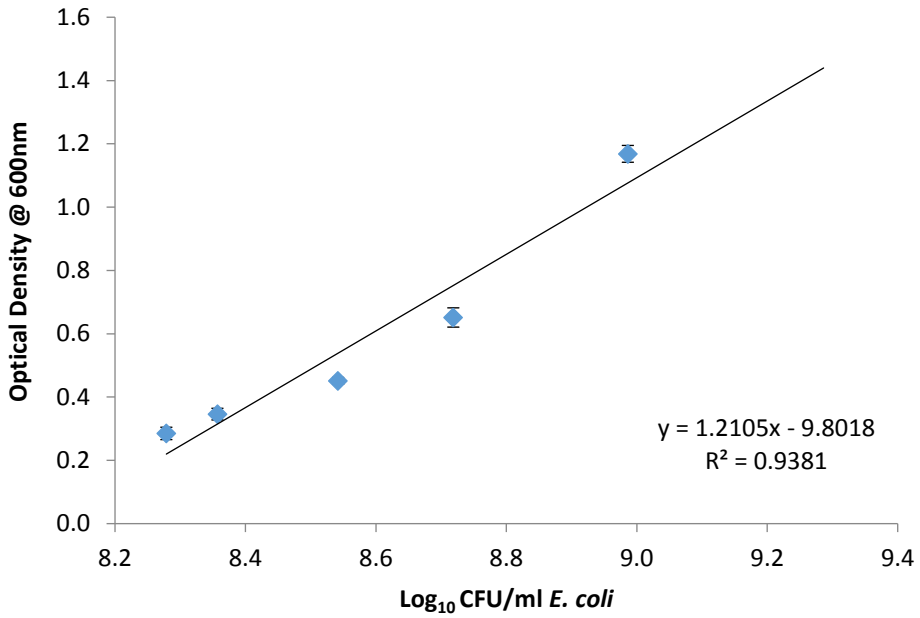

Figure 4.11 Standard curve for *E. coli* at an optical density of 600 nm (N=4).

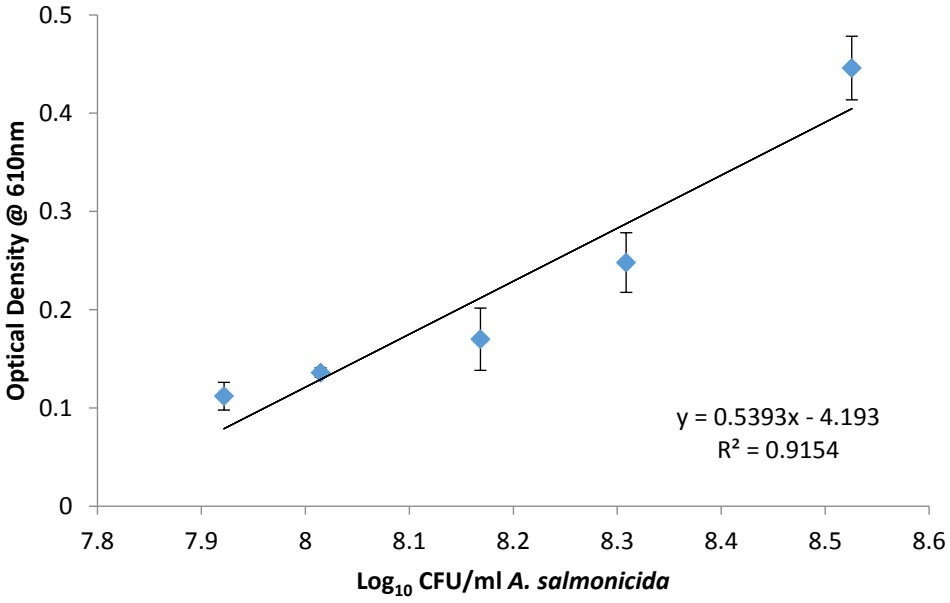

Figure 4.12 Standard curve for *A. salmonicida* at an optical density of 610 nm (N=3).

4.12 PUV inactivation of E. coli

The initial stage of this work package involved validation procedures in order to become familiar with culturing the specific test species and to become confident in operating the PUV system. The validation process involved the use of the bacterial species *E. coli* as the model organism. **Figures 4.13** and **4.14** illustrate kill curves for *E. coli* following treatment with PUV light, which display the decrease in the cell density expressed as $\log_{10}$ CFU/ml plotted against the increasing number of pulses employed at a rate of 1 pps. The bacteria were subjected to PUV light treatment at voltages of 300 V and 400 V at a distance of 8 cm from the light source, with the number of pulses ranging from 0 to 220 pulses. The initial cell density for PUV treatment at both 300 V and 400 V was 1.0×10^6 CFU/ml. Results are expressed as the mean of replicate plate counts following treatment (N=4).

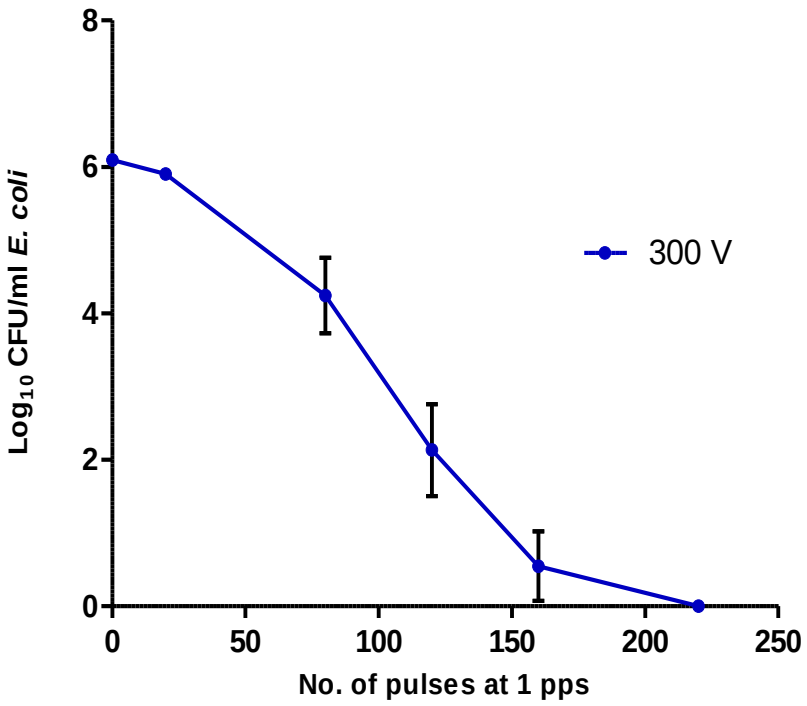

Figure 4.13 Kill curve showing inactivation of *E. coli* which was innoculated into 10 ml of QSR solution using PUV light at a voltage of 300 V at a distance of 8 cm from the light source. Results are a mean of 4 replicates ± SEM at a rate of 1 pps.

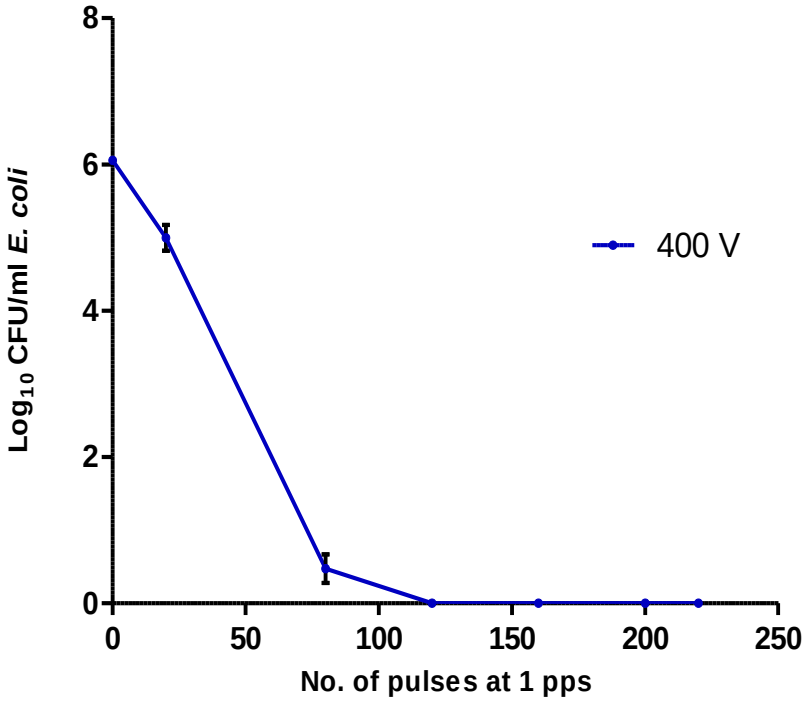

Figure 4.14 Kill curve showing inactivation of *E. coli* which was inoculated into 10 ml of QSR solution using PUV light at a voltage of 400 V at a distance of 8 cm from the light source. Results are a mean of 4 replicates ± SEM at a rate of 1 pps.

The results demonstrated a greater level of inactivation upon exposure to the higher voltage level. At 300 V 1.8 J of energy is emitted per pulse which is much lower compared to the 3.2 J of energy released per pulse at 400 V (Samtech Ltd, 2015). However, this study suggests that even operating at 300 V resulted in effective inactivation of *E. coli* denoted by the kill curve in **Figure 4.13**, although a greater number of pulses were required to achieve this inactivation compared to treatment with 400 V. To reach non-detectable numbers of *E. coli* following PUV treatment at 300 V, 220 pulses were required, whereas 120 pulses was the limit required to achieve the same result at the higher voltage of 400 V. This result led to the employment of 400 V rate as the standard setting on the PUV system throughout this study as it proved to provide a high rate of inactivation at a low energy level in terms of voltage. The results established demonstrated the bacterial inactivation efficiency of this light technology even at this lower end of the voltage range.

Upon initial comparative investigation of the results from the present study with results established by Garvey *et al.* (2009), a greater number of pulses were required

to inactivate *E. coli* to a non-detectable number in the present project. Studies carried out by Garvey (2009) resulted in almost complete inactivation of *E. coli* to a non-detectable number after 80 pulses of PUV light at 400 V and a rate of 1 pps. Whereas in this present study, 120 pulses, also at 400 V and a rate of 1 pps, were required in order to cause inactivation of *E. coli* to a non-detectable number. However, the methodology applied to both inactivation studies differed regarding the preparation of the bacterial species and the volume of suspension treated. Garvey (2009) carried out these studies using the same PUV system used in the present study. Over time, the xenon bulb in the flash lamp chamber can decrease in disinfection ability as the xenon filled flash lamp has a life of between 400 to 5000 hours depending on the repetition rate (Hamamatsu Photonics K. K., 2014). Replacement of this bulb is essential to ensure optimum efficiency of the PUV technology at all times. Future studies are recommended to incorporate bulb replacement schedules based on dosimetry studies carried out to determine whether this is a contributing factor to efficiency.

4.13 PUV inactivation of fish pathogens

Kill curves for the selected fish pathogens *A. salmonicida* and *F. psychrophilum* following treatment with PUV light are presented in **Figures 4.15** and **4.16**. These figures display the decrease in the cell density expressed as $\log_{10}$ CFU/ml plotted against the increasing number of pulses employed at a rate of 1 pps. Results are expressed in terms of triplicate plate counts per treatment. As 400 V at 1 pps gave encouraging results for *E. coli,* the same voltage parameters were used for *A. salmonicida* and *F. psychrophilum.* This was to determine whether such a low voltage would be effective at inactivating both fish pathogens. The initial starting density was 1.0×10^6 CFU/ml and 7.6×10^7 CFU/ml for *A. salmonicida* and *F. psychrophilum,* respectively (as explained in **Section 4.10**). As previously mentioned, upon a comparison of *E. coli* inactivation results in the present study with work carried out by Garvey (2009) a greater number of pulses were required to reduce the pathogenic load to a non-detectable number. Therefore, in the case of the fish pathogens a reduced number of pulses may have caused the same inactivation rates had the

xenon flash lamp been replaced. However, the objective of this study was to investigate the ability of PUV light to inactivate fish pathogens, the required rate for differing pathogens is an implication of this project and should be investigated in more depth in the future to enhance the efficacy of the PUV system.

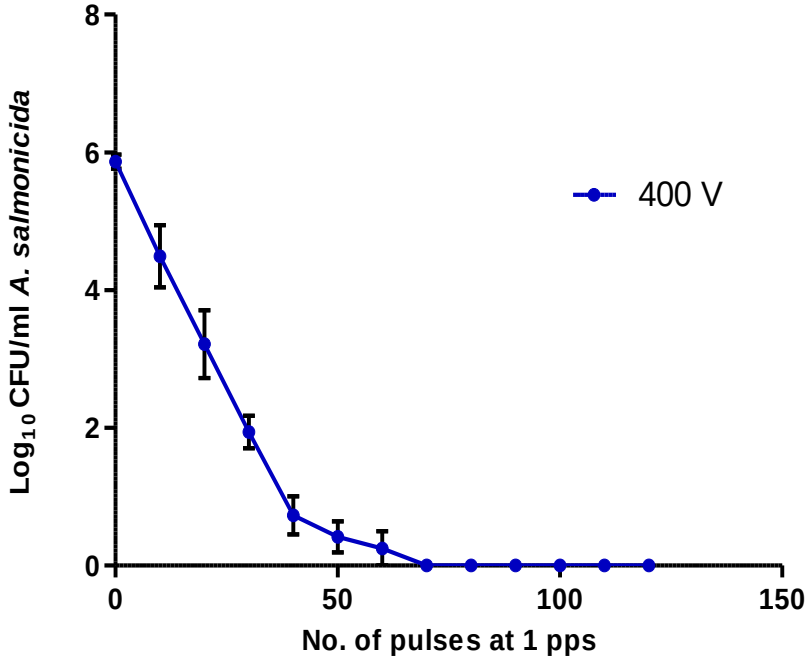

Figure 4.15 Kill curve showing the inactivation of *A. salmonicida* post exposure to PUV light at 400 V and 8 cm from light source. Results are a mean of 4 replicates ± SEM at a rate of 1 pps.

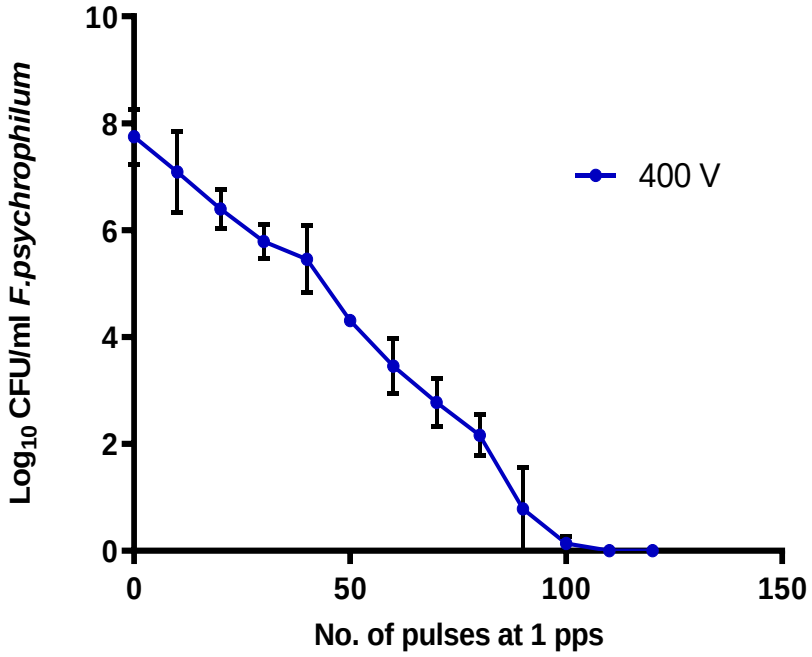

Figure 4.16 Kill curve showing the inactivation of *F. psychrophilum* post exposure to PUV light at 400 V and 8 cm from light source. Results are a mean of 4 replicates ± SEM at a rate of 1 pps.

Following range finding procedures, **Figure 4.15** and **Figure 4.16** illustrate that inactivation of *A. salmonicida* was achieved at a lower number of pulses compared to the inactivation of *F. psychrophilum*. The number of pulses applied ranged from 0 to 120 for both pathogens. In order to achieve inactivation of *A. salmonicida* 70 pulses of PUV light treatment were required at 400 V. This result is much lower than the number of pulses necessary to decrease the pathogenic load of *E. coli* i.e. 120 pulses, implying that PUV treatment is more effective at reducing the cell density of *A. salmonicida* to a non-detectable number. The inactivation to non-detectable numbers of *F. psychrophilum* required the same amount of pulses needed for inactivation of *E. coli.* This may indicate that both *E. coli* and *F. psychrophilum* are more resilient to the effects of PUV light than *A. salmonicida.* However, as the initial cell density for *F. psychrophilum* was 2 $\log_{10}$ doses higher than that for *A. salmonicida,* this may also have had an effect on the efficiency with which *F. psychrophilum* was inactivated. A standardised initial CFU/ml concentration for both pathogens would give a better insight into resilience comparisons. To the best of the researcher's knowledge, no comparative PUV studies are available for *A. salmonicida* and *F. psychrophilum,* as this is the first study in my opinion that determines the effect of PUV light on these fish pathogens.

A one-way ANOVA test was performed to investigate the significance of the mean inactivation rate achieved between all the data points, at the 95% level of confidence. For all three bacteria, the overall p-value was < 0.0001, indicating that a significant difference existed between each mean $\log_{10}$ CFU/ml value at each pulse setting from 0 to 120 pulses. The Dunnett's test indicated significant differences between the control and all treatments for both *E. coli* and *A. salmonicida*. Even after the first treatment of 10 pulses of PUV light exposure, there was a significant reduction in $\log_{10}$ CFU/ml. For example, after exposure to 10 pulses of light, the $\log_{10}$ CFU/ml for *A. salmonicida* decreased from 6.0 to 4.5 which equates to a cell density reduction from 1.0×10^6 to 3.1×10^4 CFU/ml. For *F. psychrophilum*, however, a significant difference between the control and treatment only occurred following exposure to 40 pulses or more of PUV light. However, the fact that the initial cell density of *F. psychrophilum* was 2 $\log_{10}$ doses higher than *E. coli* and *A. salmonicida* pre PUV

exposure, may explain why a higher number of pulses were required to reach the inactivation level, and it may not necessarily mean that the inactivation rate was lower. This concept is further explained in the comparison of death values i.e. inactivation rates of both microorganisms in **Section 4.13**. Regardless of the significant rates of inactivation, overall, PUV light was successful at eliminating each of the three bacterial species to a non-detectable number.

4.14 CW UV light inactivation of test microorganisms

As conventional UV is a common method of disinfection currently used in fish farms, both *E. coli* and *A. salmonicida* were subjected to inactivation *via* a CW UV light source at 254 nm. As for the PUV inactivation studies, validation and range finding were necessary initial steps for CW UV studies. In contrast to PUV light, the effect of exposure to CW UV light is based on time, which was measured in seconds in this study. The pathogens were subjected to CW UV light treatment at a distance of 8 cm from the light source, with the exposure time ranging from 0 to 35 sec of UV treatment. The initial cell density for both bacterial cultures was adjusted to $1.0x10^6$ CFU/ml using QSR as the diluent.

Kill curves for both *E. coli* and *A. salmonicida* following treatment with continuous UV light are presented in **Figures 4.17** and **4.18**, which display the decrease in the cell density expressed as $\log_{10}$CFU/ml plotted against the increasing exposure time (sec). Results are expressed in terms of triplicate plate counts per treatment. **Figures 4.17** and **4.18** demonstrate that in both cases of CW UV inactivation, 35 secs of light exposure was required in order to achieve a non-detectable number of both microorganisms. This indicates a closer relationship between the mechanism of inactivation for *E. coli* and *A. salmonicida* following CW UV exposure compared to inactivation that occured post PUV treatment. Once again, a one-way ANOVA was carried out to investigate significance in the mean rates of inactivation over time at the 95% level of confidence. A Dunnett's test for post analysis was then carried out to determine the difference in inactivation rates between the control (0 sec) and all treatments (5 – 35 sec). In both cases a p-value of < 0.0001 was achieved, with

significance in inactivation levels between control and treatments occurring after 10 secs for *E. coli* and after 5 secs for *A. salmonicida*.

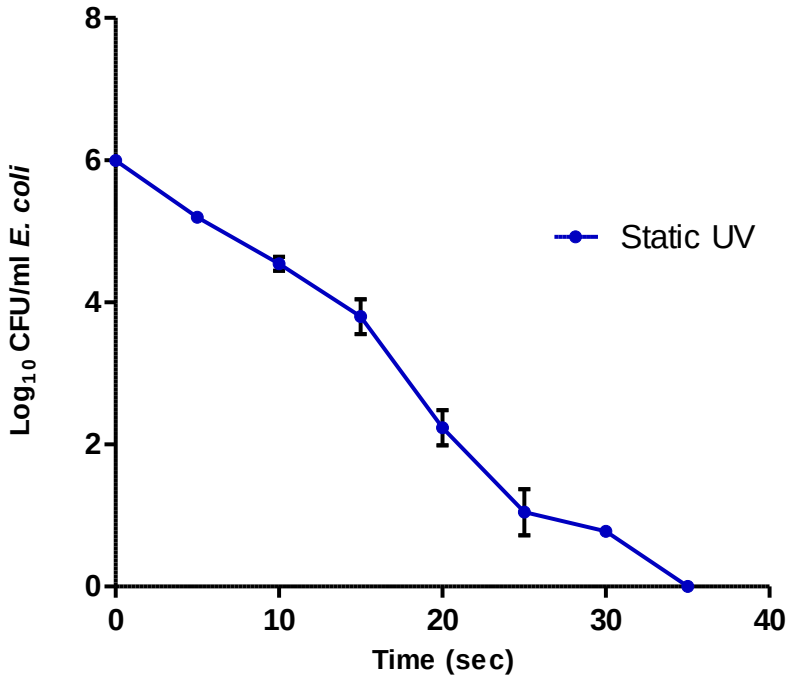

Figure 4.17 Kill curve showing inactivation of *E. coli* using CW UV light at a distance of 8 cm from the light source. Results are a mean of 4 replicates ± SEM.

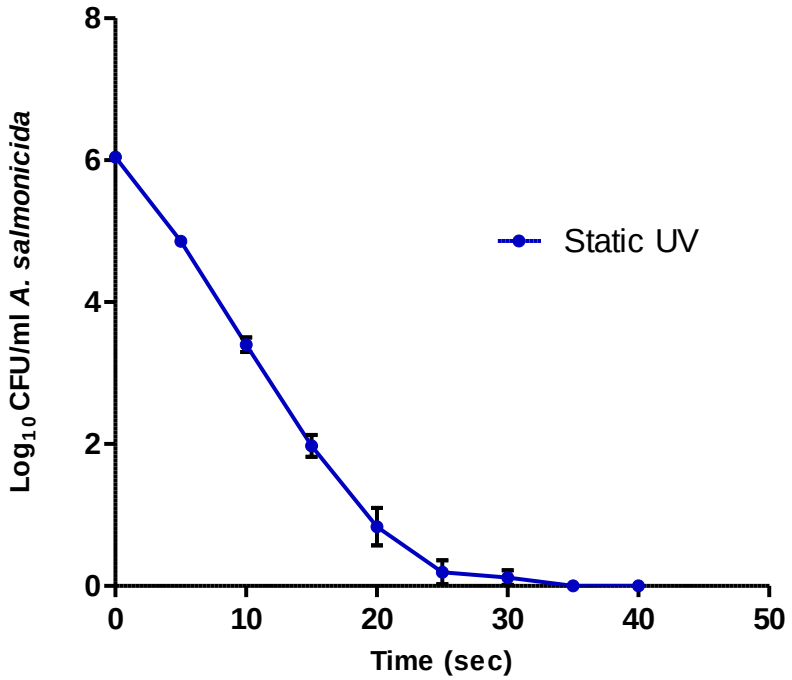

Figure 4.18 Kill curve showing inactivation of *A. salmonicida* using CW UV light at a distance of 8 cm from the light source. Results are a mean of 4 replicates ± SEM.

In terms of comparability, D-values were calculated to determine the rate of inactivation of each test microorganism by PUV or CW UV treatment. A D-value can be defined as the inactivation rate of $\log_{10}$CFU/ml of the selected pathogen per pulse (PUV) or per sec (CW UV). These results reflect the number of pulses or the amount of time required that can reduce the bacterial load by one $\log_{10}$ order. The smaller the D-value, the lower the number of pulses or the amount of time required for disinfection, indicating a greater inactivation efficacy, as mentioned in **Section 4.9**. The individual curves displaying the slope of the line and the R^2 value are illustrated in **Appendix A**. All the representative D-values for the two disinfection technologies and the three bacterial test species are outlined in **Table 4.5**.

The D-values determined for *E. coli* and *A. salmonicida* were 4.80 and 4.27 respectively in terms of CW UV inactivation. This reflects a similar disinfection efficacy of the CW UV system for both microorganisms. This was also evident in terms of required inactivation time of 35 secs for both *E. coli* and *A. salmonicida* illustrated in the kill curves in **Figures 4.17** and **4.18** respectively.

Table 4.5 Death values for inactivation studies.

Test Microorganism	Average Death Value	
	CW UV	PUV @ 400 V
E. coli	4.80	17.40
A. salmonicida	4.27	9.66
F. psychrophilum	-	13.83

For PUV, the results indicate that under the experimental conditions of this study *A. salmonicida* was more susceptible to the effects of PUV light with a D-value of 9.66, which was lower than the D-values of 17.40 and 13.83 established for *E. coli* and *F. psychrophilum*, respectively. As already denoted, the starting concentration for *F. psychrophilum* was higher than that utilised for the other test organisms. **Figure 4.16** illustrates that more pulses were required to reduce the CFU/ml of *F. psychrophilum* when compared to *A. salmonicida* illustrated in **Figure 4.15**. This would suggest that the PUV is much more efficient at inactivating *A. salmonicida* compared to *F. psychrophilum*. However, based on the D-values of 17.40 and 13.83 for *F. psychrophilum and A. salmoncida* respectively, which are close in value, should the

initial cell densities have been equal, a lower number of pulses may have inactivated *F. psychrophilum* to a non-detectable level, rather than the 120 pulses required in the experiment carried out. These results indicate that it is extremely important to have more than one method of comparability for inactivation; otherwise, some inactivation rates may be skewed. The reasons for the different inactivation susceptibilities of each pathogen warrants further investigation. The results in **Table 4.5** indicate that CW UV light is more efficient at inactivating the pathogens compared to PUV light due to the lower D-value established for CW UV, however, there are many factors to consider when comparing the two technologies.

A major influencing factor is the fact that following disinfection with PUV compared to CW UV there is a decrease in photoreactivation ability of the pathogen. As explained in **Section 4.1**, the impact endured by samples exposed to the intense energy emitted by pulses of UV light is much more damaging compared to the effects of CW UV light. Pathogens require exposure to UVA light following treatment with PUV in order to reactivate (Maclean *et al.*, 2008). As explained by Oguma *et al*. (2002) the length of time the pathogens are exposed to this UVA light is essential for the opportunity of photoreactivation to occur, i.e. 1 to 3 hours. In the case of CW UV disinfection, this is a possibility. If the culture water in a fish farm is continuously exposed to the UV light for disinfection, the bacteria have an opportunity to undergo photo repair. However, with PUV light employed as a disinfection method, there may be a lesser possibility of this occurring due to the rapid pulses of intermittent light without a build-up of continuous UVA light, which is ultimately required for the photoreactivation process to occur in this instance.

There are many difficulties in measuring the overall effectiveness of PUV versus CW UV in terms of inactivation. Different methods are utilised to measure the dose of both systems, however, energy usage is also a major factor that needs to be included especially when considering the utilisation of PUV instead of CW UV in a particular industry. If one compares CW UV to PUV in terms of time, CW UV transpires to be the more effective treatment. However, this cannot be concluded without incorporating the dose emitted onto the pathogens in mJ/cm^2 from each technology, as energy efficiency is a major factor in effectiveness. This energy usage of both

technologies and the emitting dose of each lamp needs to be clearly identified in order to make a reliable comparison between both systems in terms of their efficiency for use in an aquaculture setting. A common method for measuring the dose of PUV light emitted is chemical actinometry (Gómez-López and Bolton, 2016), where the photon flow entering a solution can be determined *via* the use of a chemical solution (Bolton *et al*., 2011). While CW UV light proved more effective at disinfection in this study, a study carried out by McDonald *et al.* in 2000 observed that PUV light was much more effective at eliminating *B. subtilis* than CW UV light. The inactivation level achieved *via* PUV at a fluence of 4 mJ/cm^2, required 8 mJ/cm^2 of light from the CW UV disinfection method. The increased efficiency of PUV over conventional UV was observed for both aqueous and surface disinfection of *B. subtilis* (McDonald *et al.*, 2000). This iterates how important it is to compare every aspect of the disinfection method in order to be able to conclusively advise on the most suitable UV method for each problematic pathogen.

Not only is it important to know the UV dose for comparative purposes but it is also important to have an understanding of the different doses required to target various pathogens present on an aquaculture site. Upon partial determination of the bacterial and phytoplankton profiles at KWF, there was no indication of problematic levels of pathogenic microbial species (*F. psychrophilium*) or toxicogenic phytoplankton species, such as *Microcystis* sp., present in the rearing water. Although this study proved the concept of using PUV light to eliminate *E. coli*, *A. salmonicida* and *F. psychrophilium*, full implementation of PUV disinfection in a fish farm may disturb the natural ecosystem, possibly leading to less efficient nutrient uptake or oxygen production by natural means. Therefore, in order to keep pathogenic bacteria and toxic phytoplankton species at bay, the dose of efficient pathogenic elimination that has minimal effects on the ecosystem balance must be determined. Another factor to consider is the use of PUV in conjunction with chemicals for a combined disinfection approach, and whether exposure of certain chemicals to PUV light may result in the formation of more toxic chemical compounds. An investigation into possible effects with selected chemical disinfectants has been conducted and is presented in the next chapter.

Chapter 5
Ecotoxicological Assessment of Disinfection Measures

Introduction

Biological assays are a critical means of determining the effects of substances on living organisms (Asker, 2011). Ecotoxicological bioassays investigate the effect of toxicants at the levels of the organism, population and community (Asker, 2011). The biological integrity of the organism is then compared to that of organisms who have not been exposed i.e. a control, and the toxic effects are evaluated (Tothill and Turner, 1996). Such ecotoxicological assays or bioassays can be utilised to investigate a specific endpoint or multiple endpoints, such as growth inhibition, mortality, behavioural abnormalities, physiological changes, etc. (Asker, 2011). Contaminants from many sources, such as WWTPs and agriculture, can end up in surface waters used in aquaculture and so the execution of such bioassays aids in the assessment of such contamination to aquatic life as well as human health (Tothill and Turner, 1996).

5.1 Ecotoxicological assessment of chemical disinfectants used in the aquaculture industry

Many chemicals are used in aquaculture for disinfection and prevention measures (Asker, 2011), as outlined in **Chapter 2, Section 2.8** of this document. Should these chemicals become remobilised or bioavailable through biological, chemical or physical activities, they can pose a serious threat to the indigenous aquatic biota (Davoren *et al.*, 2005). It is, therefore, critical to implement a water monitoring system to identify the toxicological hazard posed to fish stock, and lower trophic levels, which is a major objective of this project.

When assessing the effect of chemicals on the surrounding or receiving environment, it is not sufficient to assess the toxic effect on aquatic biota *via* chemical or physical methods alone (Tothill and Turner, 1996). This type of analysis does not reflect the interactive effects of other chemicals that may be used, nor does it demonstrate the potential bioaccumulation or bioavailability effects of toxicants on different organisms. Bioassays are more desirable, as living organisms respond to any harmful chemicals that induce a biological response (Díaz-Garduño *et al*., 2016). The effects

of exposure to poor environmental conditions are integrated into the growth of the organism which can be assessed *via* the use of bioassays.

Ecotoxicological assays can also determine the bioavailability of a substance for each test organism. However, use of a single organism may not detect the mode of action or different effects of contaminants, therefore, it is important to implement a battery of tests and test organisms so as to represent multiple trophic levels (Davoren *et al.*, 2005). This multi-trophic approach is illustrated in **Figure 5.1**, which shows where each of the test organisms are on the trophic system namely phytoplankton, water flea and brine shrimp, all of which were utilised in this study.

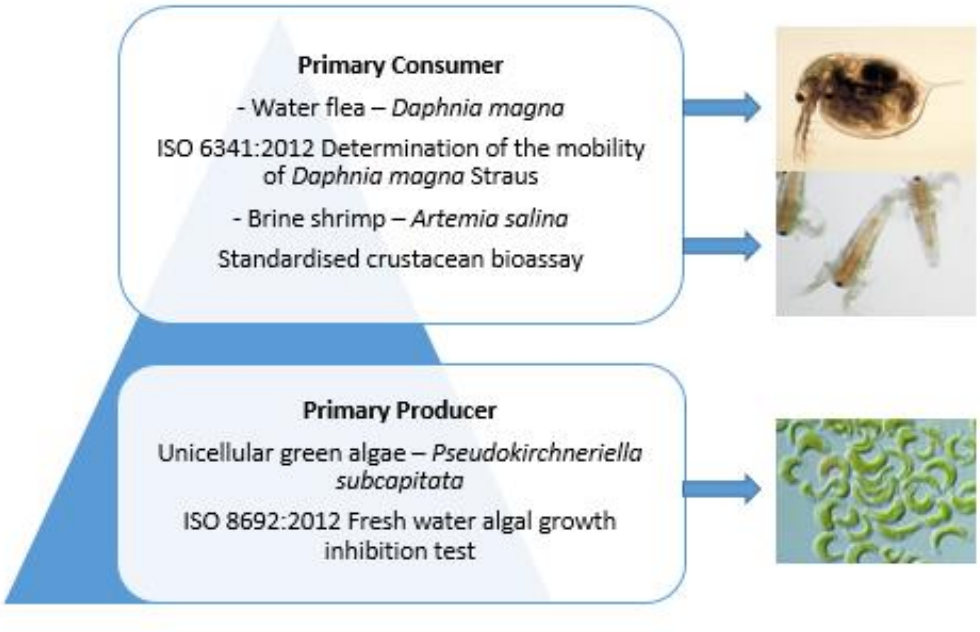

Figure 5.1 The multi-trophic test battery approach for ecotoxicological bioassays.

While the primary potential application of PUV light in aquaculture in the future is disinfection, chemicals are still widely relied on in this industry, and so there is the possibility of both disinfection methods to be used in conjunction with each other. It is, therefore, important to investigate the relationship that exists between the effects of PUV light on the chemical structure in terms of the formation of dangerous by-products, such as trihalomethanes (THM's). THM's are formed when chlorine-based chemicals are broken down and react with organic matter (Latifoglu, 2003). A series of ecotoxicological studies, illustrated in **Figure 5.1**, were carried out in order to

determine the effect of PUV light on the commonly used aquaculture disinfectants bronopol and chloramine-T.

5.1.1 Algae

Algae are commonly used for ecotoxicological testing due to their basis as a primary producer in the aquatic ecosystem, their sensitivity to a range of toxicants and low cost of culturing (Helfrich and Libey, 1990; Asker, 2011; Perosa *et al.*, 2015). Algae are also an important resource in aquaculture where they can be used for water treatment in terms of oxygenation, recycle nutrients in rearing water and act as a sustainable food source (Gao and McKinley, 1994; Muller-Feuga, 2000; Perosa *et al.*, 2015). Therefore, it was essential to utilise algae as the primary producer for the ecotoxicological testing carried out in this project, especially as an objective of this project was investigating phytoplankton communities in rearing water. The purpose of algal testing using *Pseudokirchneriella subcapitata* in this project (**Figure 5.2**) was to determine the effects of a substance on the growth of exponentially growing test organisms over a period of 72 hours.

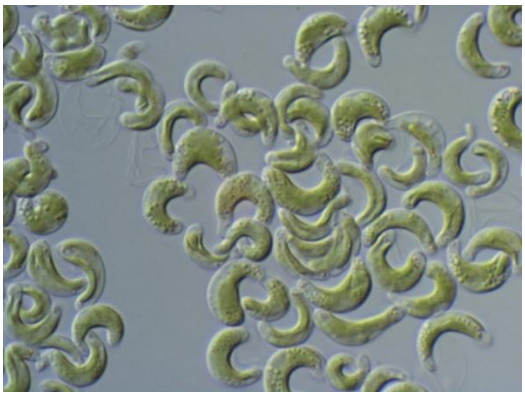

Figure 5.2 *Pseudokirchneriella subcapitata* structure (CCAP, 2014).

The test endpoint is growth inhibition expressed as the E_rC_{50}, i.e. the effective concentration that results in a 50% reduction in culture growth compared to the control, a 10% reduction and 90% reduction are also values assessed expressed as an E_rC_{10} and E_rC_{90} respectively. The lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) are also explored (OECD, 2011).

5.1.2 Brine shrimp

Artemia salina is a form of brine shrimp that is commonly incorporated into ecotoxicity battery assessments as a biological herbivorous invertebrate species representative *via* a standardised crustacean bioassay (Davoren *et al.*, 2005). *A. salina* can be used to screen for potential neurotoxic and hepatotoxic effects of cyanobacterial blooms (Tothill and Turner, 1996). *A. salina* is used for toxicity testing of marine water (Tothill and Turner, 1996), however, this organism is used as a feed source for larvae at KWF and so it was deemed necessary to utilise said organism for bioassay testing. Other reasons why *A. salina* are being more commonly utilised in toxicity testing include the simple culturing process of *A. salina* where hatching of cysts occurs easily under favourable conditions; the simple equipment that can be used for hatching and testing, and only a small volume of the organisms are required for experimentation (Svensson *et al*., 2005).

Figure 5.3 *Artemia salina* organisms.

There are approximately 15 different molting stages of *A. salina,* where they grow and develop into different larval or nauplii stages (Stappen, 1996). Instar I, II and III are the most important for this study. Instar I larvae tend to be fed to young fish larvae in aquacultural practices (Merchie, 1996), whereas instar II and III are the levels of *Artemia* required for the ecotoxicological testing (Vanhaecke and Persoone, 1984).

5.1.3 *Daphnia magna*

Daphnia magna, which is a freshwater crustacean (**Figure 5.4**), commonly known as the water flea, is one of the main organisms used for freshwater invertebrate

ecotoxicological testing due to its many advantages associated with their use. These include a short reproductive cycle, high sensitivity to toxicants and parthenogenic reproduction i.e. development of an offspring without fertilisation (Tothill and Turner, 1996; Olmstead and LeBlanc, 2000; Brennan *et al.*, 2006; Colbourne *et al.*, 2011; ISO, 2012). *D. magna* is also a major component of the aquatic ecosystem as a primary consumer (ISO, 2012) and is ecologically relevant to the freshwater ecosystem.

They are relatively easy to culture, attain large brood sizes and have a short lifespan which adds to their extensive use in toxicity testing and water quality monitoring (Brennan *et al.*, 2006; Colbourne *et al.*, 2011). Both chronic and acute tests are commonly used to assess the effects of a pollutant on *D. magna* (Tothill and Turner, 1996).

Figure 5.4 Female *Daphnia magna* (Clare, 2002).

There are many endpoints that can be investigated with daphnids such as immobilisation and moulting, which are acute endpoints, and survival and reproduction, which are chronic endpoints (Brennan *et al.*, 2006). Results achieved give an indication of the toxicological hazard that may occur in the natural freshwater environment. In the project, acute lethality tests using *D. magna* were conducted by exposing the organisms to the disinfectant chemical of interest for 24 and 48 hours (Tothill and Turner, 1996; ISO, 2012). Immobilisation was the toxicological end-point, therefore immobile organisms were counted following incubation and compared to the control to assess the effect (Gallina *et al.,* 2008; Tothill & Turner, 1996).

Methodologies

A range of ecotoxicological tests were carried out in order to determine whether or not selected chemical disinfectants commonly used in aquaculture would produce dangerous or more toxic by-products once exposed to PUV light. Each bioassay was carried out initially as per standard methods. The selected chemicals were then exposed to PUV light at 900 V for 100 pulses at a rate of 1 pps. Following treatment, the bioassay was carried out again as per standard methods to determine if there was a difference caused to the chemical toxicity by PUV exposure. The chemicals selected for investigation in the bioassays were bronopol (Pyceze™) and chloramine-T (Halamid®). These were chosen based on an extensive review of the literature (**Chapter 2, Section 2.8**) revealed these chemicals as two of the most commonly used in Irish aquaculture and they are also associated with the disinfection of *A. salmonicida* and *F. psychrophilum*. The collaborating fish farms in this study were also surveyed based on the chemical disinfectants most commonly used on their sites in the case of a disease occurrence and concurred with the choice of the test chemicals.

5.2 Algal growth inhibition assay

The algae bioassay was based on a modified version of the European Standard 'Water quality – Fresh water algal growth inhibition test with unicellular green algae (ISO 8692:2012)', to determine the effect of certain water-soluble chemicals on the growth rate of green algae. Two test organisms permitted for use in this test are *Desmodesmus subspicatus* or *P. subcapitata* (**Figure 5.2**), the latter species was used for this study. The algae cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP) in the form of liquid algae in tubes, 5 ml of which was inoculated into 50 ml of fresh culturing media immediately. The culturing media used was Jaworski's Media (JM), which was prepared as per CCAP's standard recipe for Jaworski's Medium for Freshwater Algae and was equilibrated by bubbling filtered air through for 30 minutes. The culture was covered with cotton wool to provide air permeability and incubated at 23°C in a shaking incubator containing white fluorescent lights approximately 35 cm away from the culture vessels, with an

intensity of between 60 and 120 µmol m^{-2} s^{-1}. Sub-culturing was carried out on a biweekly basis to ensure optimal growth conditions in the exponential phase when the bioassay was performed. All culture vessels, cotton wool, pipette tips and volumetric flasks were autoclaved at 121°C for 15 minutes before use for sterilisation to ensure optimal asepsis prior to assay commencement. The JM was also autoclaved prior to the addition of sodium bicarbonate ($NaHCO_3$) in order to avoid thermal destruction of this substance.

Cell number was carried out in triplicate for each test concentration and the control. The concentrations utilised for each chemical including the reference chemical, potassium dichromate ($K_2Cr_2O_7$) are outlined in **Table 5.1**.

Table 5.1 Concentrations utilised in the *P. subcapitita* growth inhibition assay for $K_2Cr_2O_7$, bronopol and chloramine-T.

Test Chemical Concentration (ppm)		
$K_2Cr_2O_7$	**Bronopol**	**Chloramine-T**
0	0	0
0.10	0.50	0.1
0.50	1.00	0.25
1.00	2.50	0.5
2.50	5.00	1.0
5.00	7.50	2.50
10.00	10.00	5.0
-	-	7.5
-	-	10.0

The initial cell density for the assay was 1 x 10^4 cells/ml as stated in the ISO guidelines. To achieve this density an algal stock density of 2 x 10^5 cells/ml was added to 19 ml of the test chemical or JM in the case of the control, resulting in a total volume of 20 ml per culture flask. All of the flasks were plugged with cotton wool to avoid contamination but to allow sufficient CO_2 to enter the vessels. The flasks were incubated as per initial culture at 25°C in a shaking incubator with fluorescent white lights for a period of 72 and 96 hours. Following the incubation time, a cell count determining the number of algal cells per ml of media/test chemical was counted in

triplicate with a haemocytometer, using a Neubauer Improved (Bright-Line) chamber as per Davoren *et al*. (2005). Once the cell density was determined the specific growth rate ($\boldsymbol{\mu}$) was established for each sample *via* the following equation:

$$\mu = \frac{\mathbf{l}_n \, \boldsymbol{n}_L - \boldsymbol{l}_n \, \boldsymbol{n}_0}{\boldsymbol{t}_L \; - \; \boldsymbol{t}_0}$$

where $\boldsymbol{n}_0$ = the initial cell density, $\boldsymbol{n}_L$ = the cell density measured at time $\boldsymbol{t}_L$, $\boldsymbol{t}_0$ = the time of test start and $\boldsymbol{t}_L$ = the time of test termination. For each treatment, the percentage inhibition ($\%\boldsymbol{I}_r$) was calculated using the equation:

$$\% \, \boldsymbol{I}_r = \frac{\mu_C \; - \; \mu_i}{\mu_c} \times \mathbf{100}$$

where $\boldsymbol{\mu}_c$ = the mean growth rate for the control algal cultures and $\boldsymbol{\mu}_i$ = the growth rate of the test culture.

A non-linear regression plot was then established as the percentage inhibition against the test concentration in ppm on a logarithmic scale, and the E_rC_{50}, the concentration which causes a 50% inhibition in growth rate, was calculated for each test substance. These results give an indication of the potential toxicological hazard that may occur in the natural environment. In order for the test to be considered as valid, there must be a 67 fold increase over the 72 hour test period in the control compared to the initial algal seeding density of 1 x 10^4 cells/ml of culture media (ISO, 2012).

It is also important to test the validity of the experimental setup *via* the use of a reference substance, the results of which were compared to the ISO established results for said reference chemical. An initial assay based on $K_2Cr_2O_7$, was carried out in order to prove the validity of the test system. The mean value for the E_rC_{50} outlined by the ISO standard at 72hrs for $K_2Cr_2O_7$ is 1.19 ppm (C.I. = 0.92 – 1.46 ppm) to which results carried out in this study have been compared.

5.3 Artemia salina crustacean bioassay

An adapted version of the *Artemia salina* crustacean bioassay was carried out as per the 'Standardised Short-Term Toxicity Test with *Artemia* Nauplii (ARC-Test)', established by Vanhaecke & Persoone of the Aquaculture and Artemia Reference Centre (1981). This bioassay involves the hatching of *Artemia* cysts in artificial seawater to obtain instar II-III nauplii within 24 – 48 hours, followed by treatment with the selected chemical to establish the toxic effect. The equipment used to hatch the *Artemia* cysts was the JBL Artemio Set – Breeding Set for *Artemia* nauplii sourced from Seahorse Aquariums, **Figure 5.5**. This is a funnel-shaped container with gentle aeration applied in the form of an air hose powered by an air pump, to maintain the nauplii in continuous suspension.

Figure 5.5 JBL Artemio Set apparatus.

The purpose of the funnel shape is to ensure no organisms are trapped in inaccessible areas of the apparatus, therefore, providing optimal hatching success; and allows for easy collection of the population *via* an outlet tap function at the end of the funnel (JBL, 2016). The cysts were hatched in the Artemio Set by adding 1 – 2 g of brine shrimp cysts to 500 ml of artificially prepared seawater. Following 24 – 48 hours of culturing the hatched *A. salina* organisms were removed *via* the outlet tap into a receiving sieve to allow any media to be removed (JBL, 2016) and then placed into a petri dish containing fresh media. This process was also carried out after hatching for

18 hours to obtain instar I nauplii. The reason for this was to ascertain the difference in sensitivity of the organisms hatched in a shorter time period as *Artemia* on fish farms are often fed to larvae after 18-24 hours of hatching (Merchie, 1996). Therefore, it is important to be aware of the different sensitivity levels based on the hatching period.

All ecotoxicological bioassays were prepared in 24-well multi-well plates with a control included each time. A series of dilutions of the test chemicals, namely, bronopol and chloramine-T, were initially prepared for range finding purposes before carrying out the definitive tests. Following range finding procedures the definitive tests were carried out as per the method described. The concentrations utilised for the test chemicals and the reference chemical are outlined in **Table 5.2**. Firstly, 1 ml of each test chemical concentration, was placed into the four wells vertically on the multi-well plate. A volume of *Artemia* larvae containing approximately 50 organisms was removed from the petri dish and placed into a rinsing well for each concentration. A dissecting microscope was then utilised in the transferring of 10 *A. salina* from the control well to the remaining wells to achieve triplicate counts for each concentration as outlined in **Figure 5.6**. This assay was carried out for each of the aforementioned chemicals.

Table 5.2 Concentrations utilised in the A. salina crustacean bioassay for $K_2Cr_2O_7$, bronopol and chloramine-T.

Test Chemical Concentration (ppm)		
$K_2Cr_2O_7$	**Bronopol**	**Chloramine-T**
0	0	0
10	10	250
18	25	500
32	50	750
56	75	1000
100	100	2500
-	125	-
-	150	-
-	200	-

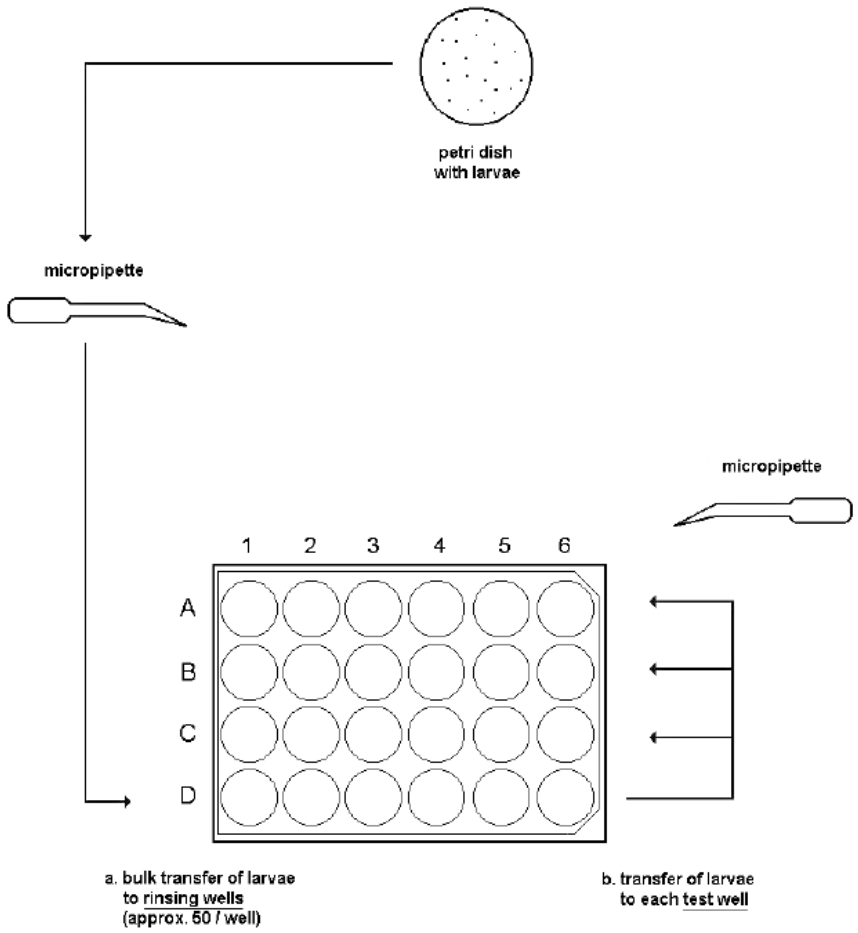

Figure 5.6 Procedure for the transfer of *Artemia salina* to the 24-well multi-well plate for testing (MicroBio Tests Inc, 2015).

When all the test wells contained the test chemical and the correct number of organisms, the plate was covered with parafilm to avoid evaporation of any liquid and then the lid was placed on the plate. The plate was then wrapped in tinfoil before placing in the incubator at 25°C for 24 hours as described by Davoren *et al.* (2005) and for 48 hours. The larvae were not fed during the test. Following incubation, the number of dead and living organisms was recorded for each test well. The larvae were considered dead if no movement of the appendages was observed after 10 seconds. The results were then expressed as % mortality for each concentration *via* the following equation:

$$\%\ \boldsymbol{Mortality} = \frac{\boldsymbol{No.\ of\ dead\ larvae}}{\mathbf{30}} \times \mathbf{100}$$

A non-linear regression curve was plotted displaying the percentage mortality of the organisms plotted against each concentration. The LC_{50} for each chemical was established, which is the concentration that causes lethality to 50% of the organisms after a single exposure. A preliminary test using $K_2Cr_2O_7$ as the reference chemical was carried out to ensure the validity of the test method. The concentrations used are outlined in **Table 5.2**, with artificial seawater as the control. The reference test was considered valid if there was less than 10% mortality in the controls and if the LC_{50} value achieved was within the range of 30 and 50 ppm.

5.4 Inhibition of mobility test using D. magna

The *D. magna* bioassay was based on an adapted version of the European Standard 'Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea)* – Acute toxicity test (ISO 6341:2012). This test is used to determine the immobilisation of the water flea *D. magna* Straus following exposure to substances contained in water and wastewater, as well as toxicity following exposure to chemicals. In order to culture the test specimens and expand the brood stock, the daphnids were placed in 2 litre glass beakers, which were filled with 1.8 L of aerated spring water as the culture media. Filtered natural water is acceptable for culturing as long as the *D. magna* acclimatise and survive the culturing process without any signs of stress, otherwise, the M4 medium outlined in Annex A of the ISO is recommended. The ISO (6341:2012) recommends culturing water in the range of pH 6 to pH 9, and hardness as $CaCO_3$ between 140 to 275 ppm. Daily culture conditions required a dark photoperiod of 16 hours of light and 8 hours of darkness, at a temperature of 20°C (ISO, 2012), in order to ensure parthenogenic reproduction of daphnids as per conditions outlined by Olmstead & LeBlanc (2000). Media changes were performed three times weekly, where the test specimens were separated based on size into adults, juveniles and neonates *via* the use of the JBL Artemio 4 sieve combination set, using the 0.6 mm, 0.3 mm and 0.15 mm sieves, respectively. The organisms were then transferred to the respective glass beaker culture vessels containing 50% fresh aerated culture media and 50% old media.

All tests were carried out using less than 24 hours old neonates, which were separated as per methods previously described. The two chemicals selected as per all the ecotoxicology tests were bronopol and chloramine-T. Initial range finding studies were carried out for both test chemicals to determine the definitive concentration range. A stock solution of each test chemical was prepared and the relevant dilutions were prepared from this stock using the aerated spring water as the diluent. Five concentrations and a control (fresh culture water) were prepared for each chemical as outlined in **Table 5.3**. A reference test was also carried out using the $K_2Cr_2O_7$ to ensure the validity of the test.

Table 5.3 Concentrations utilised in the *D. magna* inhibition of mobility bioassay for $K_2Cr_2O_7$, bronopol and chloramine-T.

Test Chemical Concentration (ppm)		
$K_2Cr_2O_7$	**Bronopol**	**Chloramine-T**
0	0	0
0.10	0.10	0.10
0.50	0.50	1.00
1.00	1.00	2.50
2.50	2.00	5.00
5.00	3.00	10.00

For each concentration, four 50 ml glass beakers with 20 ml of the test chemical and 5 neonates per beaker were required, giving a total of 20 test organisms per concentration. During the course of the experiments, the test organisms remained unfed. Following incubation for both 24 and 48 hour periods, the mobility of test specimens was investigated, where the *D. magna* were considered immobile if they showed the inability to swim during 15 seconds post gentle agitation of the test vessel, even if the antennae still showed signs of movement. The validity criteria for the test was that mortalities were less than 10% in the control wells. The number of immobile organisms in each beaker was counted for each concentration and the percentage inhibition was determined. The EC_{50} i.e. the effective initial inhibitory concentration that results in immobilisation to 50% of the *D. magna* for a given chemical, was determined by plotting the percentage inhibition of mobility against the test chemical concentration.

Results & Discussion

Exposure of organisms in the rearing water, as well as receiving waters, to toxic substances can lead to disease, modification of biological processes e.g. reproduction, impairment of fish flesh and fish kills (Tothill and Turner, 1996). Many measures are implemented in the aquaculture industry to ensure an environment free from disease, such as chemical disinfection, UV light, oxygenation, biofilters etc. (Sharifi-Yazdi and Darghahi, 2006; Crab *et al.*, 2007; Agarwal *et al.*, 2011; Picón-Camacho *et al.*, 2012). It is important to assess whether the use of two simultaneous disinfectant systems will have a negative influence on the rearing water or the discharge water. It is important to understand the relationship that exists between PUV light and typical chemical disinfectants that are used on aquaculture farms, remnants of which may remain in the outgoing water body. Therefore, the effect of PUV on selected chemical disinfectants, namely bronopol and chloramine-T, was investigated.

A number of ecotoxicological bioassays were carried out in order to determine the effect of PUV light on bronopol and chloramine-T. Each test consisted of two parts: firstly, the assay was carried out using both chemicals as per the relevant ISO or standard methodology in all cases. Secondly, the chemical was subjected to PUV exposure and the assay was carried out again to determine the effect of PUV light on the selected chemical disinfectants. In each of the different ecotoxicological assays carried out, a stock concentration of each chemical was exposed to 100 pulses of PUV light at 900 V and at a rate of 1 pps. This assessment was carried out in order to ascertain the effects that PUV light have on the selected disinfectants if both are utilised in conjunction on a fish farm. Exposure to PUV light may cause the breakdown of the parent compound to more toxic by-products, which would increase the toxic effects on fish stock, on fish farm organisms, such as phytoplankton, or on the natural receiving ecosystem upon the discharge of the rearing water. With phytoplankton as the principal production base in the aquatic food chain (Geis *et al.*, 2000) an increase in chemical toxicity caused by PUV exposure would inevitably have negative effects on oxygen production, nutrient removal and

as a food source for higher trophic levels, disrupting natural aquatic populations, hence this ecotoxicological investigation.

5.5 Algal growth inhibition assay

For the 'Fresh water algal growth inhibition test with unicellular green algae (ISO 8692:2012)' results were calculated as the algal number at varying concentrations of the test substance as a function of time. *P. subcapitata* was utilised as the algal species in all tests. The reference test was carried out using $K_2Cr_2O_7$ as the standard chemical to ensure the validity of the test method. Bronopol and chloramine-T were selected as the test chemicals. Results are illustrated in **Figures 5.7** to **5.11**, and expressed in all cases, as the effective concentration that causes a 50% reduction in the growth rate for each time point, known as the E_rC_{50}. The test was carried out over a period of 72 and 96 hours in all cases.

5.5.1 *P. subcapitata* bioassay – Reference test

Figure 5.7 illustrates the growth inhibition curve following exposure to increasing concentrations of $K_2Cr_2O_7$ used as a reference chemical for 72 and 96 hours, respectively, with the final result of an E_rC_{50} being established. The E_rC_{50} established for 72 hours was 0.79 ppm and for 96 hours was 0.87 ppm. The result obtained for 72 hours is lower than the ISO standard value recommended for potassium dichromate of 1.19 ppm, with confidence interval values at 95% ranging from 0.92 ppm to 1.46 ppm. One of the reasons for the difference in the result may be due to the use of a static incubator rather than a shaking one, as those facilities were not available at the time the test was carried out. Another aspect of the methodology that may have contributed to the difference in the E_rC_{50} value for 72 hours is the composition of Jaworski's media. The recipe for Jaworski's media utilised in the present study was that recommended by CCAP, the source of the algal stocks, to ensure the optimum growth of the algae, which differs slightly from the recipe outlined in the ISO standard.

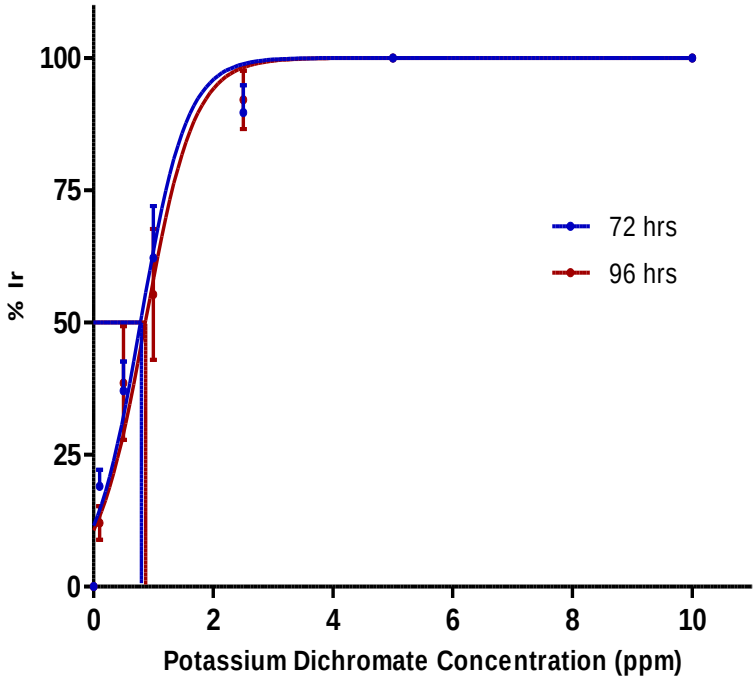

Figure 5.7 Dose response curve for *P. subcapitata* exposed to $K_2Cr_2O_7$ for 72 and 96 hrs. **72 hrs E_rC_{50} = <u>0.79 ppm</u>** [95% C.I = 0.66 – 0.91]; **96 hrs E_rC_{50} = <u>0.87 ppm</u>** [95% C.I = 0.69 – 1.05].

The E_rC_{50} result obtained in the present study was in close comparison to the same result standard for the ALGALTOXKIT F, 'Freshwater Toxicity Test with Microalgae' a kit for algal growth inhibition manufactured by MicroBio Tests Inc. The established E_rC_{50} result for the reference test using potassium dichromate is 0.84 ppm for the ALGALTOXKIT. The Kurume Laboratory at the Chemicals Evaluation and Research Institute, Japan, have a range of between 0.698 to 1.08 ppm as the standard range limit for the algal growth inhibition test using $K_2Cr_2O_7$ as the reference chemical *via* the same methodology outlined in this present study (CERI, 2007). The E_rC_{50} of 0.79 ppm for 72 hours in this current study is within the range established by Kurume Laboratory. The confidence intervals of 0.66 to 0.91 established in this present study are in good agreement with the range of values for the reference test according to the Kurume Laboratory.

In order to determine whether significant differences occurred between the means of all the data points for both the 72 hour and 96 hour tests, a one-way ANOVA was performed. In both cases a significant difference existed ($p < 0.0001$), implying that

the percentage growth inhibition rate of algae was significantly different for each concentration. The post-test analysis in the form of a Dunnett's multiple comparison test was carried out to determine significant differences that occurred between the control and any of treatment concentrations. For the 72 hour test, significant differences existed between the control and all the treatments from 0.1 to 10.0 ppm. The 96 hour test showed significant differences between the treatments from 0.5 to 10.0 ppm, however, there was not a significant difference between the control and the 0.1 ppm treatment.

5.5.2 *P. subcapitata* bioassay – Bronopol

The second analysis carried out regarding the algal growth inhibition assay was to determine the toxicity of the chemical disinfectant bronopol pre and post PUV treatment. This chemical was investigated as it is commonly used for disinfection, mainly of eggs, in aquaculture (Birkbeck *et al.*, 2006; Oono *et al.*, 2007; Noga, 2010). Following range finding procedures, the standard assay was carried out over a period of 72 and 96 hours, with results illustrated in **Figures 5.8** and **5.9**, respectively.

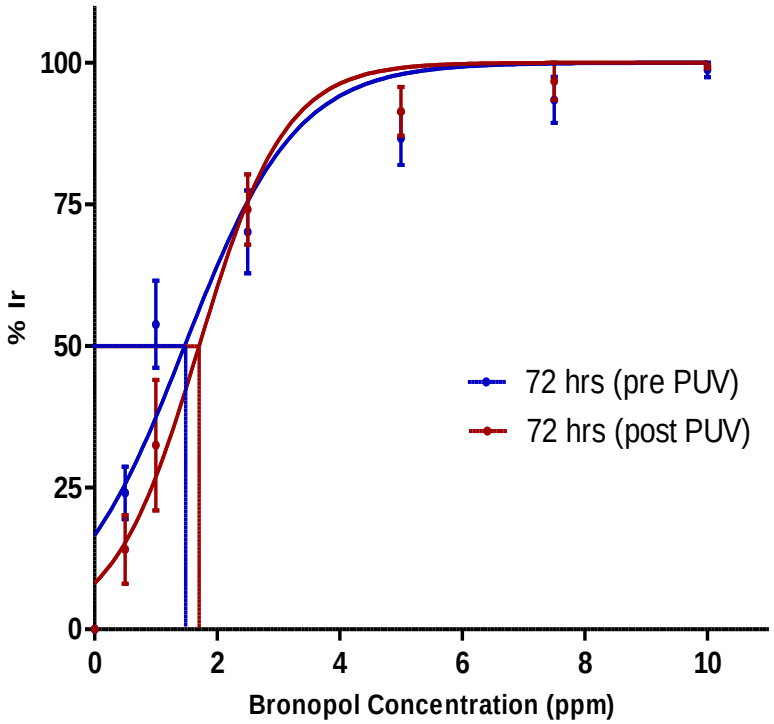

Figure 5.8 Dose response curve for *P. subcapitata* exposed to bronopol for 72 hrs pre and post PUV light exposure. **Pre PUV E_rC_{50}= 1.47 ppm** [95% C.I = 1.16 – 1.78]; Post **PUV E_rC_{50} = 1.71 ppm** [95% C.I = 1.42 – 1.99].

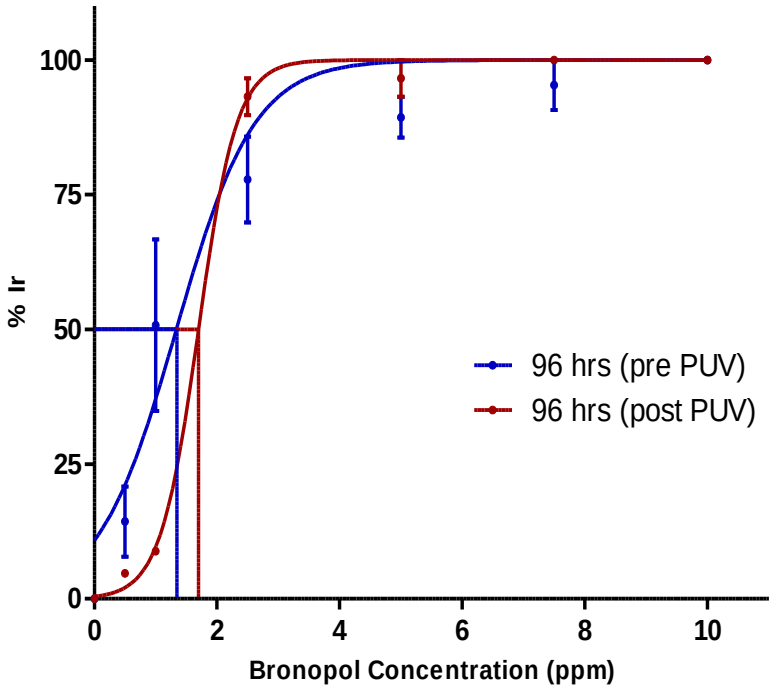

Figure 5.9 Dose response curve for *P. subcapitata* exposed to bronopol for 96 hrs pre and post PUV light exposure. **Pre PUV E_rC_{50}= 1.34 ppm** [95% C.I = 1.12 – 1.56]; Post **PUV E_rC_{50} = 1.70 ppm** [95% C.I =1.59 – 1.81].

The E_rC_{50} values determined pre PUV treatment of the bronopol were 1.47 and 1.34 ppm for 72 and 96 hours respectively, illustrated in **Figures 5.8** and **5.9**. There are contradictory reports in reference to the E_rC_{50} value using bronopol. Three different chemical suppliers were investigated, the first being Sigma Aldrich as this was the source of the bronopol product used in the present study. The establish E_rC_{50} for bronopol outlined in the ecotoxicological information section of the material safety data sheet (MSDS) provided by Sigma Aldrich is 0.37 ppm. The same result is outlined in the MSDS for bronopol provided by CDH Fine Chemical. An MSDS provided by Berger Seidle refers to the E_rC_{50} within the range of 0.4 - 2.8 ppm for bronopol for use with green algae. The value of 1.47 ppm (95% C.I. = 1.16 - 1.78) achieved in the present study lies within this range. It was noted on all of the aforementioned MSDS documents that the algae inhibition test was carried out in accordance with the OECD guidelines; whereas the present study was based on an adapted version of the ISO 8692:2012. Additionally, as previously mentioned, the CCAP composition for culture media was used rather than the one outlined in the ISO or OECD standards, which may have led to the contrast in 72 hour E_rC_{50} values.

A one-way ANOVA performed on the data for both the 72 and 96 hour tests showed significant differences between the means of the data points ($p < 0.0001$), implying the occurrence of significant differences in algal growth inhibition rates. The Dunnett's multiple comparison post analysis test determined significant differences existed between the control and all the treatments from 0.5 to 10 ppm for the 72 hour test. The 96 hour test showed significant differences between the control and bronopol concentrations from 1.0 to 10.0 ppm, however, there was not a significant difference between the control and the 0.5 ppm treatment.

The algal growth inhibition test produced an E_rC_{50} of 1.71 ppm for 72 hours (95% C.I. = 1.42 – 1.99) following PUV treatment of bronopol; the E_rC_{50} for 96 hours was 1.698 ppm (95% C.I. = 1.59 – 1.81), both illustrated in **Figures 5.8** and **5.9**, respectively. A one-way ANOVA determined that the means of all the data were significantly different with $p < 0.0001$ for both the 72 and 96 hour tests. For the 72 hour time point, the Dunnett's test identified that there was a significant difference between the control and concentrations from 1.0 to 10.0 ppm; the same result was observed for the 96 hour test.

Upon initial comparison of the results pre and post PUV exposure of bronopol, the results look different, with a 72 hour E_rC_{50} of 1.47 ppm pre PUV exposure compared to an E_rC_{50} of 1.70 ppm post PUV exposure. This gives the impression that the toxicity of bronopol was reduced following PUV treatment. However, an unpaired t-test resulted in a p-value of 0.902, which is not significant at the 95% level of confidence with p > than 0.05. For the 96 hour test, the unpaired t-test revealed that there was also no significant difference between the data set achieved prior to PUV exposure of bronopol compared to treated bronopol as $p = 0.888$. This means that the difference in the toxicity of bronopol pre and post PUV exposure on algal growth inhibition was not significant enough to cause a major difference in algal growth at this level of effect.

5.5.3 *P. subcapitata* bioassay – Chloramine-T

The algal growth inhibition assay was carried out on the second test chemical chloramine-T, as it is another commonly used chemical disinfectant in the aquaculture industry (Grasteau *et al.*, 2015). Range finding procedures led to a concentration range for the definitive test of between 0.1 and 15.0 ppm. An E_rC_{50} value of 2.29 ppm (95% C.I. = 2.05 – 2.53) was obtained for the 72 hour test period and a value of 1.63 ppm (95% C.I. = 1.34 – 1.92) for the 96 hour period pre PUV treatment, illustrated in **Figures 5.10** and **5.11**.

To the best of the researcher's knowledge, very limited data is available from other sources regarding the 72 hour E_rC_{50} for use with chloramine-T, as most data tend to focus on the 96 hour value in reference to this chemical. The 96 hour E_rC_{50} value obtained by Schmidt *et al*. (2007) for the freshwater algal growth inhibition assay was 4.5 ppm which is much higher than the result obtained in the present study. Differences in results obtained may be once again due to the test methodology utilised.

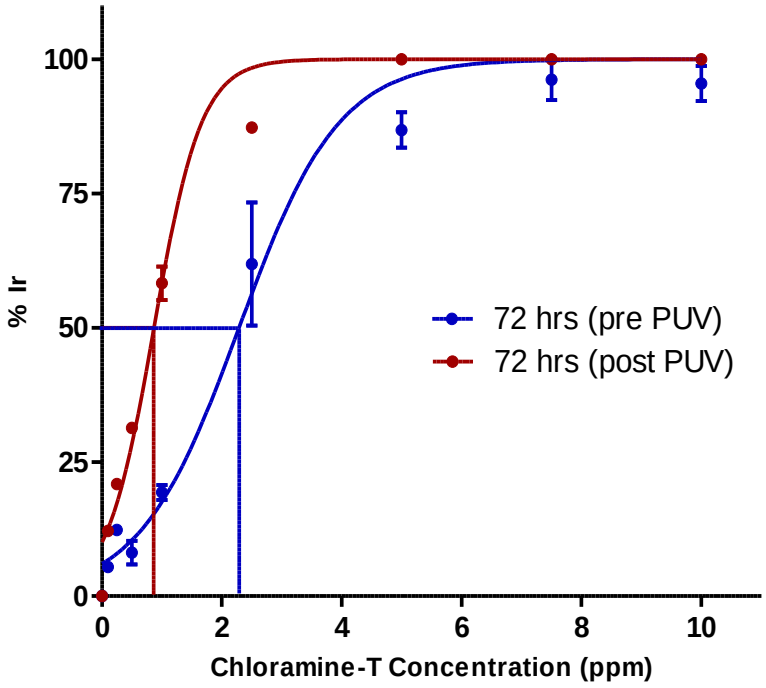

Figure 5.10 Dose response curve for *P. subcapitata* exposed to chloramine-T for 72 hrs pre and post PUV light exposure. **Pre PUV E_rC_{50}= 2.29 ppm** [95% C.I = 2.05 – 2.53]; **Post PUV E_rC_{50} = 0.87 ppm** [95% C.I = 0.74 – 1.00].

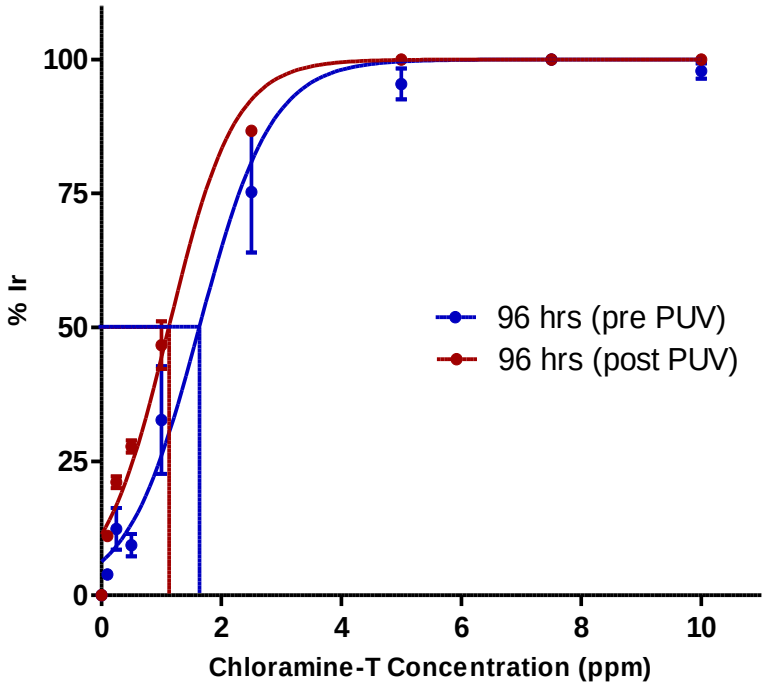

Figure 5.11 Dose response curve for *P. subcapitata* exposed to chloramine-T for 96 hrs pre and post PUV light exposure. **Pre PUV E_rC_{50}= 1.63 ppm** [95% C.I = 1.34 – 1.92]; **Post PUV E_rC_{50} = 1.12 ppm** [95% C.I = 0.94 – 1.31].

For chloramine-T, $p < 0.0001$ following a one-way ANOVA for both 72 and 96 hours, implying a significant difference existed between the means of the data points. The Dunnett's post analysis test for 72 hours identified significant differences between the control and the treatments from 1.0 ppm to 15.0 ppm, but not between the control and the 0.1, 0.25 or 0.5 ppm concentrations. This would suggest that for future testing the initial treatment concentration could start at 0.5 ppm. For the 96 hour chloramine-T test, the Dunnett's test revealed significant differences between the control and test concentrations from 1.0 ppm, however, no significant differences were obtained between the control and treatments below 1.0 ppm.

The test was then carried out using PUV treated chloramine-T, which yielded an E_rC_{50} of 0.87 ppm at 72 hours, **Figure 5.10**, with 95% C.I.'s ranging from 0.74 to 1.00. The 96 hour E_rC_{50} illustrated in **Figure 5.11** was 1.12 ppm (95% C.I. = 0.94 – 1.31). A one-way ANOVA determined that the means of all the data points were significantly different with $p < 0.0001$ in the case of both 72 and 96 hours. A Dunnett's test

revealed that the control was significantly different to all treatments (0.1 to 5.0 ppm) for both the 72 and 96 hour tests.

In comparison to the test pre PUV treatment of chloramine-T, there was a major increase in the effect of growth inhibition of the algae, as the E_rC_{50} decreased from 2.29 ppm pre PUV to 0.87 ppm post PUV for 72 hours. This suggests that there was a major increase in the toxic effects of the chemical on the algae growth rate post exposure of chloramine-T to PUV light. The 96 hour tests resulted in relatively similar E_rC_{50}s pre and post PUV exposure of chloramine-T. An unpaired t-test comparing the 72 hour data pre and post PUV exposure resulted in a p-value of 0.490 which suggests that the two sets of results were not significantly different, even though upon initial observation the E_rC_{50}'s suggested otherwise. There was no a significant difference between the two sets of data for the 96 hour test either, which resulted in a p-value of 0.720. Therefore, the toxicity of chloramine-T post PUV was not significantly different from that of the chemical prior to PUV treatment. The analysis carried out on the effect of bronopol and chloramine-T on the growth inhibition rate of algae *via* the use of an adapted version of ISO 8692:2012, demonstrated that bronopol had a higher toxic effect against the algal growth rate pre treatment of the chemicals with PUV light. Whereas, chloramine-T exerted higher toxicity on the growth inhibition rate of algae post treatment of the chemicals with PUV light.

5.6 A. salina crustacean bioassay

The *A. salina* crustacean bioassay was carried out in accordance with the 'Standardised short-term toxicity test' with *Artemia* nauplii (ARC-test). This test determined the percentage mortality of the test organism as a function of time following incubation with selected test chemicals. The standard method requires that the test is carried out utilising *Artemia* in instar II-III stages of growth. This method was employed for the present study, however, a variation of the method was also carried out where the bioassay was performed using *Artemia* in the instar I stage of growth to determine the difference in the effect of toxicity. The assay was initially carried out using the *A. salina* species (referred to in this study as the lab strain).

The bioassay was then carried out on an unknown strain of *Artemia* obtained from the collaborating fish farm, KWF, that utilises this batch of *Artemia* as a food source for perch larvae for two weeks post-hatching, referred to as the wild strain. As previously mentioned, bronopol is used for egg disinfection (Noga, 2010; Oono et al., 2007; Birkbeck et al., 2006) and because *Artemia* are fed to the larvae, eggs harbouring residues of the disinfectant may have an effect on the *Artemia*. If there are lingering residues of bronopol in the culture water, it is important to ascertain whether the concentration used to disinfect the eggs will have an impact on the *Artemia*, therefore, potentially reducing the food source available for the larvae. Invertebrate bioassays usually involve the establishment of results *via* microscopic or visual examination (Tothill and Turner, 1996); in this case, the latter method was used. Results are illustrated in **Figures 5.12** to **5.20** and expressed, in all cases, as the concentration that causes mortality in 50% of the test organisms for each time point, known as the LC_{50}.

5.6.1 *A. salina* crustacean bioassay – Reference test

To ensure the validity of the test procedure, an initial bioassay was required using $K_2Cr_2O_7$ as the reference chemical. **Figure 5.12** illustrates the dose response curve for *A. salina* following exposure to increasing concentrations of $K_2Cr_2O_7$ used as a reference chemical for 24 and 48 hours. The test was performed on the lab strain on the instar II-III stage. The 24 hour LC_{50} value obtained for the reference chemical was 36.92 ppm with confidence intervals at the 95% level of between 30.54 and 43.30 ppm. The results achieved in this study were in good agreement with results obtained by Toğulga (1998) who obtained a mean LC_{50} value range for exposure to $K_2Cr_2O_7$ of between 32 and 42 ppm. The original establishment of the ARC-test involved intercalibration exercises to ensure a standardised test method. This involved investigating the effect of $K_2Cr_2O_7$ on *Artemia* nauplii to determine variability between different testing facilities. The mean results achieved for the 24 hour LC_{50} were 38.87 and 42.39 ppm. The result of 36.92 ppm achieved in the present study is in close correspondence to the LC_{50} values established during the standardisation of the ARC-test. Svensson *et al.* (2005) achieved an EC_{50} value of 39.90 ppm for the

reference test using $K_2Cr_2O_7$. Even though the result expressed by Svensson *et al.* (2005) was a different investigative parameter, the same effect of toxicity was established.

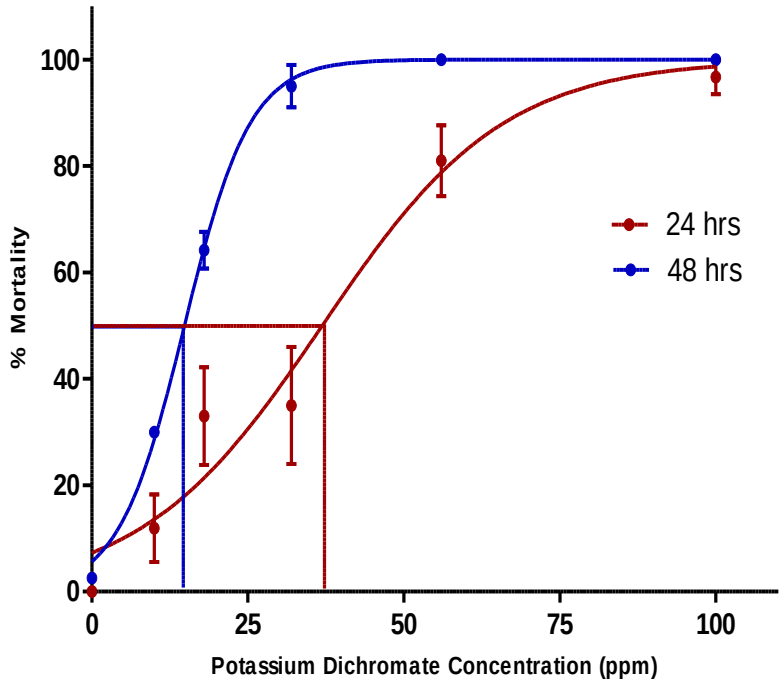

Figure 5.12 Dose response curve for *A. salina* exposed to $K_2Cr_2O_7$ for 24 and 48 hrs. **24 hrs LC_{50} = 36.92 ppm** [95% C.I = 30.54 – 43.30]; **48 hrs LC_{50} = 14.84 ppm** [95% C.I = 14.10 – 15.59].

The LC_{50} value obtained by Davoren *et al.* (2005) yielded a lower LC_{50} of 22.70 ± 2.7 ppm after 24 hours. This may be due to the difference in the media utilised for the experiment, as Davoren *et al.* 2005 used seawater sourced from Sigma Aldrich, whereas the artificial seawater prepared in this project was prepared every two weeks in the lab as per the composition list obtained from the Artoxkit M for toxicity screening using *Artemia*. The media in this assay was also aerated for 24 hours prior to testing increasing the oxygen availability, which may have prolonged the viability and increased the survival strength of the *A. salina* against the toxicant. The LC_{50} achieved for the 48 hour test was 14.84 ppm with the confidence intervals ranging between 14.10 and 15.59 ppm. In most bioassays, the 48 hour results tend to be lower, and therefore more toxic as the organisms are exposed to the chemical for a longer period of time.

Statistical analysis in the form of a one-way ANOVA resulted in a p value of < 0.0001 for both time periods suggesting significant differences were present between data means. The Dunnett's post analysis test revealed that for the 24 hour test significant differences occurred between the control and treatment concentrations from 18 ppm to 100 ppm. However, the difference in means between the control and the lowest concentration of 10 ppm was not significant. Analysis of the 48 hour results revealed significant differences between the control and all treatment concentrations of $K_2Cr_2O_7$.

5.6.2 *A. salina* crustacean bioassay (lab strain) – Bronopol

The same methodology was adhered to for bronopol. Testing and range finding procedures led to a range of concentrations from 0.0 to 200.0 ppm for the definitive test. **Figures 5.13** and **5.14** illustrate the dose response curves for the percentage mortality of the lab strain of *A. salina* following exposure to increasing concentrations of bronopol for 24 and 48 hours respectively. The 24 hour LC_{50} achieved for bronopol was 87.49 ppm (95% C.I. = 79.86 – 95.13) and the 48 hour LC_{50} was 42.46 ppm (95% C.I. = 37.03 – 47.90) pre PUV treatment of the chemical.

A one-way ANOVA was performed on the data to determine whether significant differences occurred between the means of all test concentrations for the 24 and 48 hour tests for bronopol. Both the 24 and 48 hour tests showed significant differences between the means of the data points ($p < 0.0001$), implying the occurrence of significant differences in percentage mortality of *A. salina*. The Dunnett's post analysis test determined significant differences existed between the control and treatments from 50 to 200 ppm of bronopol for the 24 hour test; no significant difference existed between the control and the concentrations from 10 to 25 ppm. The 48 hour test showed significant differences between the control and bronopol concentrations from 25 to 200 ppm, however, there was not a significant difference between the control and the 10 ppm treatment.

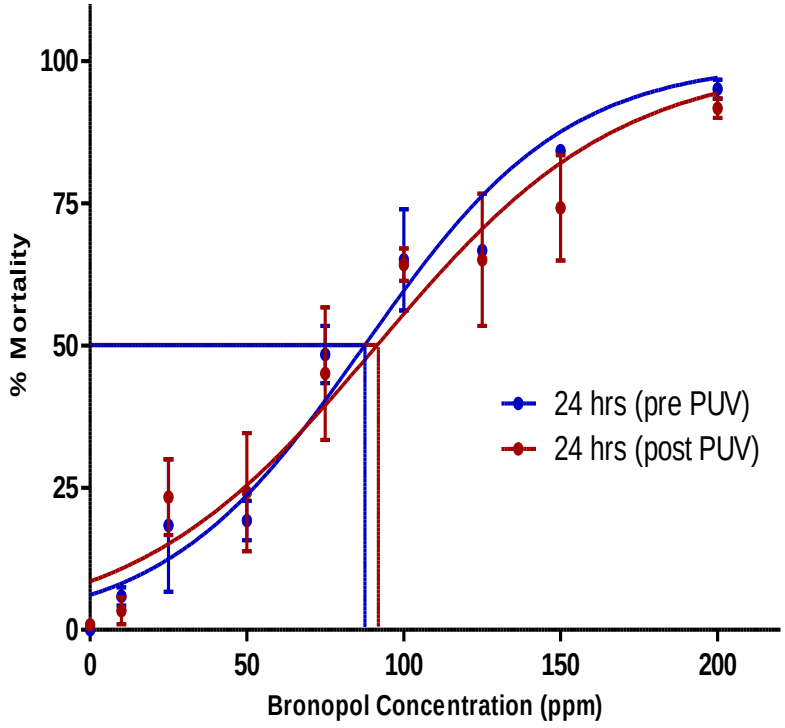

Figure 5.13 Dose response curve for *A. salina* exposed to Bronopol for 24 hrs pre and post PUV light exposure. **Pre PUV LC_{50}= 87.49 ppm** [95% C.I = 79.86 – 95.13]; **Post PUV LC_{50} = 91.45 ppm** [95% C.I = 79.60 – 103.30].

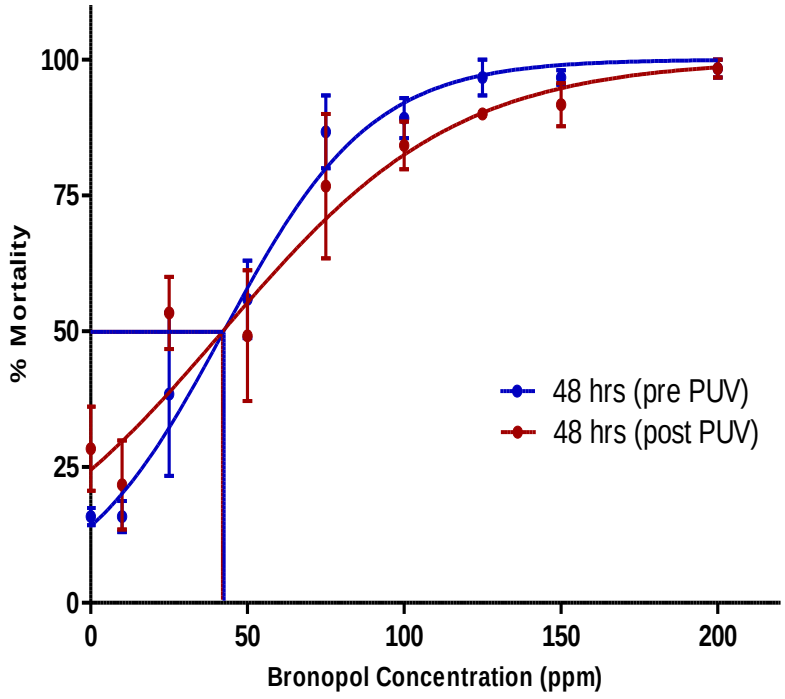

Figure 5.14 Dose response curve for *A.salina* exposed to Bronopol for 48 hrs pre and post PUV light exposure. **Pre PUV LC_{50}= 42.46 ppm** [95% C.I = 37.03 – 47.90]; **Post PUV LC_{50} = 42.15 ppm** [95% C.I = 30.50 – 53.80].

For the *A. salina* mortality bioassay an LC_{50} of 91.45 ppm was established for 24 hours (95% C.I. = 79.60 – 103.30) following PUV treatment of bronopol and the LC_{50} for 48 hours was 42.15 ppm (95% C.I. = 30.50 – 53.80). A one-way ANOVA determined that the means of all the data were significantly different with $p < 0.0001$ for both the 24 and 48 hour tests. However, the means were only significantly different from the control from treatments 75 ppm to 200 ppm following analysis *via* a Dunnett's test. There were no significant differences between the control and the treatment concentrations of 10, 25 or 50 ppm for both the 24 and 48 hour results.

Results achieved pre and post PUV exposure of bronopol were very similar, indicating the little effect that PUV light exposure had on bronopol. An unpaired t-test identified a p value of 0.940 for the 24 hour data sets indicating that no significant difference existed pre and post PUV exposure and revealed that there was only a difference of 1.21 ppm between the means of the two data sets. There was no significant difference between the 48 hour data sets ($p = 0.999$), with an even smaller difference between the means of the data sets of 0.01 ppm. These results, similar to the conclusion obtained regarding the algae bioassay, indicate that the difference in the toxicity of bronopol pre and post PUV exposure was not significant. Therefore, the effect on *A. salina* mortality pre and post PUV exposure was similar and not significant enough to cause a major difference in mortality rates at this effect level.

5.6.3 *A. salina* crustacean bioassay (lab strain) – Chloramine-T

The concentration range established for use in the definitive test for chloramine-T was 0 to 2500 ppm following range finding procedures. **Figures 5.15** and **5.16** illustrate the non-linear regression curve for both the 24 hour and 48 hour time points of the crustacean bioassay. The 24 hour LC_{50} achieved was 687.90 ppm with the 95% confidence intervals ranging from between 674.00 and 701.90 pre PUV treatment. The 48 hour LC_{50} was 656.60 ppm and the 95% confidence intervals ranged from between 628.20 to 685.10, also pre PUV treatment.

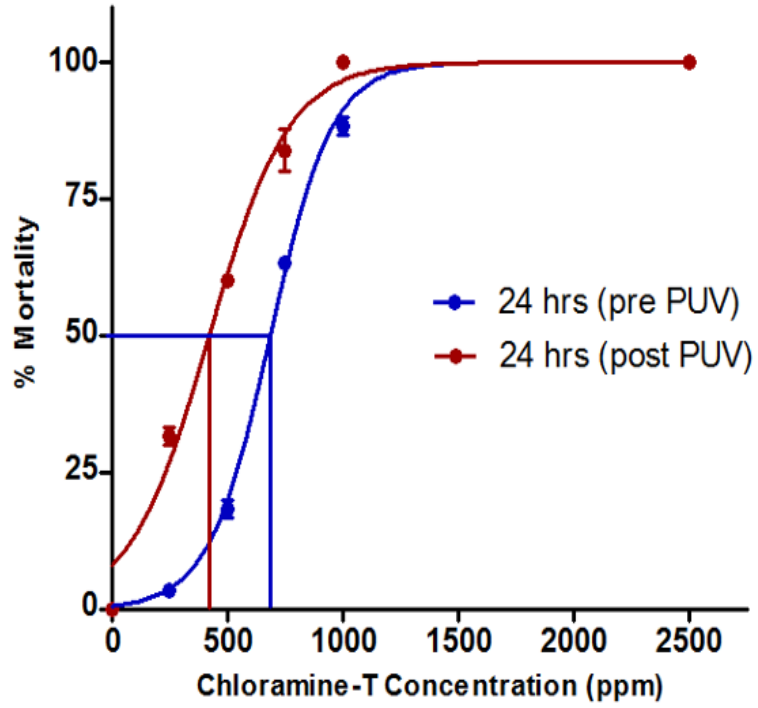

Figure 5.15 Dose response curve for *A. salina* exposed to chloramine-T for 24 hrs pre and post PUV light exposure. **Pre PUV LC_{50} = 687.90 ppm** [95% C.I = 674.00 – 701.90]; **Post PUV LC_{50} = 424.50 ppm** [95% C.I = 384.30 – 464.70].

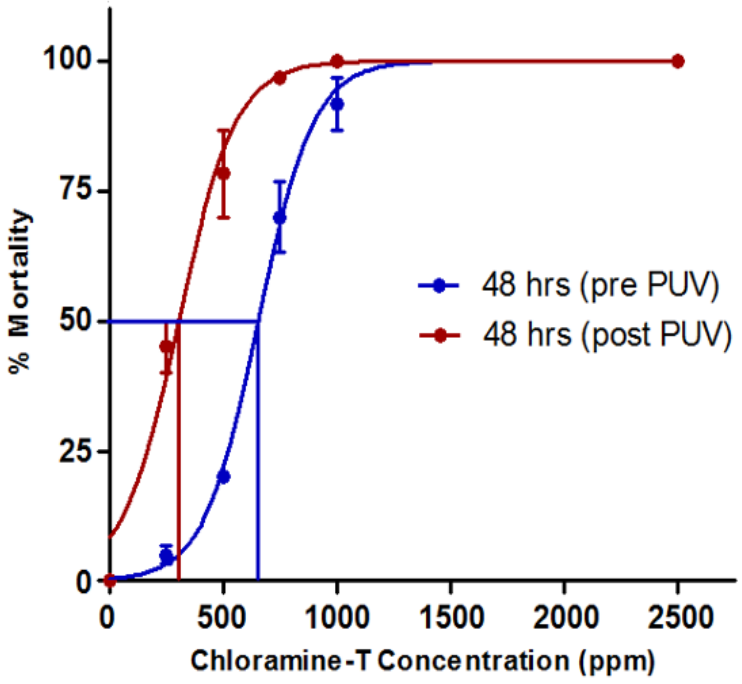

Figure 5.16 Dose response curve for *A. salina* exposed to chloramine-T for 48 hrs pre and post PUV light exposure. **Pre PUV LC_{50} = 656.60 ppm** [95% C.I = 628.20 – 685.10]; **Post PUV LC_{50} = 302.80 ppm** [95% C.I = 258.30 – 347.40].

For chloramine-T, $p < 0.0001$ following a one-way ANOVA for both 24 and 48 hours, implying a significant difference existed between the means of the data points and therefore percentage mortality of *A. salina*. The Dunnett's post analysis test, for both the 24 and 48 hour tests, identified significant differences between the control and the treatments from 500 ppm to 2000 ppm, but not between the control and the 250 ppm treatment concentration.

Once again, the exposure of chloramine-T to PUV light resulted in much lower end results, indicating an increase in the toxicity of chloramine-T post PUV treatment. For the 24 hour test, the LC_{50} result achieved post PUV exposure of chloramine-T was 424.50 ppm (95% C.I. = 384.30 – 464.70) compared to an LC_{50} of 687.90 ppm prior to PUV treatment, illustrated in **Figure 5.15**. The 48 hour LC_{50} was 302.80 ppm, two times lower than the LC_{50} of 656.60 ppm achieved pre PUV treatment, illustrated in **Figure 5.16**. A one-way ANOVA for both 24 and 48 hours determined that the means of all the data points were significantly different ($p < 0.0001$). A Dunnett's test revealed that all the treatment concentrations were significantly different from the control for both time points.

An unpaired t-test revealed that although the results seem more toxic following PUV exposure, p values of 0.501 and 0.387 were established for comparison of the 24 and 48 hour data sets, respectively. This indicates that the differences between the PUV treated and untreated chemical were not enough to be significant and, therefore, the difference between the mortality rates of each test, albeit different, was not statistically significant.

5.6.4 *A. salina* crustacean bioassay (KWF wild strain) – Bronopol

Once all tests were carried out using the lab strain of *Artemia*, tests were then carried out on a strain of *Artemia* obtained from KWF to determine whether a difference in toxicity was observed between the two strains upon exposure to bronopol. The ARC test was carried out as previously described and **Figures 5.17** and **5.18** illustrate the dose response curves for the percentage mortality of this wild strain of *A. salina*, as a comparison to the lab strain. The resulting LC_{50} values for the wild strain were 90.74

ppm (95% C.I. = 76.60 – 104.90) and 37.72 ppm (95% C.I. = 22.77 – 52.67) for the 24 hour and 48 hour tests respectively. This result was in close proximity with the results achieved for the lab strain of 87.49 ppm (24 hour) and 42.46 ppm (48 hour), which suggests similarity in the two strains of *Artemia*; it may even be possible that both strains are the same. This warrants the need for further investigation into identifying the genetic makeup of both strains.

Statistical analysis in the form of a one-way ANOVA identified a significant difference between the means of the data where $p < 0.0001$ for 24 hours and $p = 0.0005$ for the 48 hour test. A Dunnett's post analysis test identified that significant differences existed between the control and the treatment concentrations of bronopol from 75 to 150 ppm for both time periods. There was no significant difference between the control and the treatments from 10 to 50 ppm, indicating that the difference in mortality levels of *Artemia* was significant compared to the control after the concentration reached 75 ppm.

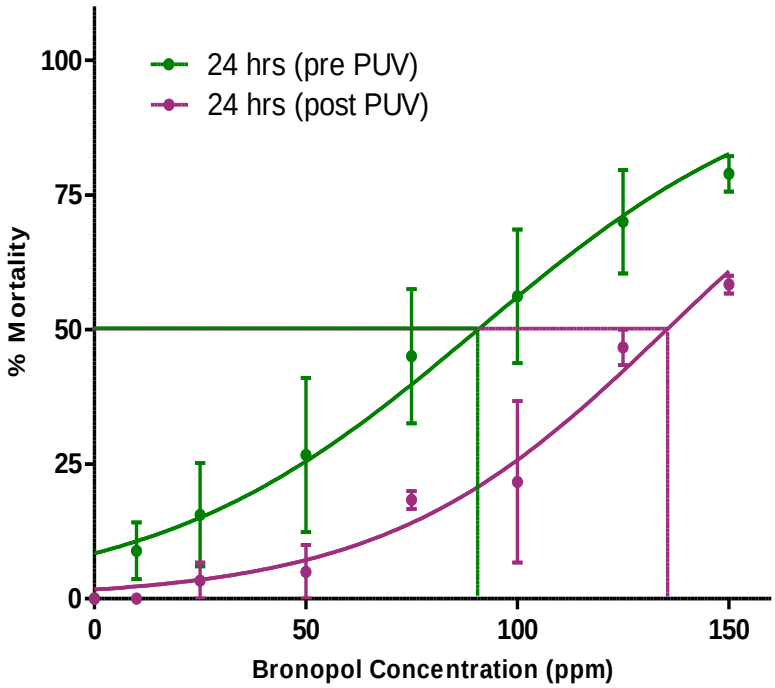

Figure 5.17 Dose response curve for *A. salina* (wild strain from KWF) exposed to bronopol for 24 hrs pre and post PUV light exposure. **Pre PUV LC_{50}= 90.74 ppm** [95% C.I = 76.60 – 104.90]; **Post PUV LC_{50} = 135.40 ppm** [95% C.I = 125.70 – 145.00].

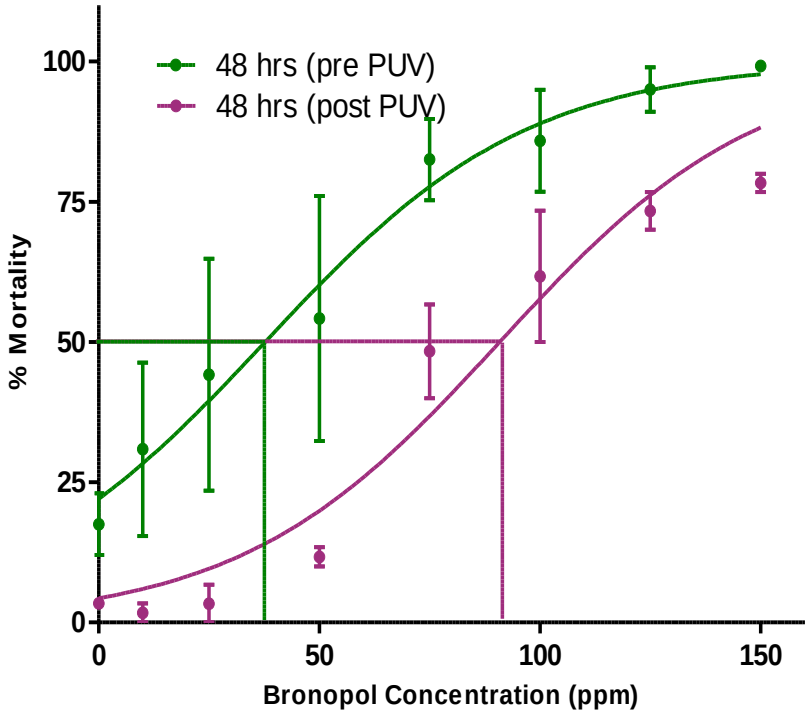

Figure 5.18 Dose response curve for *A. salina* (wild strain from KWF) exposed to bronopol for 48 hrs pre and post PUV light exposure. **Pre PUV LC_{50}= 37.72 ppm** [95% C.I = 22.77 – 52.67]; **Post PUV LC_{50} = 90.88 ppm** [95% C.I = 81.39 – 100.40].

This test was then repeated on the wild strain of *A. salina* post treatment of bronopol with PUV light. Unlike results achieved for the lab strain of *A. salina*, which showed very little difference pre and post treatment of bronopol, the wild strain resulted in great differences pre and post PUV treatment. The LC_{50} established for the 24 hour test post PUV treatment of the chemical was 135.40 ppm (95% C.I. = 125.70 – 145.00) compared to 90.74 ppm for pre PUV treatment, which is illustrated in **Figure 5.17**. The difference in the 48 hour LC_{50} pre and post PUV treatment of bronopol was also much higher for the wild strain. An LC_{50} of 90.88 ppm (95% C.I. = 81.39 – 100.40) post PUV was established in comparison to an LC_{50} of 37.72 ppm achieved pre PUV treatment, illustrated in **Figure 5.18**. These results as previously discussed suggest that the toxicity of bronopol is decreased or altered following PUV treatment at the described parameters. A one-way ANOVA was carried out on both the 24 hour and 48 hour tests post PUV treatment of bronopol to determine whether significant differences were present between the means of all data points. For the 24 hour test p = 0.0006, indicating a significant difference between the means, with a Dunnett's

test confirming that only significant differences exited between the control and the 125 and 150 ppm treatment concentrations. The means of the percentage mortality of *A. salina* resulting from the treatments of 10 to 100 ppm were not significantly different from the control. The 48 hour test resulted in a p value of < 0.0001 with the Dunnett's test indicating that treatments from 75 to 150 ppm of bronopol post PUV exposure were significantly different from the control.

The results achieved for the *A. salina* crustacean bioassay utilising the wild strain of *A. salina* from KWF suggest that upon exposure to PUV treated bronopol this strain of *Artemia* are less susceptible to mortality effects compared to the lab strain for which pre and post PUV results were very similar. An unpaired t-test was carried out in order to verify the differences resulting in p values for the 24 and 48 hour tests of 0.177 and 0.101 respectively, which was greater than the significant level of 0.05. This suggests that in both cases there was no significant difference between the data sets obtained pre and post PUV treated bronopol for the 24 and 48 hour time points for the strain of *Artemia* obtained from KWF.

5.6.5 *A. salina* crustacean bioassay (KWF wild strain & reduced hatching period) – Bronopol

The bioassay carried out on instar I organisms involved 18 hours of hatching compared to between 24 and 48 hours for instar II-III larvae, utilising the strain of *Artemia* obtained from KWF and bronopol as the test chemical. **Figures 5.19** and **5.20** illustrate the dose response curves for the percentage mortality of this strain of *A. salina*, following exposure to increasing concentrations of bronopol for 24 and 48 hours respectively. Experiments were carried as per the methodology utilised for the previous ARC tests. The susceptibility of the *A. salina* to mortality utilising instar I test organisms was significantly reduced. The 24 hour LC_{50} was unobtainable even through estimation, as the toxicity in terms of percentage mortality was so low. The 48 hour LC_{50} was 143.80 ppm (95% C.I. = 122.90 – 164.60), which would indicate an even higher 24 hour LC_{50} value.

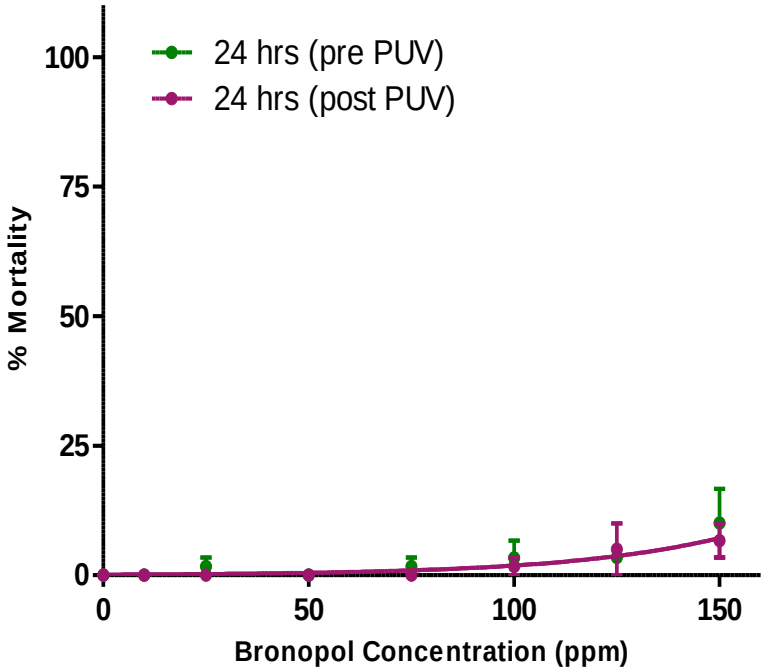

Figure 5.19 Dose response curve for *A. salina* (wild strain from KWF), after an 18 hour hatching period, exposed to bronopol for 24 hrs pre and post PUV light exposure. **Pre PUV LC_{50} = unobtainable estimation**; **Post PUV LC_{50} = <u>241.60 ppm</u>** [95% C.I = 144.00 – 339.30].

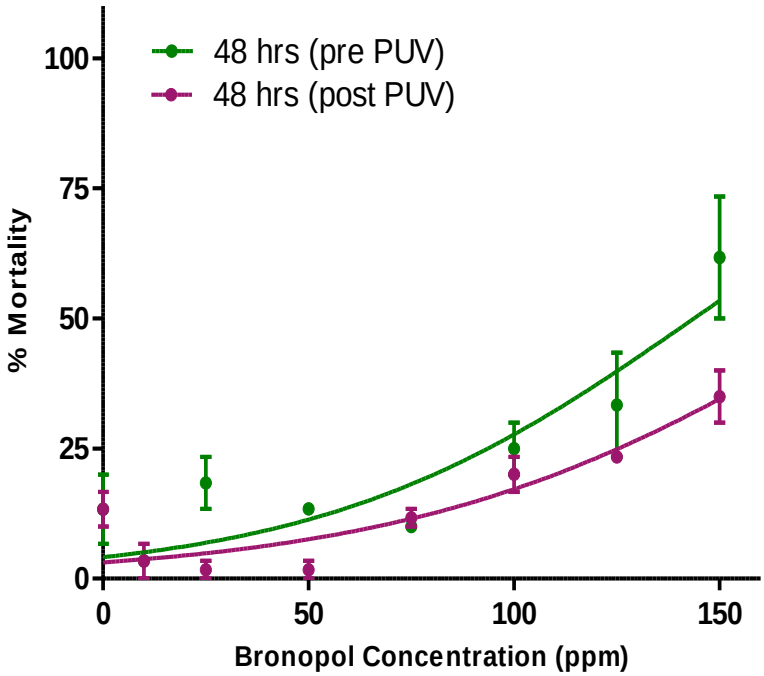

Figure 5.20 Dose response curve for *A. salina* (wild strain from KWF), after an 18 hour hatching period, exposed to bronopol for 48 hrs pre and post PUV light exposure. **Pre PUV LC_{50} = <u>143.80 ppm</u>** [95% C.I = 122.90 – 164.60]; **Post PUV LC_{50} = <u>184.30 ppm</u>** [95% C.I = 156.70 – 212.00].

The results achieved implies a great difference in sensitivity between instar I and instar II-III. The same effect was observed by Toğulga (1998), who found that following exposure to the ionic detergent sodium lauryl sulphate (SLS), toxic effects occurred at a lower concentration of 14 ppm for instar II-III nauplii. Whereas toxic effects were reported at 35 ppm for instar I. Based on the results obtained, it seems that at the early larval stage the nauplii are less susceptible to toxicants. This is apparently due to the different anatomical and morphological features of the instar levels of *Artemia* nauplii (Toğulga, 1998).

A one-way ANOVA established that there were no significant differences between the means of the data points for the 24 hour test ($p = 0.3005$), implying that there were no significant differences in the percentage mortality of test organisms as the concentrations increased from 10 to 150 ppm of bronopol. This was confirmed by a Dunnett's Multiple Comparison test which found no significance in the percentage mortality between the control and any of the treatments. This result is evident in **Figure 5.19** where there is only a slight increase in the curve which only reaches a percentage mortality value of 10.05% at the highest concentration of 150 ppm. For the 48 hour test, a one-way ANOVA resulted in a p value of 0.0041 which implies a significant difference existed between the means, however post analysis *via* a Dunnett's test confirmed that there was only a significant difference between the control and the highest concentration of 150 ppm. The same concentration range was employed for this bioassay as for the ARC-test using organisms from the instar II-III stage when comparing the age of the organism to establish a true comparison. Going forward, further range finding for the younger test organisms would enable a more accurate LC_{50} result.

Davoren *et al.* (2005) state that the *A. salina* has too low of a sensitivity threshold to be utilised as an appropriate organism for the screening of chemicals. However, the major advantage of the utilisation of *Artemia* nauplii compared to other organisms for ecotoxicological and aquaculture tests is the ease of use and storage due to the availability of dry cysts (Toğulga, 1998).

5.7 D. magna inhibition of mobility bioassay

The acute toxicity *D. magna* test was utilised to determine the effect of bronopol and chloramine-T on the mobility of the water flea *D. magna*. The test was carried out *via* an adapted version of the ISO 6341:2012. Results are illustrated in **Figures 5.21** to **5.25** and are expressed in all cases as the effective initial inhibitory concentration that immobilises 50% of the *D. magna* population known as the EC_{50}.

5.7.1 *D. magna* inhibition of mobility bioassay – Reference test

The initial reference test was performed to ensure the validity of the test method using $K_2Cr_2O_7$ as the reference chemical. The test organisms were exposed to concentrations of $K_2Cr_2O_7$ ranging from 0.0 to 5.0 ppm. The data was plotted on a non-linear regression curve to establish results in the form of an EC_{50}, illustrated in **Figure 5.21**.

The 24 hour EC_{50} result determined for the reference test was 1.76 ppm with confidence intervals at the 95 % level ranging from 1.36 to 2.16. The ISO guideline results established to ensure test validity range from 0.6 – 2.7 ppm if using $K_2Cr_2O_7$ as the reference chemical. The EC_{50} for the reference test carried out in this present study falls within the range of the ISO guidelines. There are many conflicting results in the literature regarding the EC_{50} for the reference chemical. Kühn *et al.* (1989) established an EC_{50} value of 0.35 ppm for the acute toxicity *D. magna* test using $K_2Cr_2O_7$. Brennan *et al.* (2006) achieved an EC_{50} of 1.05 ppm (C.I. = 0.97 – 1.13) for the 24 hour acute test using $K_2Cr_2O_7$ as the reference chemical. One possibility for this is the fact that there is no standardised feeding methodology and so the food provided for the *Daphnia* may influence the results from one researcher to another. The EC_{50} established for the 48 hour test was 0.97 ppm (95% C.I. = 0.76 – 1.18).

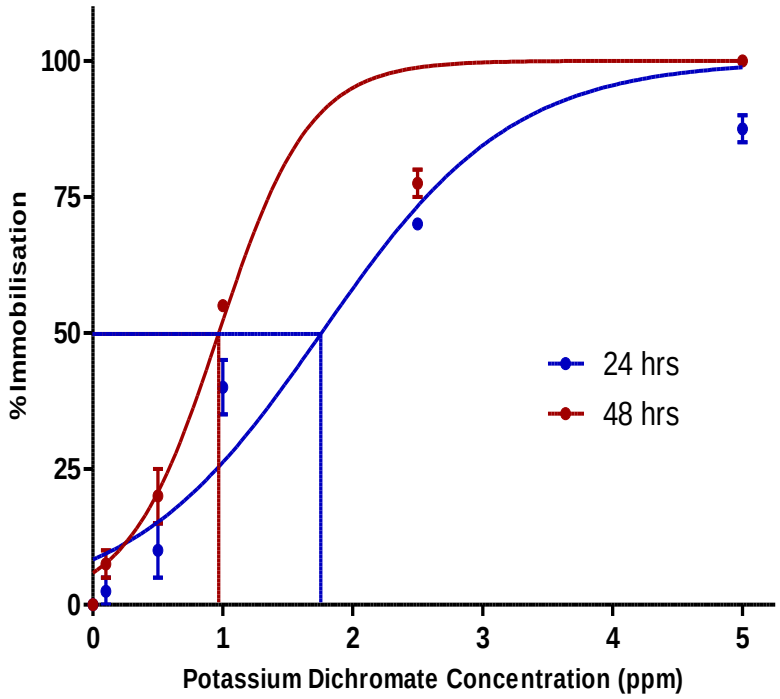

Figure 5.21 Dose response curve for *D. magna* exposed to $K_2Cr_2O_7$ for 24 and 48 hrs. 24 hrs **EC_{50} = 1.76 ppm** [95% C.I = 1.36 – 2.16]; 48 hrs **EC_{50} = 0.97 ppm** [95% C.I = 0.76 – 1.18].

A one-way ANOVA was applied to determine significant variances between the means of the data. With a resulting p value of < 0.0001, significant differences between the means existed between the data points. A Dunnett's multiple comparison post analysis test identified significant differences in the means between the control and the concentrations of $K_2Cr_2O_7$ from 1.0 to 5.0 ppm. However, no significant difference existed between the control and the 0.1 or 0.5 ppm concentrations. This indicated that the mobility of the *D. magna* was not significantly inhibited until they were exposed to concentrations of higher than 0.5 ppm of $K_2Cr_2O_7$. The same result was achieved for the one-way ANOVA applied for the 48 hour test (p < 0.0001), however, the Dunnett's test revealed that differences between the control and the treatments were significant from 0.5 ppm.

5.7.2 *D. magna* inhibition of mobility bioassay – Bronopol

Range finding procedures led to the implementation of a concentration range from 0.0 to 3.0 ppm for the definitive acute toxicity test for bronopol. A non-linear

regression curve was established where the bronopol concentration (ppm) was plotted against the percent inhibition of mobility in the test organisms, **Figure 5.22.** An EC_{50} of 1.65 ppm (95% C.I. = 1.55 – 1.76) was determined for the 24 hour test pre PUV treatment of bronopol. Most data in the literature tends to express the toxicity of bronopol on *D. magna* as a 48 hour EC_{50}. The USEPA (1994) achieved a 48 hour EC_{50} of 1.4 ppm, as did Sigma Aldrich and CDH Fine Chemical denoted in both MSDS's. The 48 hour EC_{50} established in the present study was 0.82 ppm pre PUV treatment with confidence intervals at the 95% level ranging from 0.76 to 1.18, illustrated in **Figure 5.23**. This is almost half the value that is reported in the literature. This may be due to a number of reasons. As previously mentioned, there is no standardised method for feeding the *D. magna,* which may affect the toxic effect exerted upon the organisms. It was noted on the MSDS's that both Sigma Aldrich and CDH Fine Chemical followed the OECD 202 guidelines for the bioassay rather than the ISO 6341:2012, which was followed in the present study.

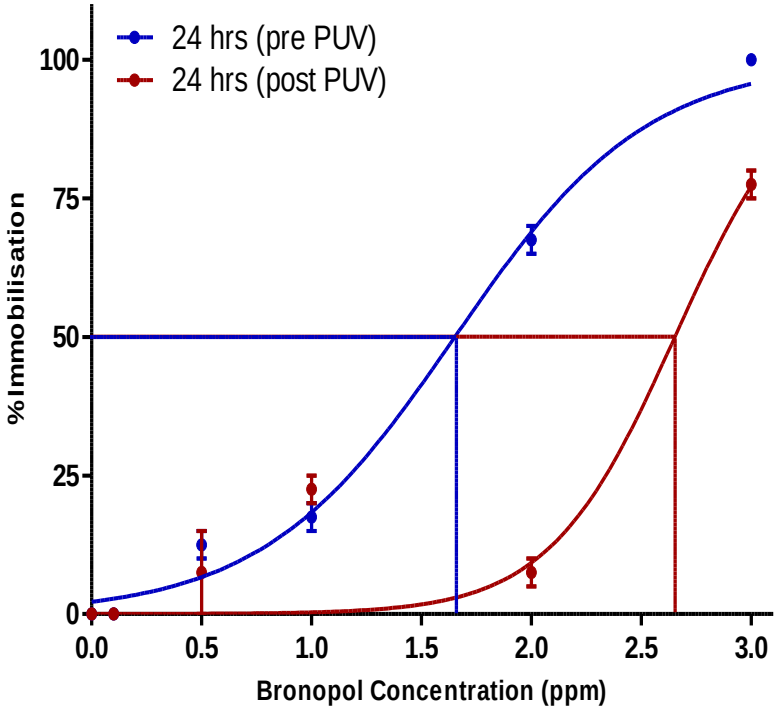

Figure 5.22 Dose response curve for *D. magna* exposed to bronopol for 24 hrs pre and post PUV light exposure. **Pre PUV EC_{50} = 1.65 ppm** [95% C.I = 1.55 – 1.76]; **Post PUV EC_{50} = 2.65 ppm** [95% C.I = 2.37 – 2.93].

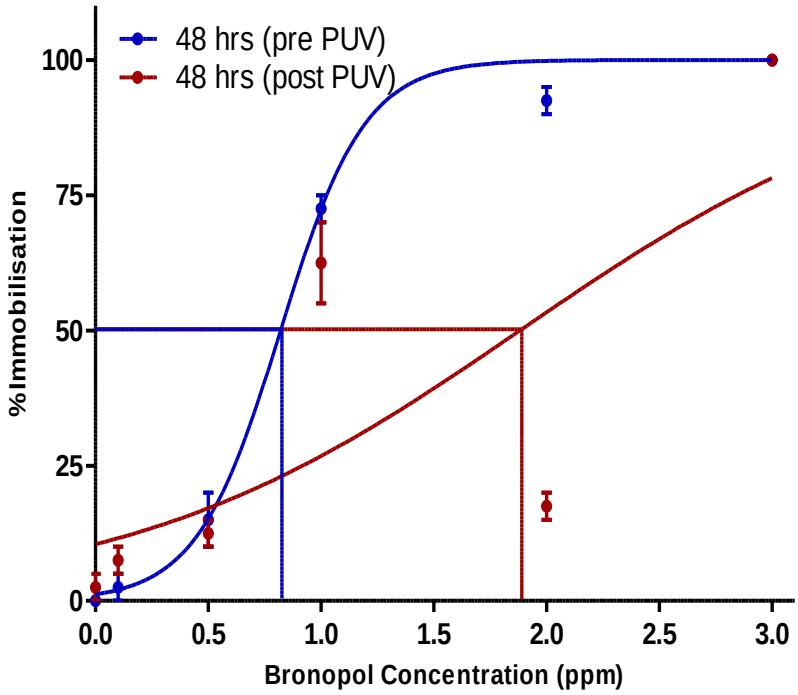

Figure 5.23 Dose response curve for *D. magna* exposed to bronopol for 48 hrs pre and post PUV light exposure. **Pre PUV EC_{50} = 0.82 ppm** [95% C.I =0.77 – 0.88]; **Post PUV EC_{50} = 1.88 ppm** [95% C.I = 0.98 – 2.79].

For both the 24 and 48 hour tests $p < 0.0001$ for the one-way ANOVA, indicating significant differences between the means of the data points. The Dunnnett's test identified the treatment concentrations from 1.0 to 10.0 ppm were significantly different from the control for both the 24 hour and 48 hour tests. This, therefore, revealed the 0.5 ppm treatment as the NOEC value, the concentration where there was no observed level of effect.

The *D. magna* inhibition of mobility assay produced an EC_{50} of 2.65 ppm for 24 hours (95% C.I. = 2.37 – 2.93) following PUV treatment of bronopol and the EC_{50} for 48 hours was 1.88 ppm (95% C.I. = 0.98 – 2.79). A one-way ANOVA determined that the means of all the data were significantly different, with $p < 0.0001$ for both the 24 and 48 hour tests. For both time points, the Dunnett's test identified that only significant differences with the control existed for the 1.0 and the 3.0 ppm concentrations. There were no significant differences between the mean of the control and the means achieved in terms of percentage inhibition of mobility for the 0.1, 0.5 or 2.0 ppm concentrations. As with the previous ecotoxicological bioassays carried out, the

results established give the impression that the toxicity of bronopol was reduced following PUV treatment. Prior to PUV treatment, the EC_{50} for the 24 and 48 hour tests were 1.65 and 0.82 ppm respectively, which is much lower than the ppm results required to achieve 50% inhibition of mobility of the daphnids compared to PUV treated bronopol. However, an unpaired t-test resulted in a p value of 0.523 for the 24 hour data sets and 0.602 for the 48 hour test data sets, which is not significant at the 95% level of confidence as p was > than 0.05. This means that the difference in the toxicity of bronopol pre and post PUV exposure on the inhibition of *D. magna* mobility was not significant enough to cause a major difference at a laboratory scale.

For the majority of the data points exposed to PUV treated bronopol the percentage immobilisation increased as the treatment concentration increased. However, upon exposure to the 2.0 ppm concentration, the results displayed an irregular trend. At this concentration, the percentage immobilisation decreased again to 7.5%, the same percentage immobilisation achieved for the 0.5 ppm concentration. This may be classified as an outlier, however, the same result was achieved following the execution of two independent tests with triplicate replicates per treatment. This suggests that the *D. magna* may obtain a defence mechanism that initiates upon exposure to bronopol at this concentration following a possible structural change in the chemical after PUV treatment, as the same response was not observed following exposure to non-treated bronopol. The percentage immobilisation increased once again as the concentration increased to 3.0 ppm. This pattern occurred for both the 24 and 48 hour tests for PUV treated bronopol, and so warrants further investigation into the chemical structure of PUV treated bronopol and an investigation to the *D. magna* responses at a molecular level.

5.7.3 *D. magna* inhibition of mobility bioassay – Chloramine-T

The treatment range for the bioassay with chloramine-T was 0.0 to 10.0 ppm for the definitive test following range finding procedures. The EC_{50} achieved for the 24 hour test was 3.14 ppm (95% C.I. = 2.62 – 3.65) pre PUV treatment of the chemical. An EC_{50} of 4.8 ppm for 24 hours was established by Kühn *et al.* (1989) also using chloramine-T as the test chemical. For the 48 hour test, an EC_{50} of 0.95 ppm was

obtained pre PUV treatment with the 95% confidence intervals ranging from 0.80 to 1.11. Both the 24 hour and 48 hour non-linear regression curves for this test are illustrated in **Figures 5.24** and **5.25**, respectively.

A one-way ANOVA resulted in a p value of < 0.0001, which indicated that significant differences existed for both the 24 and 48 hour tests, with a Dunnett's post analysis test identifying that there were significant differences between the control and the 1.0 to 10.0 ppm concentrations in both cases. This revealed the 0.5 ppm treatment as the NOEC value once again, the concentration where there was no observed level of effect. As with the other ecotoxicological bioassays, upon exposure to PUV, chloramine-T seemed to increase in toxicity. An EC_{50} of 2.18 ppm was obtained for the 24 hour test (95% C.I. = 1.76 - 2.60). This is lower than the EC_{50} achieved pre PUV treatment of the chemical. The 48 hour test was, however, much closer in the EC_{50} value to the pre treatment result. The 48 hour EC_{50} value obtained post PUV treatment of chloramine-T was 0.82 ppm (95% C.I. = 0.72 – 0.92), in close proximity to 0.95 ppm achieved pre PUV treated chloramine-T.

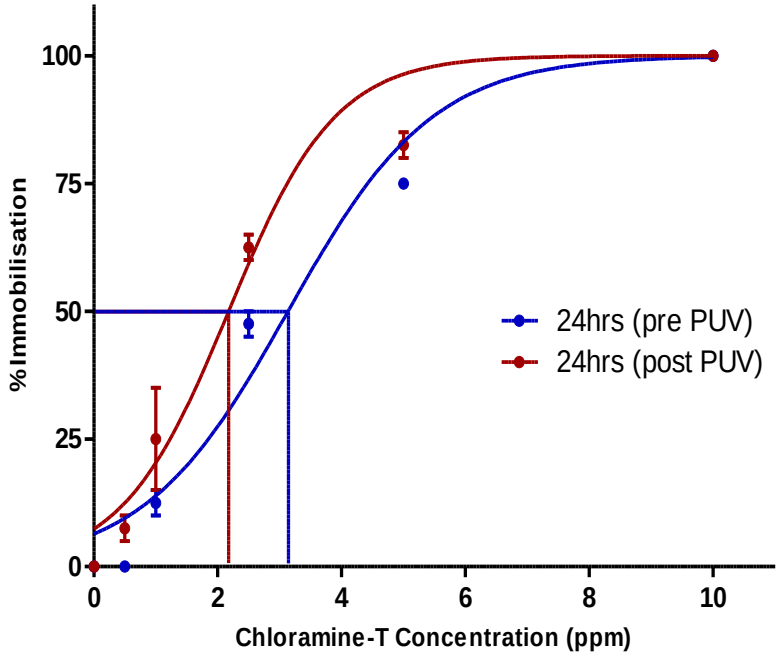

Figure 5.24 Dose response curve for *D. magna* exposed to chloramine-T for 24 hrs pre and post PUV light exposure. **Pre PUV EC_{50} = 3.14 ppm** [95% C.I = 2.62 – 3.65]; **Post PUV EC_{50} = 2.18 ppm** [95% C.I = 1.76 – 2.60].

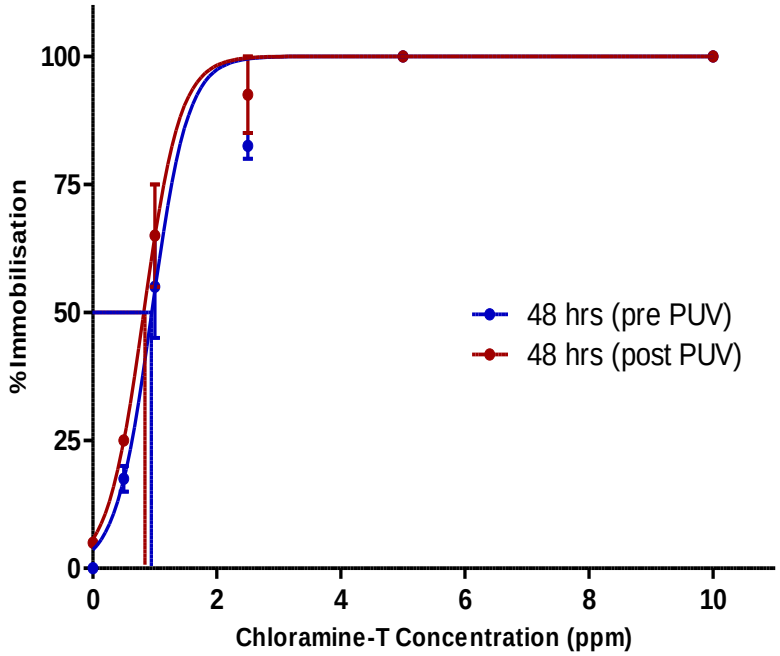

Figure 5.25 Dose response curve for *Daphnia magna* exposed to chloramine-T for 48 hrs pre and post PUV light exposure. **Pre PUV EC_{50} = 0.95 ppm** [95% C.I = 0.80 – 1.11]; **Post PUV EC_{50} = 0.82 ppm** [95% C.I = 0.72 – 0.92].

A one-way ANOVA identified that there was a significant difference between means of the data points for both the 24 hour and 48 hour tests ($p < 0.0001$). The Dunnett's test identified these differences to exist between the control and the 1.0 to 10.0 ppm treatment concentrations in both cases, making 0.5 ppm the NOEC concentration. An unpaired t-test revealed that there was no significant difference between the effects of chloramine-T on *D. magna* pre and post PUV exposure of the chemical for both the 24 and 48 hour tests, where p values of 0.7746 and 0.8272 were achieved, respectively.

5.8 Summary tables of ecotoxicological bioassays

The results and statistical analysis for each ecotoxicological bioassay are summarised in **Tables 5.4** to **5.6** including the relevant endpoint parameter for each assay.

Table 5.4 Summary of results and statistics for the algal growth inihibition assay for potassium dichromate, bronopol and chloramine-T using *Pseudokirchneriella subcapitata*.

Results Analysis	72 pre PUV	72 post PUV	96 pre PUV	96 post PUV
	Potassium dichromate			
E_rC_{50}	0.79 ppm	-	0.87 ppm	-
Confidence Intervals (95%)	0.66 – 0.91	-	0.69 – 1.05	-
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	-	Yes $p < 0.0001$	-
Control vs. Treatment Analysis: Dunnett's Test	0.1 – 10.0 ppm	-	0.5 – 10.0 ppm	-
	Bronopol			
E_rC_{50}	1.47 ppm	1.71 ppm	1.34 ppm	1.70 ppm
Confidence Intervals (95%)	1.16 – 1.78	1.42 – 1.99	1.12 – 1.56	1.59 – 1.81
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$
Control vs. Treatment Analysis: Dunnett's Test	0.5 – 10.0 ppm	1.0 – 10.0 ppm	1.0 – 10.0 ppm	1.0 – 10.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No $p = 0.902$		No $p = 0.888$	
	Chloramine-T			
E_rC_{50}	2.29 ppm	0.87 ppm	1.63 ppm	1.12 ppm
Confidence Intervals (95%)	2.05 – 2.53	0.74 – 1.00	1.34 – 1.92	0.94 – 1.31
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$
Control vs. Treatment Analysis: Dunnett's Test	1.0 – 10.0 ppm	0.1 – 5.0 ppm	1.0 – 10.0 ppm	0.1 – 5.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No $p = 0.490$		No $p = 0.720$	

Table 5.5 Summary of results and statistics for the *Artemia salina* crustacean bioassay for potassium dichromate, bronopol and chloramine-T.

Results Analysis	24 pre PUV	24 post PUV	48 pre PUV	48 post PUV
	Potassium dichromate			
LC_{50}	36.92 ppm	-	14.84 ppm	-
Confidence Intervals (95%)	30.54 – 43.30	-	14.10 – 15.59	-
Significant Difference: One-way ANOVA	Yes p < 0.0001	-	Yes p < 0.0001	-
Control vs. Treatment Analysis: Dunnett's Test	18.0 – 100.0 ppm	-	10.0 – 100.0 ppm	-
	Bronopol (Lab Artemia)			
LC_{50}	87.49 ppm	91.45 ppm	42.46 ppm	42.15 ppm
Confidence Intervals (95%)	79.86 – 95.13	79.60 – 103.30	37.03 – 47.90	30.50 – 53.80
Significant Difference: One-way ANOVA	Yes p < 0.0001	Yes p < 0.0001	Yes p < 0.0001	Yes p < 0.0001
Control vs. Treatment Analysis: Dunnett's Test	50.0 – 200.0 ppm	75.0 – 200.0 ppm	25.0 – 200.0 ppm	75.0 – 200.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No p = 0.940		No p = 0.999	
	Chloramine-T (Lab Artemia)			
LC_{50}	687.90 ppm	424.50 ppm	656.60 ppm	302.80 ppm
Confidence Intervals (95%)	674.00 – 701.90	384.30 – 464.70	628.20 – 685.10	258.30 – 347.40
Significant Difference: One-way ANOVA	Yes p < 0.0001	Yes p < 0.0001	Yes p < 0.0001	Yes p < 0.0001
Control vs. Treatment Analysis: Dunnett's Test	500 – 2500 ppm	250 – 2500 ppm	500 - 2500 ppm	250 - 2500 ppm
Significant Difference: T-test (pre vs. post PUV)	No p = 0.501		No p = 0.387	
	Bronopol (Boyle Artemia – Instar II-III)			
LC_{50}	90.74 ppm	135.40 ppm	37.72 ppm	90.88 ppm
Confidence Intervals (95%)	76.60 – 104.90	125.70 – 145.00	22.77 – 52.67	81.39 – 100.40
Significant Difference: One-way ANOVA	Yes p < 0.0001	Yes p = 0.0006	Yes p = 0.0005	Yes p < 0.0001
Control vs. Treatment Analysis: Dunnett's Test	75.0 – 150.0 ppm	125.0– 150.0 ppm	75.0 – 150.0 ppm	75.0 – 150.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No p = 0.177		No p = 0.101	
	Bronopol (Boyle Artemia – Instar I)			
LC_{50}	-	241.60	143.80 ppm	184.30 ppm
Confidence Intervals (95%)	-	144.00 – 339.30	122.90 – 164.60	156.70 – 212.00
Significant Difference: One-way ANOVA	No p = 0.3005	No p = 0.2952	Yes p = 0.0041	Yes p = 0.0003
Control vs. Treatment Analysis: Dunnett's Test	NS	NS	150.0 ppm	150.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No p = 0.584		No p = 0.288	

Table 5.6 Summary of results and statistics for the *Daphnia magna* inhibition of mobility assay for potassium dichromate, bronopol and chloramine-T.

Results Analysis	24 pre PUV	24 post PUV	48 pre PUV	48 post PUV
	Potassium dichromate			
EC_{50}	1.76 ppm	-	0.97 ppm	-
Confidence Intervals (95%)	1.36 – 2.16	-	0.76 – 1.18	-
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	-	Yes $p < 0.0001$	-
Control vs. Treatment Analysis: Dunnett's Test	1.0 - 5.0 ppm	-	0.5 – 5.0 ppm	-
	Bronopol			
EC_{50}	1.65 ppm	2.65 ppm	0.82 ppm	1.88 ppm
Confidence Intervals (95%)	1.55 – 1.76	2.37 – 2.93	0.77 – 0.88	0.98 – 2.79
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$
Control vs. Treatment Analysis: Dunnett's Test	0.5 – 3.0 ppm	1.0 – 3.0 ppm (excl. 2.0 ppm)	0.5 – 3.0 ppm	1.0 – 3.0 ppm (excl. 2.0 ppm)
Significant Difference: T-test (pre vs. post PUV)	No $p = 0.523$		No $p = 0.602$	
	Chloramine-T			
EC_{50}	3.14 ppm	2.18 ppm	0.95 ppm	0.82 ppm
Confidence Intervals (95%)	2.62 – 3.65	1.76 – 2.60	0.80 – 1.11	0.72 – 0.92
Significant Difference: One-way ANOVA	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$	Yes $p < 0.0001$
Control vs. Treatment Analysis: Dunnett's Test	1.0 – 10.0 ppm	1.0 – 10.0 ppm	1.0 – 10.0 ppm	1.0 – 10.0 ppm
Significant Difference: T-test (pre vs. post PUV)	No $p = 0.775$		No $p = 0.827$	

Based on the results achieved in each bioassay, the test organisms were less susceptible to chloramine-T, suggesting that bronopol is more toxic. This would explain why the latter is administered in lower concentrations than chloramine-T in aquaculture settings. Bronopol is usually administered in doses of 20 ppm and 50 ppm for fish and egg treatment, respectively; whereas chloramine-T is most commonly administered in a dose of 12 ppm (Marine Institute, 2017). The toxicity data established in these set of bioassays indicate that algae are more sensitive to antibacterial chemicals than crustaceans. This is in agreement with results established by Holten Lützhøft *et al*. (1999) who found that algae were indeed more sensitive to antibacterial agents than crustaceans and fish.

The results achieved in the present study indicate that, if the chemical disinfectants bronopol or chloramine-T were used in conjunction with PUV disinfection, there will most likely be minimal danger of a considerably significant increase in the toxicity of either chemical. This suggests that if chemical residues remain in rearing water before being discharged, PUV light can also be used to reduce the microbial load entering the receiving natural environment, without any major effects to the status of the rearing water. However, these results were obtained within the confinements of a laboratory and at a small-scale level.

It is evident, that while the toxicity of bronopol remained the same or slightly decreased following PUV light exposure, the toxicity of chloramine-T increased in each assay following PUV exposure. Although, the data sets pre and post PUV exposure may not be statistically significant enough to assume a change in chemical toxicity, this may have much more of an affect in an aquaculture site, where the volumes of water and chemicals used are much higher. The reason for this may be due to the formation of more toxic breakdown products than the parent. As chloramine-T is a chlorine-based product, the formation of THMs is a high possibility in an aquaculture pond as it is an organically rich matrix, which poses a serious threat to fish stock or wild stock in receiving waters. Another possible reason for the increase in toxicity of chloramine-T following PUV treatment is the increase in bioavailability as the product breaks down, allowing the test organisms to uptake more of the chemical. Therefore, in order to properly ascertain the effects of PUV on specific chemical disinfectants, an *in situ* study on a pilot aquaculture site is required. This would allow for a realistic environment, with the physicochemical and biological aspects of a fish farm included, as well as requisite chemical analysis. Nevertheless, based on the pilot experiments carried out, PUV light is safe to use in combination with the selected chemicals bronopol and chloramine-T. Their combined use has the potenital to enhance aquaculture disinfection measures or to manipulate the bacterial/phytoplankton populations for improved efficiency.

Chapter 6
Conclusion

Overarching Conclusions and Future Implications

This chapter presents a summary of overall findings from this project, how it impacts the aquaculture industry and where future development and investigation is required or favourable.

6.1 Main findings

Recognising the baseline levels (biological, physical and chemical) of an aquaculture system allows for rapid corrective actions to be undertaken if sudden deviations from the norm are observed. Problems can be immediately recognised, acted upon quickly and resolved, without any major loss of stocks and associated profits. This was the first study to utilise the Irish freshwater natural environment over a 10-month period as a baseline model for ecosystem monitoring. The population dynamics from an Irish pill-pond system were investigated from both a qualitative and quantitative perspective. In-depth knowledge of the biological community present and dynamics involved in the rearing water allows for manipulation of populations when necessary, i.e. in the case of increased nitrification requirements.

Sophisticated technologies such as FCM and DGGE PCR were used to determine biological profiles of freshwater aquaculture rearing water alongside correlations with *in situ* methods in the form of the AlgaeTorch. This constitutes the first study that reported good agreement between the use of real-time laboratory-based techniques and *in situ* monitoring technologies for enumerating phytoplankton and bacterial communities. It is envisaged that use of these combinational approaches will aid the future development of aquaculture processes. During the monitoring period, there was heavy snow and several months of drought, leading to a hosepipe ban, which introduced the impact of unusual meteorological conditions on the ecosystem dynamic.

Small aquaculture farms and companies in Ireland do not have the financial resources to employ highly intensive energy systems for production, which are also often damaging to the environment. This study was the first to focus on novel, green

innovations in the form of PUV light for disease mitigation to help progress the Irish freshwater aquaculture industry.

Microscopic analysis determined the dominance of Chlorophyta, Bacillariophyta and Cryptophyta for the phytoplankton community. The unusual weather conditions that occurred during the monitoring period resulted in a variation in phytoplankton species form month to month reflected in the results. The parameters that had the most profound influence on phytoplankton growth/community diversity were temperature, nitrates and bacterial numbers.

The most relevant bacterial communities observed from the DGGE analysis carried out were Proteobacteria, Cyanobacteria and unclassified bacteria. Similarly to the phytoplankton communities, the bacterial diversity changed monthly, with June being the most changed, based on DGGE gel banding patterns. There was a microbial balance between the FP and the TP, and the fish remained healthy over the course of the study. This was possibly due to the presence of k-selected bacteria that remained constant in the rearing water but at levels low enough to avoid overgrowth and potential disease breakouts.

Although there were no records of *Flavobacterium* related mortalities or cyanobacterial blooms, the identification of *Flavobacterium* species and Cyanobacteria from the sequencing analysis may be a cause for concern, as both bacteria may pose a potential threat in the future. For this reason, the use of specific diagnostic probes for the detection of each could be implemented to recognise and resolve any problems before they arise.

A. salmonicida has previously been detected in fish gill samples from mortalities that occurred in the aquaculture site. Bands that corresponded to *A. salmonicida* 16S rDNA in the DGGE gels were sequenced but no match with *Aeromonas* species was observed. This may indicate that the DGGE method with ubiquitous 16S rDNA analysis for bacterial profiling and identification is not a suitable method for the detection of *Aeromonas* species. Use of a more specific *A. salmonicida* virulence gene with DGGE or detection methods such as real-time PCR of pathogenic determinants

or metagenomic analysis with min-ion technology may provide a better means for rapid detection of pathogenic species.

6.1 Future implications

Identification of the most influential biological species provides the opportunity of transplantation of specific microbial assemblages when required for certain processes, i.e. the addition of a specific bacteria for nitrification or the fertilisation of a specific phytoplankton species for nutrient removal or oxygen supplementation during the daytime. However, for this to be possible and beneficial, the function of each species needs to be determined.

With increasing temperatures due to global warming, there is every possibility that harmful bacterial and phytoplankton species from warmer climates that thrive on higher temperatures will encroach in the freshwater systems in Ireland, leading to cyanobacterial blooms and eutrophication. Pathogenic bacteria also tend to replicate more rapidly when the weather is warmer. The water becomes shallower which can lead to disease outbreaks resulting in severe mortalities and financial loss. That makes it even more important to monitor and try to manipulate the biological community in the rearing water of aquaculture systems for the prevention of unwanted toxicants and disease breakouts.

The development of a diagnostic system based on the microbiome within the water would be a useful indicator for the presence of toxic phytoplankton or pathogenic bacteria. A metagenomics approach to diagnostic kits can provide identification of all pathogens present. The use of min-ion sequencing of full-length 16S rDNA could provide a more accurate and in-depth picture of microbial diversity.

Different phytoplankton species can aid in the optimization and sustainability of freshwater aquaculture practices in Ireland and elsewhere, with their uses ranging from increasing oxygen production, a sink for CO_2 and a food source for higher organisms and in turn fish, which can recycle money back into the industry. However, based on growing attention and research, and the small-scale study carried out in this project, specifically the dominating populations, the use of phytoplankton as a

natural method of nutrient removal has massive potential for water treatment in aquaculture systems, thereby enhancing the water quality without chemical treatment.

FCM serves as a potential tool for bio-monitoring of phytoplankton through cell sorting of phytoplankton into different target groups with separate specific culture media for growth promotion. This sorting process may serve as a method of isolating and identifying toxic species in order to determine the risk and implement preventative measures. Sorting of phytoplankton cells may also be used for growth and upscaling of phytoplankton cultures for use of species as a natural means of oxygen, a CO_2 sink and a food source for human nutraceuticals as well as fish, which can be input back into the aquaculture process and increase sustainability in this industry. Additionally, FCM can be used in a more in-depth approach where a range of extensive information from certain samples can be obtained e.g. target nucleic acids. Fluorochrome-labelled nucleic acid probes can target the rRNA region of specific taxa and so different phytoplankton in samples can be easily identified.

The ecosystem data obtained in this study would be very useful in the situation of disinfection by PUV light in times of disease breakouts. However, as both beneficial and pathogenic species are present in the rearing water, there are many parameters to consider optimising for appropriate use of the PUV light system to maintain a balanced ecosystem dynamic. The photoreactivation process has the potential to cause unexpected disease outbreaks in fish farms and so the effect of PUV light on the decreased ability of microorganisms to undergo photoreactivation is a potential area of investigation for the future research. Real-time PCR techniques provide an opportunity to investigate, at a molecular level, the real capability of photoreactivation of microorganisms once exposed to PUV light.

There is pressing need to use this knowledge to inform a reliable and repeatable process for low cost wastewater remediation and recirculation using resident phytoplankton and bacterial communities that exploit organic principles. In conclusion, this was a cross cutting project across multiple disciplines, with the data obtained acting as a repository of information focused on future sustainability to inform the freshwater aquaculture industry in Ireland.

Chapter 7

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Appendix A

Phylogenetic Trees For Bacterial Analysis – Neighbor Joining Format

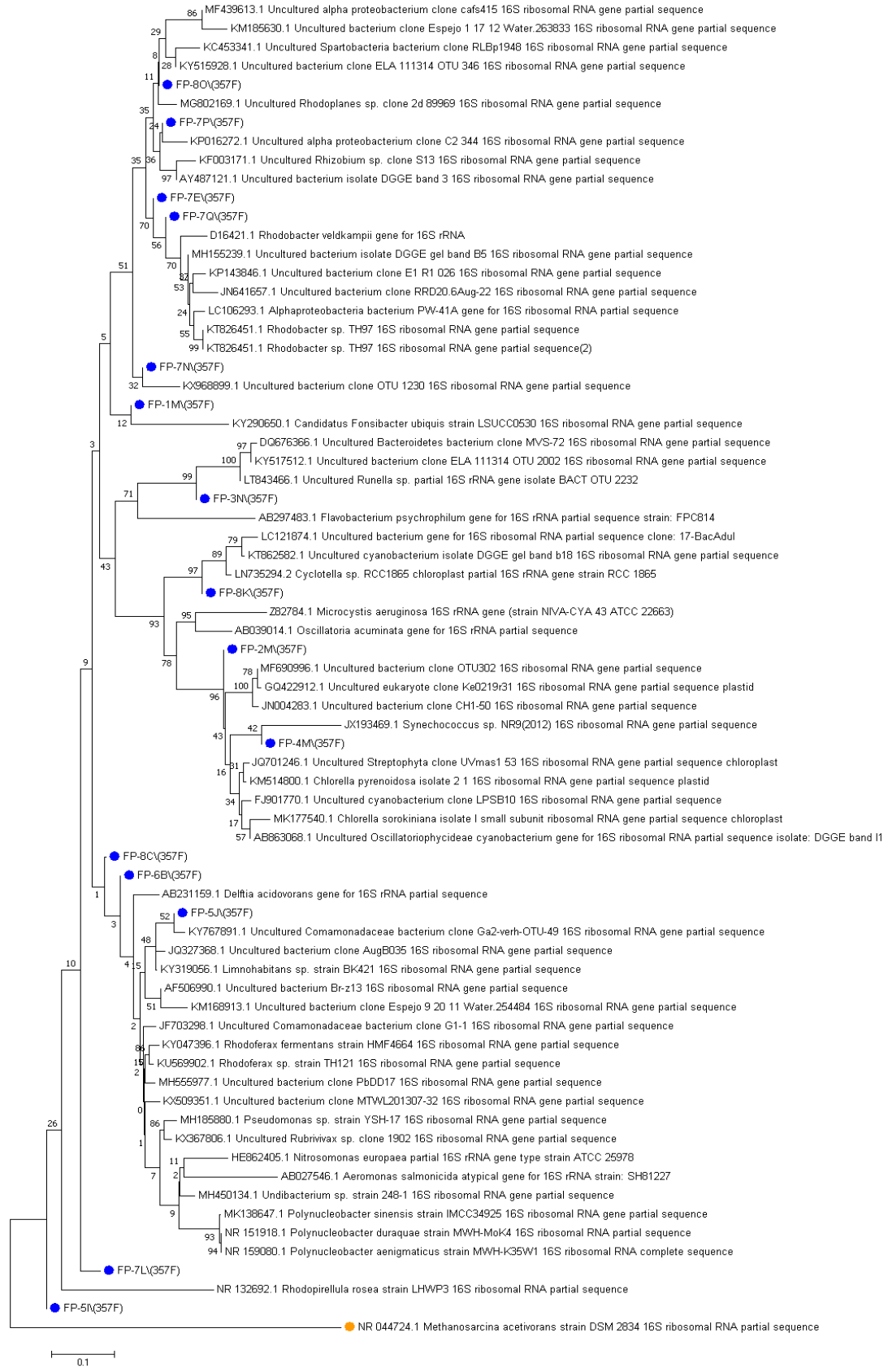


Figure A1 Phylogenetic tree of bacterial sequences for the FP from March to June using neighbor joining analysis.

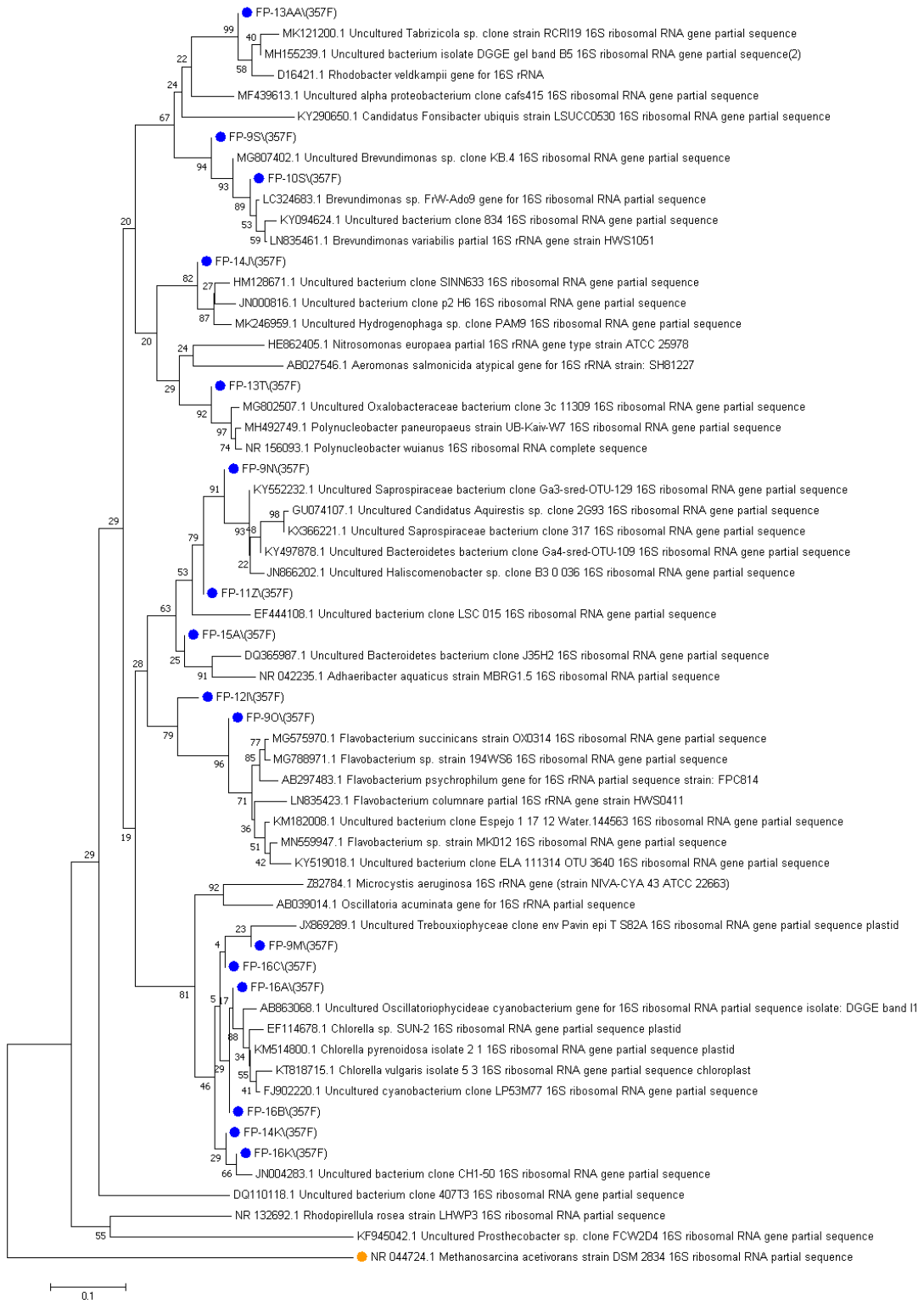


Figure A2 Phylogenetic tree of bacterial sequences for the FP from July to October using neighbor joining analysis.

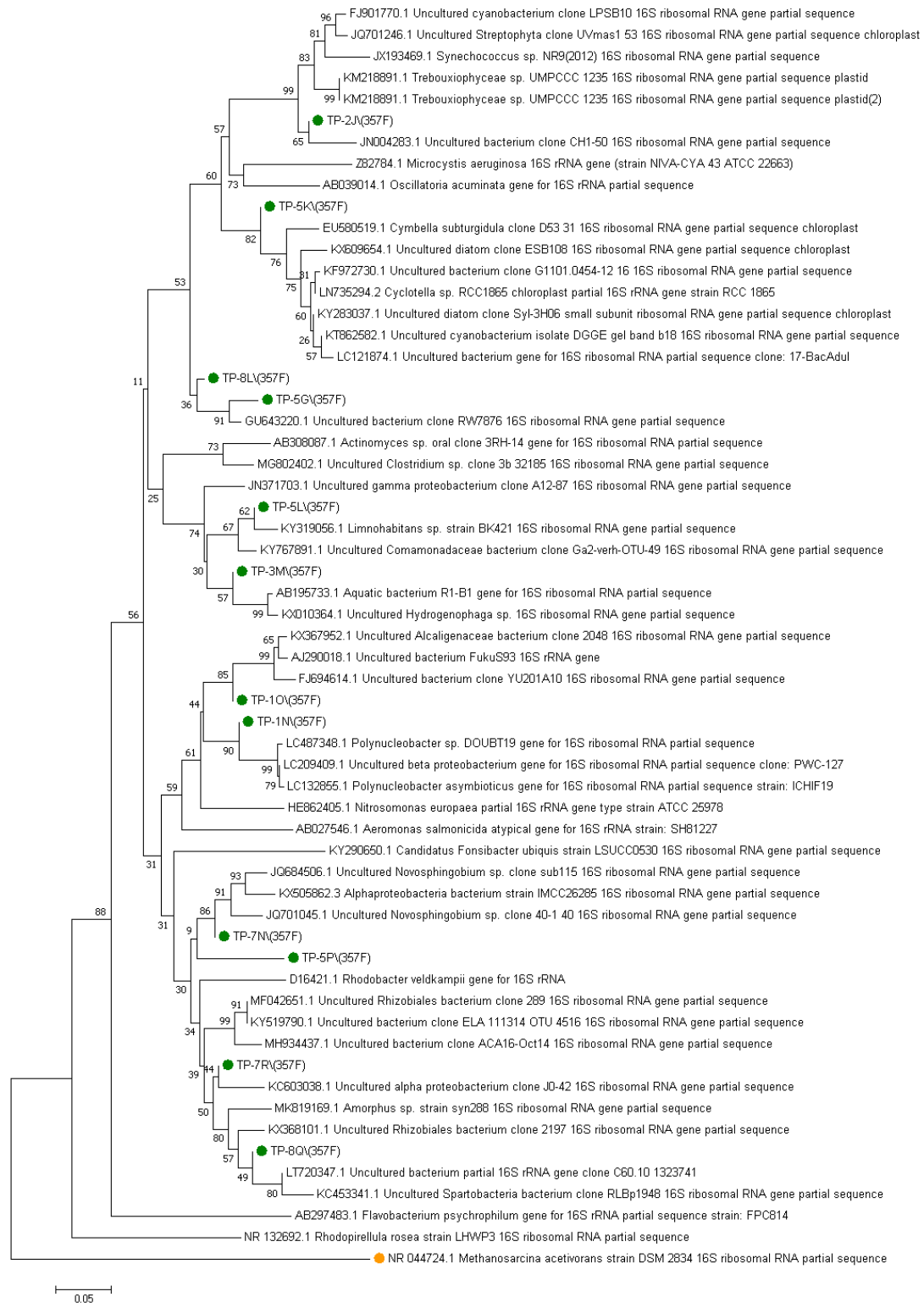


Figure A3 Phylogenetic tree of bacterial sequences for the TP from March to June using neighbor joining analysis.

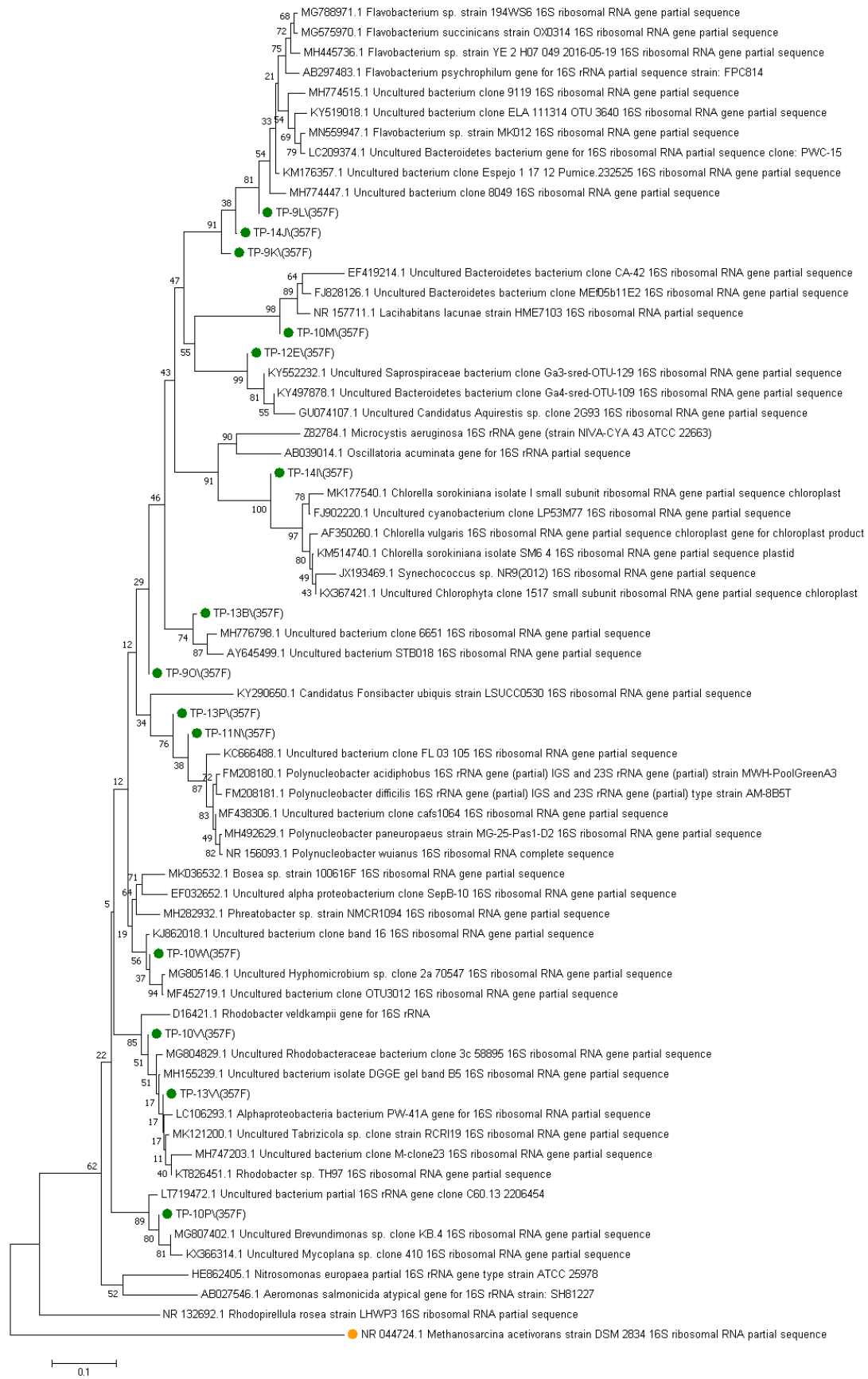
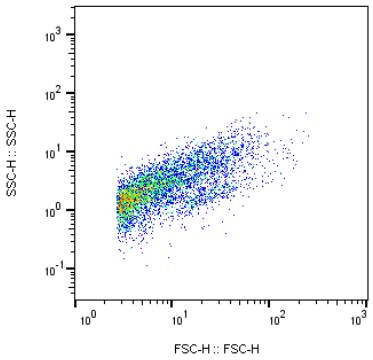


Figure A4 Phylogenetic tree of bacterial sequences for the TP from July to October using neighbor joining analysis.

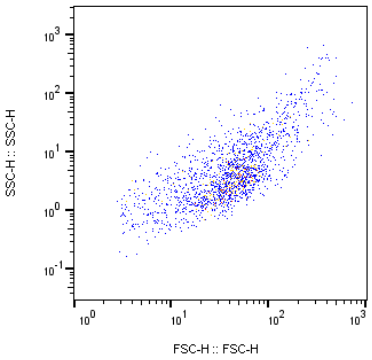
Phytoplankton Enumeration Data – Flow Cytometry Cytograms

The cytograms presented illustrate a representative graph for the desired phytoplankton population, the cyanobacterial population and triplicate phytoplankton counts in the form of a histogram, for each sample analysed from KWF; FP = Fish Pond, TP = Treatment Pond.

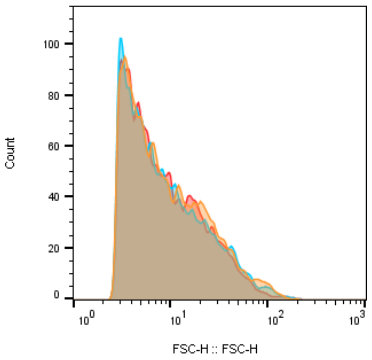
FP 1



FP 1
Chlorophyll Pos, Phycocyanin Neg

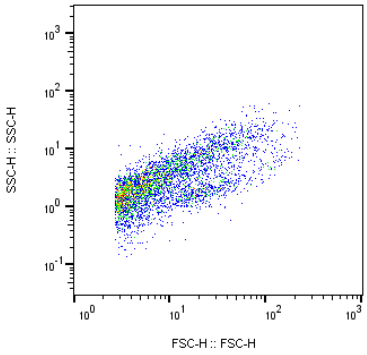


FP 1
Phycocyanin Pos

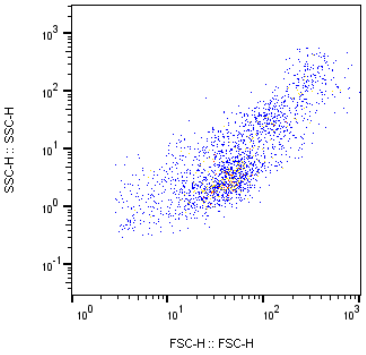


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5709
Chlorophyll Pos, Phycocyanin Neg	5518
Chlorophyll Pos, Phycocyanin Neg	5472

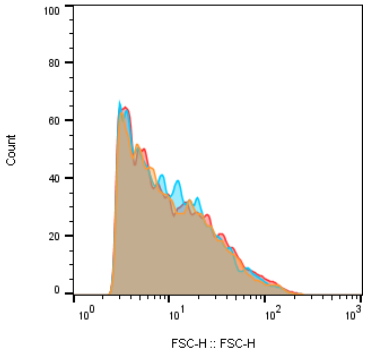
TP 1



TP 1
Chlorophyll Pos, Phycocyanin Neg

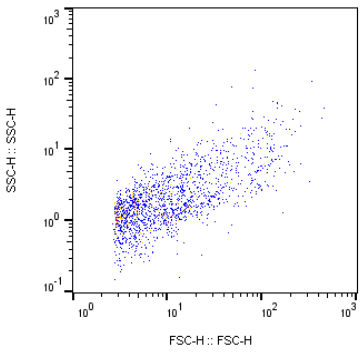


TP 1
Phycocyanin Pos

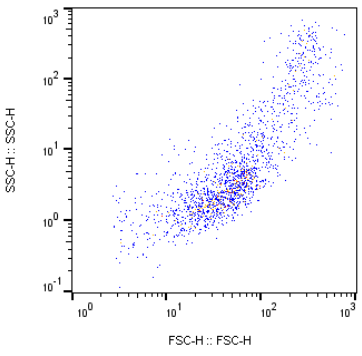


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4138
Chlorophyll Pos, Phycocyanin Neg	4339
Chlorophyll Pos, Phycocyanin Neg	4326

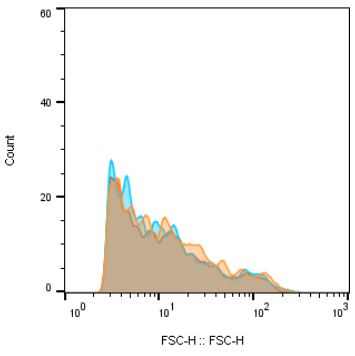
FP 2



FP 2
Chlorophyll Pos, Phycocyanin Neg

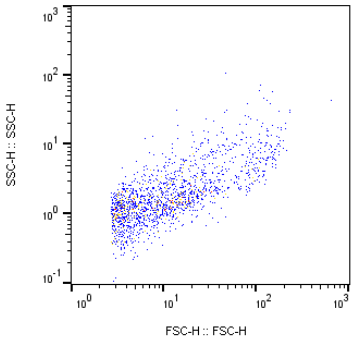


FP 2
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1718
Chlorophyll Pos, Phycocyanin Neg	1685
Chlorophyll Pos, Phycocyanin Neg	1528

TP 2



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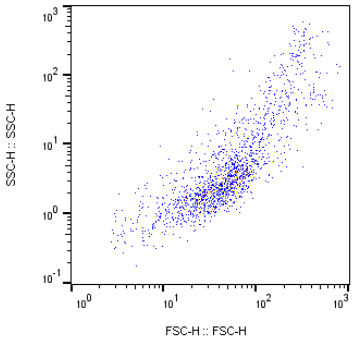
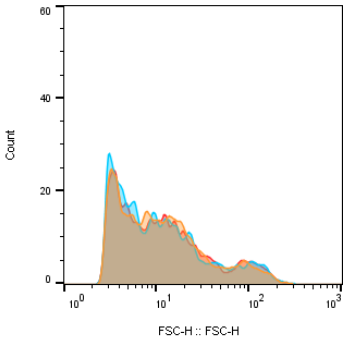
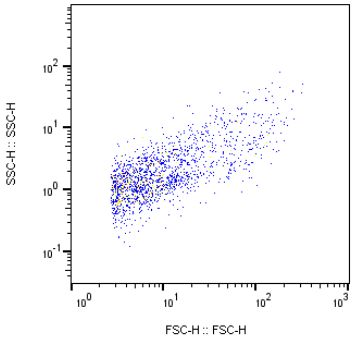


TP 2
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1667
Chlorophyll Pos, Phycocyanin Neg	1699
Chlorophyll Pos, Phycocyanin Neg	1634

FP 3



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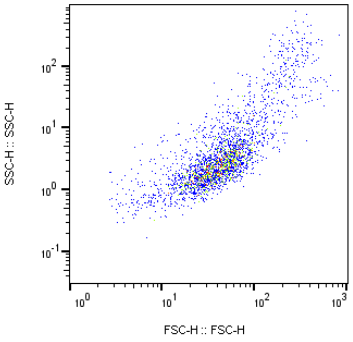
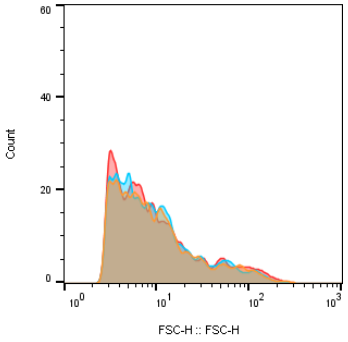
Chlorophyll Pos, Phycocyanin Neg

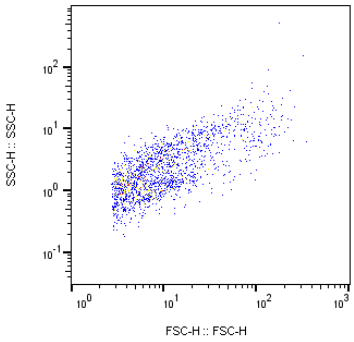


FP 3
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1598
Chlorophyll Pos, Phycocyanin Neg	1679
Chlorophyll Pos, Phycocyanin Neg	1722

TP 3



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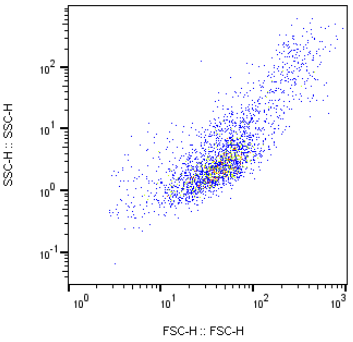
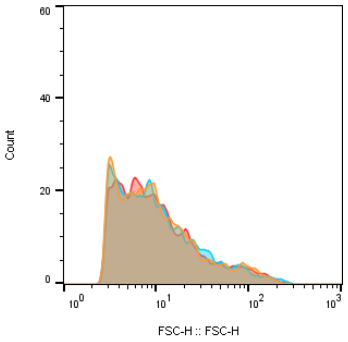
Chlorophyll Pos, Phycocyanin Neg

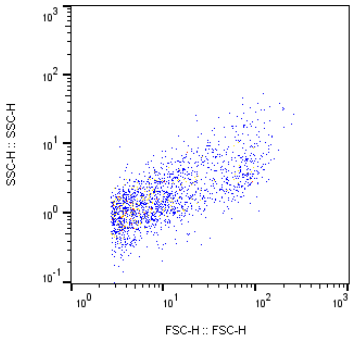


TP 3
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1863
Chlorophyll Pos, Phycocyanin Neg	1848
Chlorophyll Pos, Phycocyanin Neg	1822

FP 4



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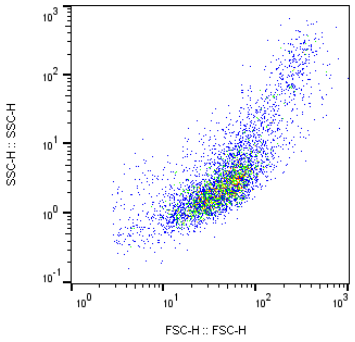
Chlorophyll Pos, Phycocyanin Neg



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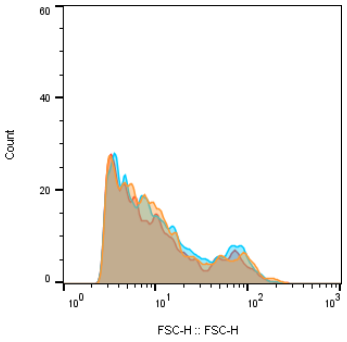
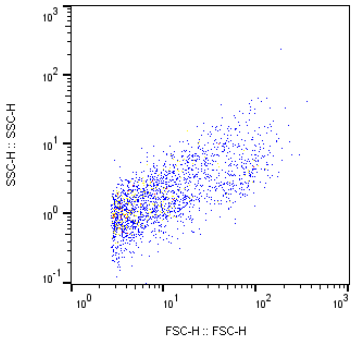
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1787
Chlorophyll Pos, Phycocyanin Neg	1826
Chlorophyll Pos, Phycocyanin Neg	1617

TP 4



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Chlorophyll Pos, Phycocyanin Neg
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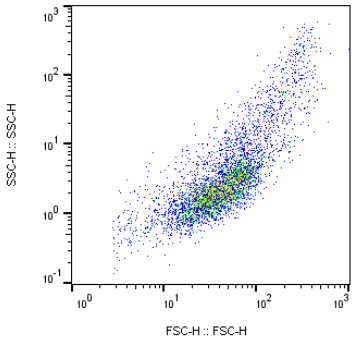
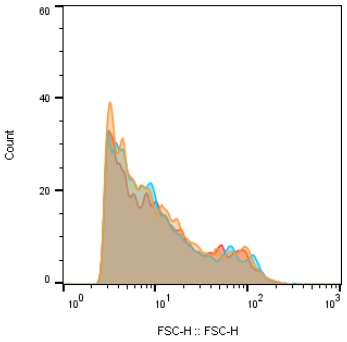
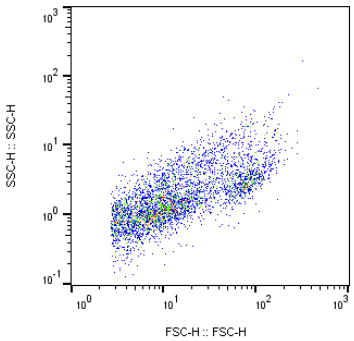


TP 4
Phycocyanin Pos
Count = 4640

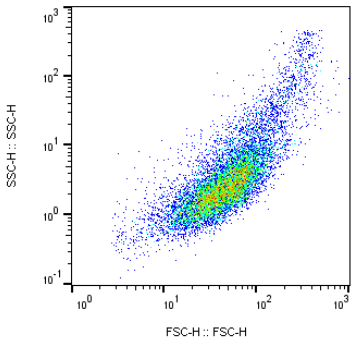


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2247
Chlorophyll Pos, Phycocyanin Neg	2129
Chlorophyll Pos, Phycocyanin Neg	2031

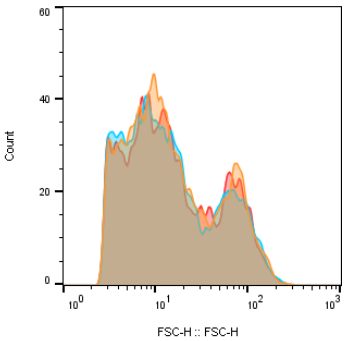
FP 5



FP 5
Chlorophyll Pos, Phycocyanin Neg

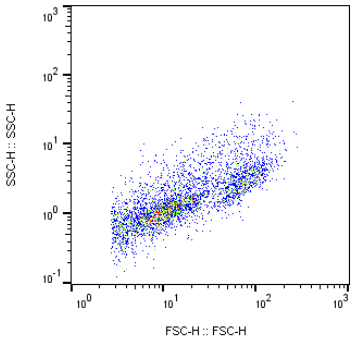


FP 5
Phycocyanin Pos

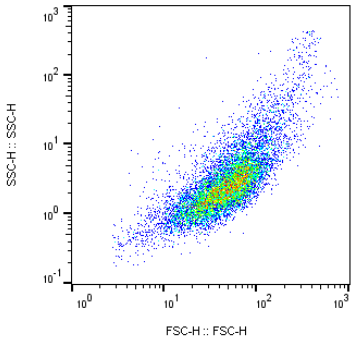


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4008
Chlorophyll Pos, Phycocyanin Neg	3853
Chlorophyll Pos, Phycocyanin Neg	3814

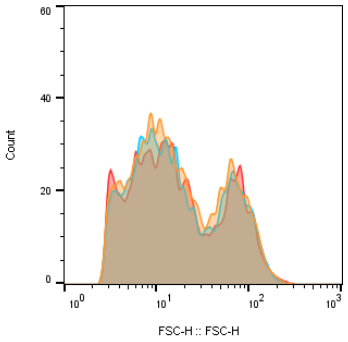
TP 5



TP 5
Chlorophyll Pos, Phycocyanin Neg

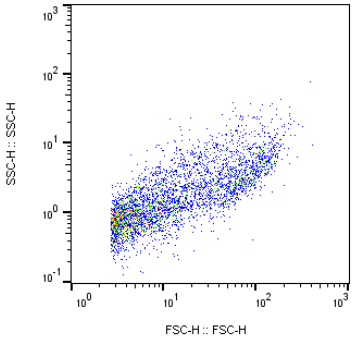


TP 5
Phycocyanin Pos

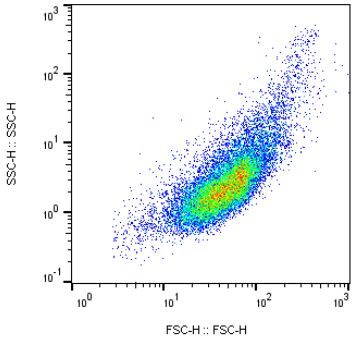


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3485
Chlorophyll Pos, Phycocyanin Neg	3226
Chlorophyll Pos, Phycocyanin Neg	3164

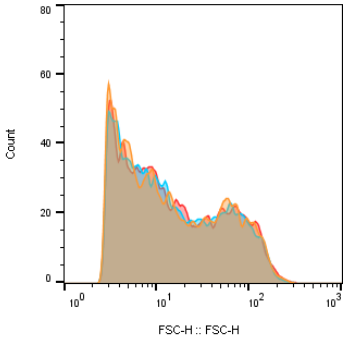
FP 6



FP 6
Chlorophyll Pos, Phycocyanin Neg

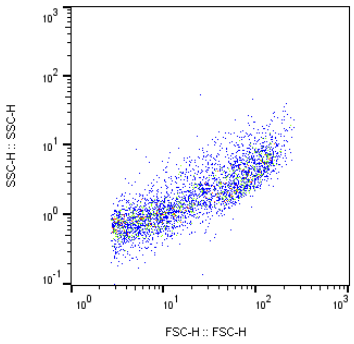


FP 6
Phycocyanin Pos



Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4075
Chlorophyll Pos, Phycocyanin Neg	4006
Chlorophyll Pos, Phycocyanin Neg	4031

TP 6



TP 6
Chlorophyll Pos, Phycocyanin Neg
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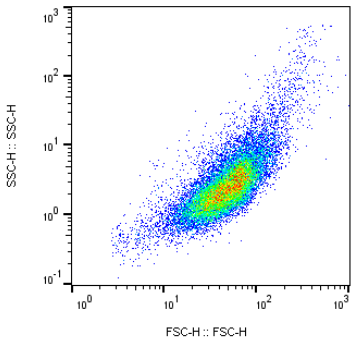

TP 6
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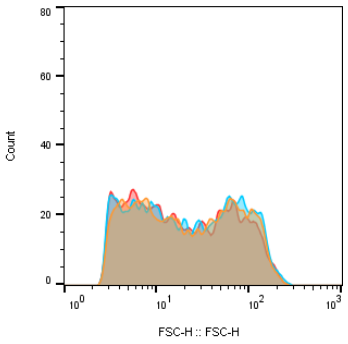
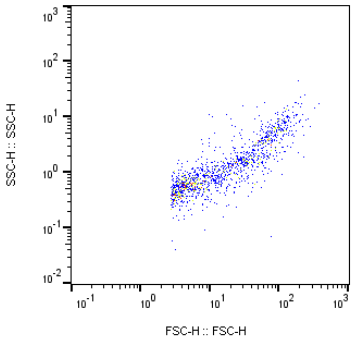
Count = 19179

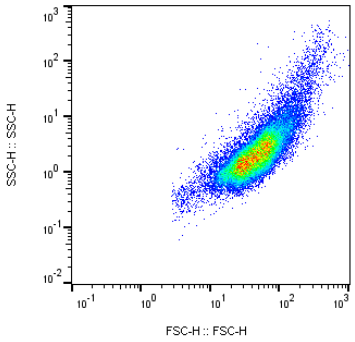


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3213
Chlorophyll Pos, Phycocyanin Neg	3357
Chlorophyll Pos, Phycocyanin Neg	3237

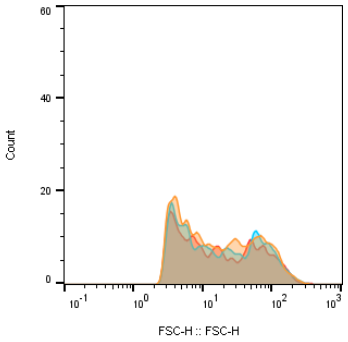
FP 7



FP 7
Chlorophyll Pos, Phycocyanin Neg

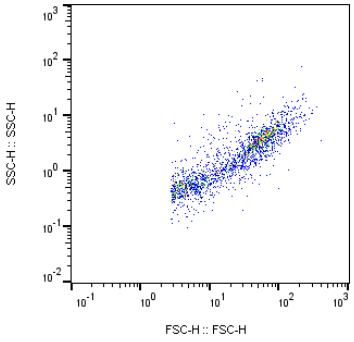


FP 7
Phycocyanin Pos

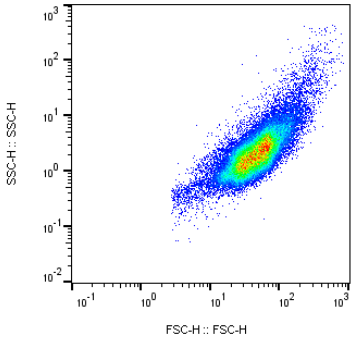


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1240
Chlorophyll Pos, Phycocyanin Neg	1078
Chlorophyll Pos, Phycocyanin Neg	982

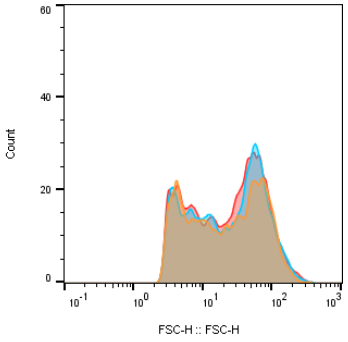
TP 7



TP 7
Chlorophyll Pos, Phycocyanin Neg

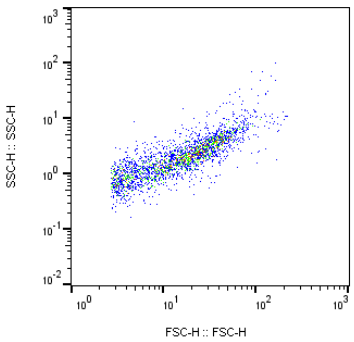


TP 7
Phycocyanin Pos

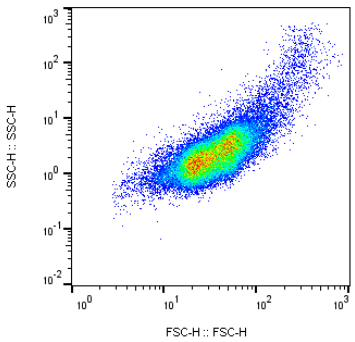


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1799
Chlorophyll Pos, Phycocyanin Neg	1960
Chlorophyll Pos, Phycocyanin Neg	2082

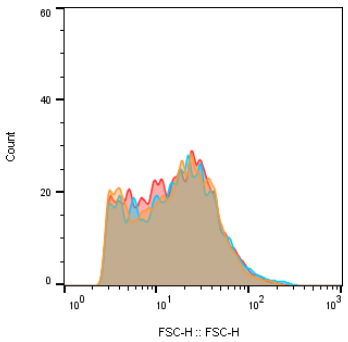
FP 8



FP 8
Chlorophyll Pos, Phycocyanin Neg

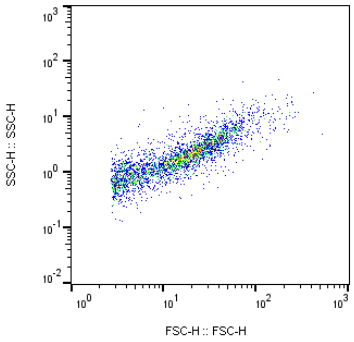


FP 8
Phycocyanin Pos

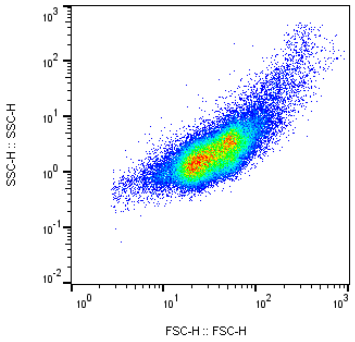


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2477
Chlorophyll Pos, Phycocyanin Neg	2453
Chlorophyll Pos, Phycocyanin Neg	2621

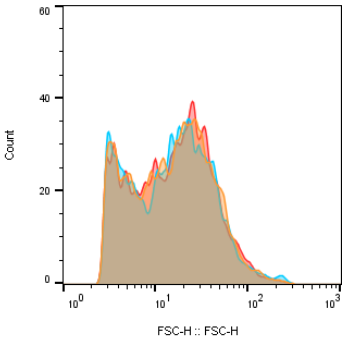
TP 8



TP 8
Chlorophyll Pos, Phycocyanin Neg

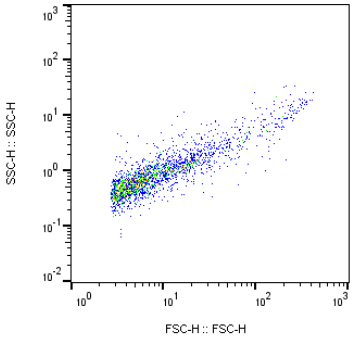


TP 8
Phycocyanin Pos

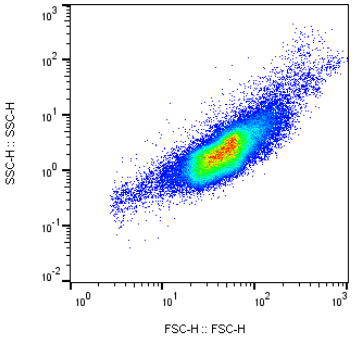


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3350
Chlorophyll Pos, Phycocyanin Neg	3255
Chlorophyll Pos, Phycocyanin Neg	3311

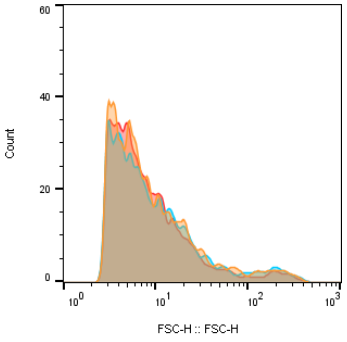
FP 9



FP 9
Chlorophyll Pos, Phycocyanin Neg

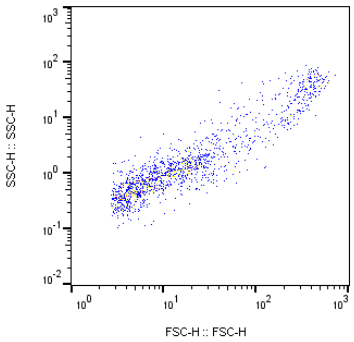


FP 9
Phycocyanin Pos

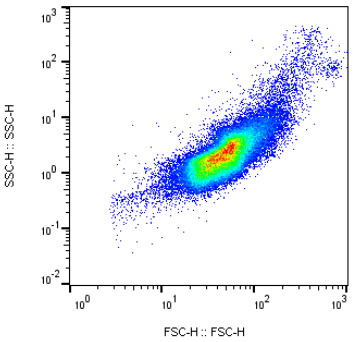


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2307
Chlorophyll Pos, Phycocyanin Neg	2149
Chlorophyll Pos, Phycocyanin Neg	2163

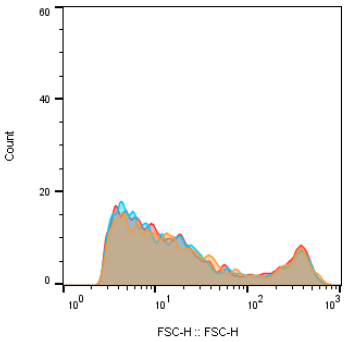
TP 9



TP 9
Chlorophyll Pos, Phycocyanin Neg

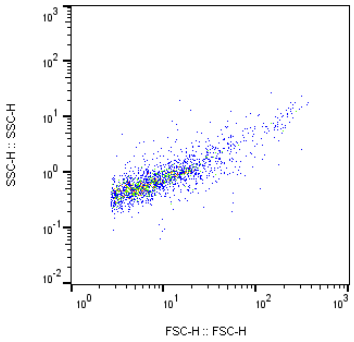


TP 9
Phycocyanin Pos

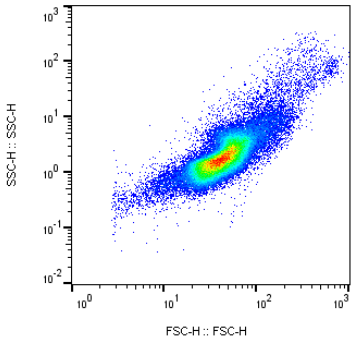


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1479
Chlorophyll Pos, Phycocyanin Neg	1537
Chlorophyll Pos, Phycocyanin Neg	1573

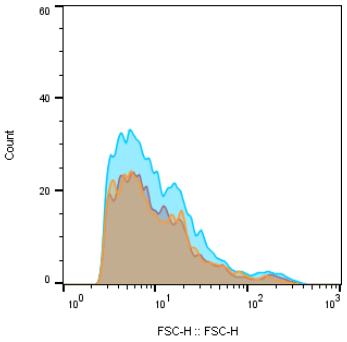
FP 10



FP 10
Chlorophyll Pos, Phycocyanin Neg

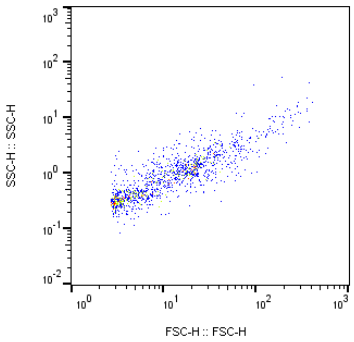


FP 10
Phycocyanin Pos

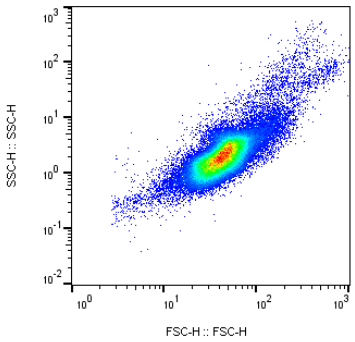


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1788
Chlorophyll Pos, Phycocyanin Neg	2587
Chlorophyll Pos, Phycocyanin Neg	1800

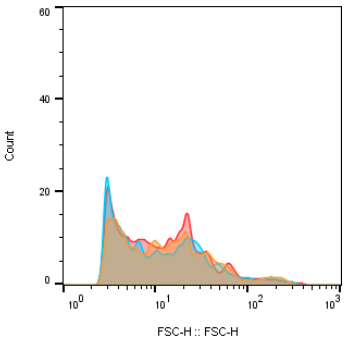
TP 10



TP 10
Chlorophyll Pos, Phycocyanin Neg

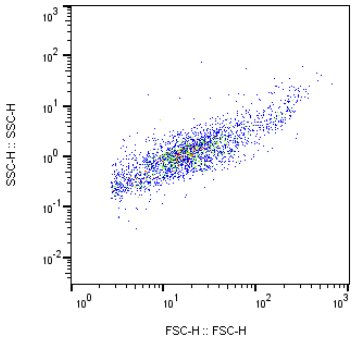


TP 10
Phycocyanin Pos

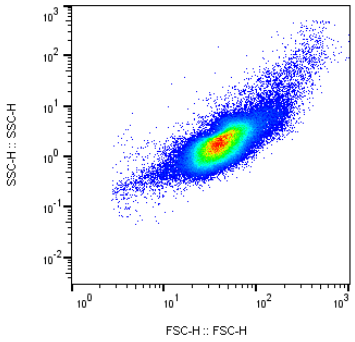


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1151
Chlorophyll Pos, Phycocyanin Neg	1167
Chlorophyll Pos, Phycocyanin Neg	1287

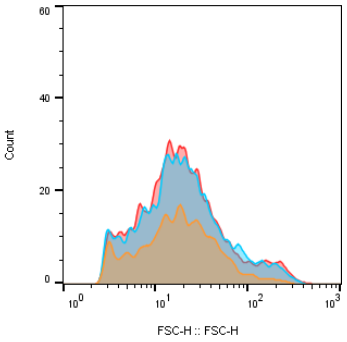
FP 11



FP 11
Chlorophyll Pos, Phycocyanin Neg

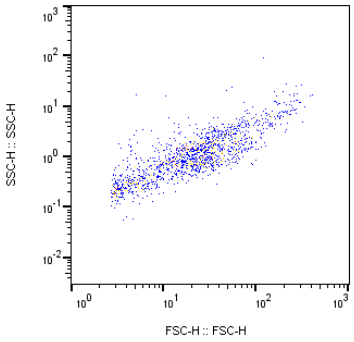


FP 11
Phycocyanin Pos

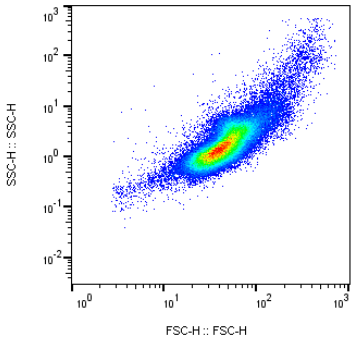


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1345
Chlorophyll Pos, Phycocyanin Neg	2408
Chlorophyll Pos, Phycocyanin Neg	2617

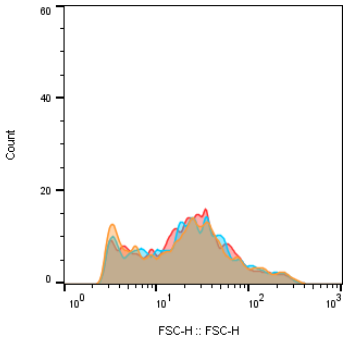
TP 11



TP 11
Chlorophyll Pos, Phycocyanin Neg

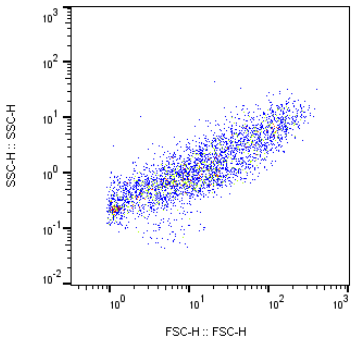


TP 11
Phycocyanin Pos

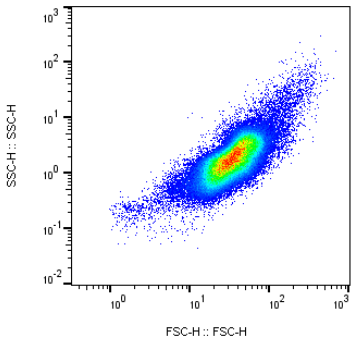


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1342
Chlorophyll Pos, Phycocyanin Neg	1350
Chlorophyll Pos, Phycocyanin Neg	1388

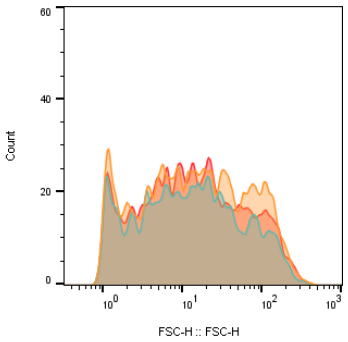
FP 12



FP 12
Chlorophyll Pos, Phycocyanin Neg

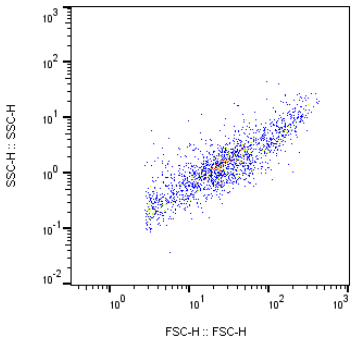


FP 12
Phycocyanin Pos

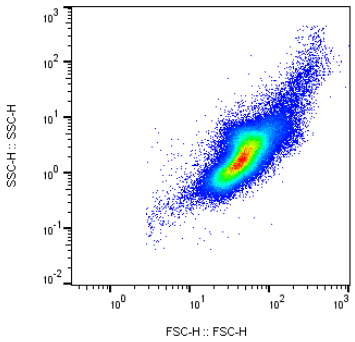


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3751
Chlorophyll Pos, Phycocyanin Neg	2963
Chlorophyll Pos, Phycocyanin Neg	3392

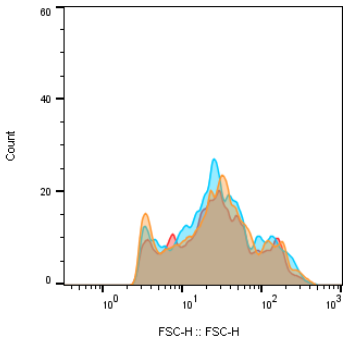
TP 12



TP 12
Chlorophyll Pos, Phycocyanin Neg

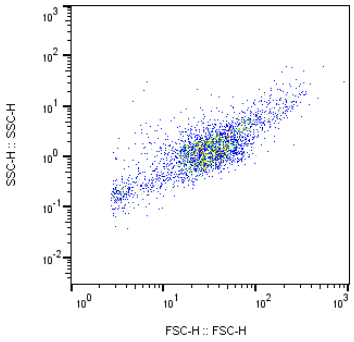


TP 12
Phycocyanin Pos

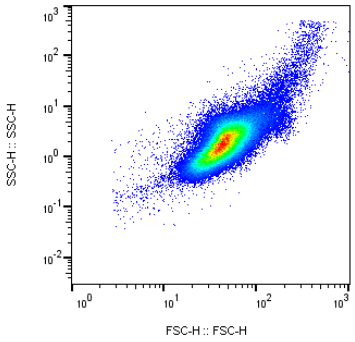


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1833
Chlorophyll Pos, Phycocyanin Neg	1967
Chlorophyll Pos, Phycocyanin Neg	1622

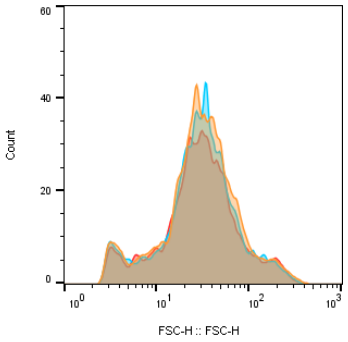
FP 13



FP 13
Chlorophyll Pos, Phycocyanin Neg

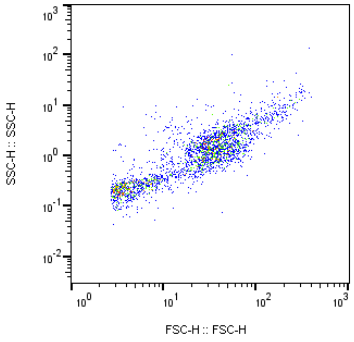


FP 13
Phycocyanin Pos

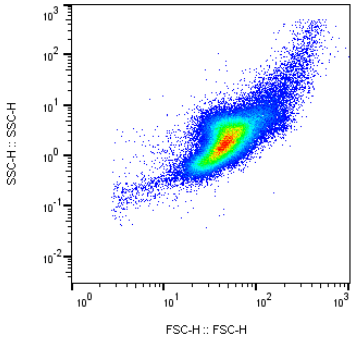


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2689
Chlorophyll Pos, Phycocyanin Neg	2501
Chlorophyll Pos, Phycocyanin Neg	2302

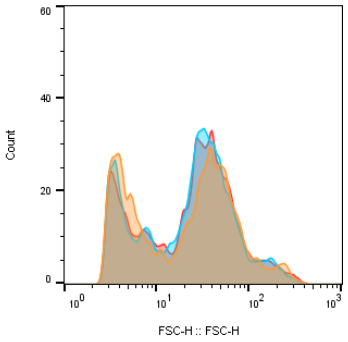
TP 13



TP 13
Chlorophyll Pos, Phycocyanin Neg

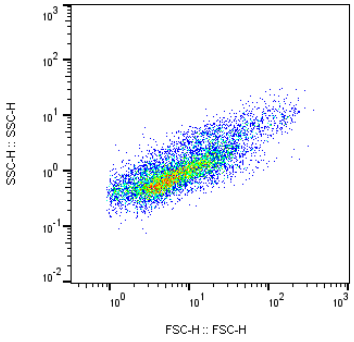


TP 13
Phycocyanin Pos

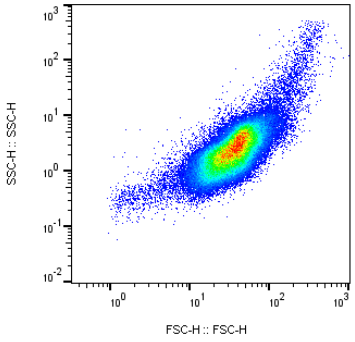


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2487
Chlorophyll Pos, Phycocyanin Neg	2591
Chlorophyll Pos, Phycocyanin Neg	2548

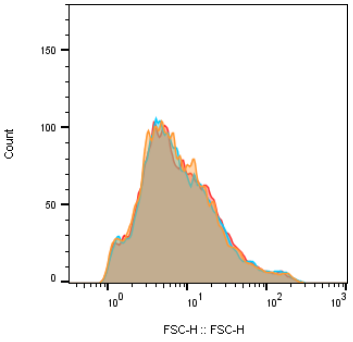
FP 14



FP 14
Chlorophyll Pos, Phycocyanin Neg

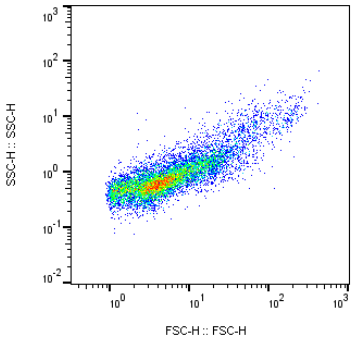


FP 14
Phycocyanin Pos

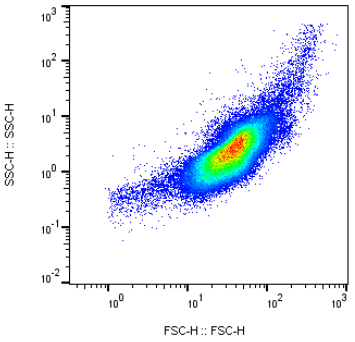


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8095
Chlorophyll Pos, Phycocyanin Neg	7804
Chlorophyll Pos, Phycocyanin Neg	7915

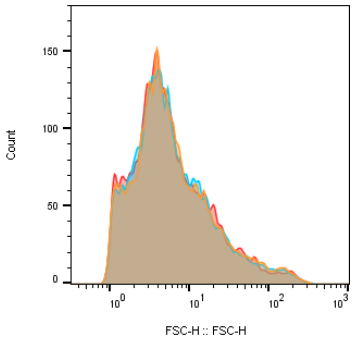
TP 14



TP 14
Chlorophyll Pos, Phycocyanin Neg

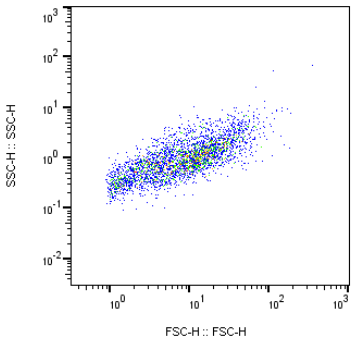


TP 14
Phycocyanin Pos

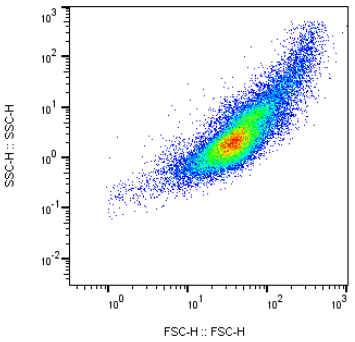


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	9822
Chlorophyll Pos, Phycocyanin Neg	9821
Chlorophyll Pos, Phycocyanin Neg	9979

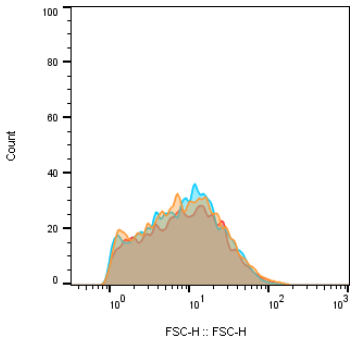
FP 15



FP 15
Chlorophyll Pos, Phycocyanin Neg

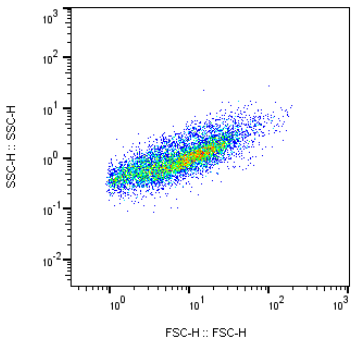


FP 15
Phycocyanin Pos

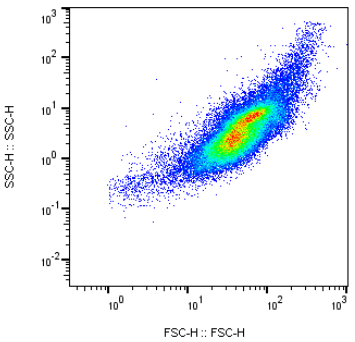


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3108
Chlorophyll Pos, Phycocyanin Neg	3074
Chlorophyll Pos, Phycocyanin Neg	2708

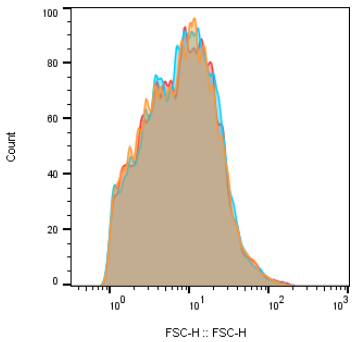
TP 15



TP 15
Chlorophyll Pos, Phycocyanin Neg

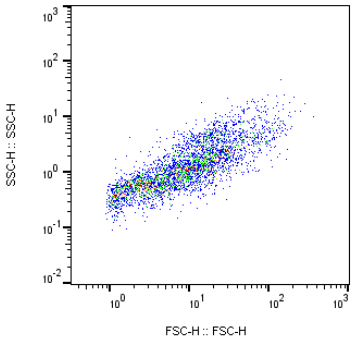


TP 15
Phycocyanin Pos

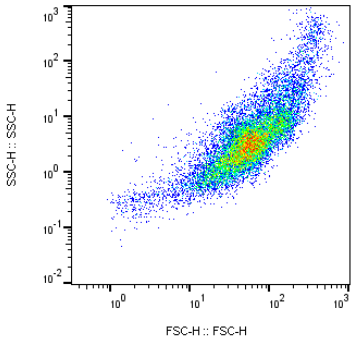


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	7923
Chlorophyll Pos, Phycocyanin Neg	8071
Chlorophyll Pos, Phycocyanin Neg	7873

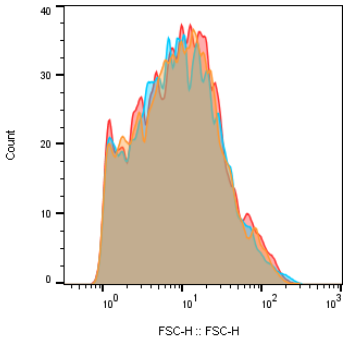
FP 16



FP 16
Chlorophyll Pos, Phycocyanin Neg

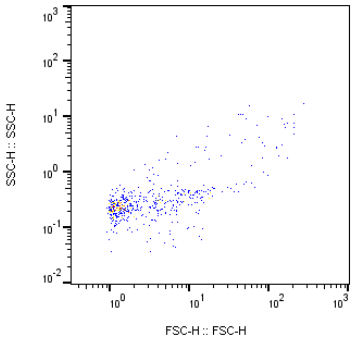


FP 16
Phycocyanin Pos

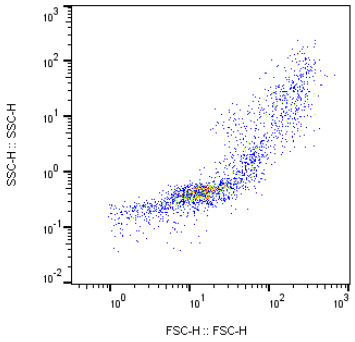


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3583
Chlorophyll Pos, Phycocyanin Neg	3555
Chlorophyll Pos, Phycocyanin Neg	3739

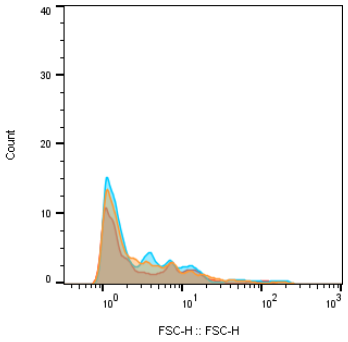
TP 16



TP 16
Chlorophyll Pos, Phycocyanin Neg

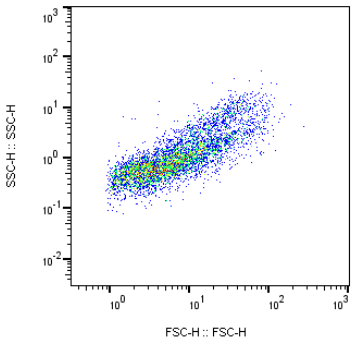


TP 15
Phycocyanin Pos

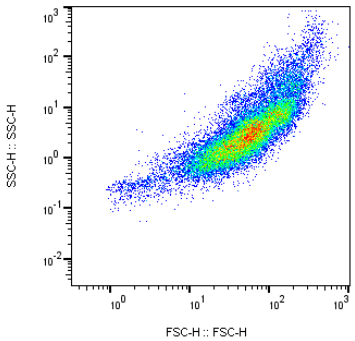


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	485
Chlorophyll Pos, Phycocyanin Neg	542
Chlorophyll Pos, Phycocyanin Neg	384

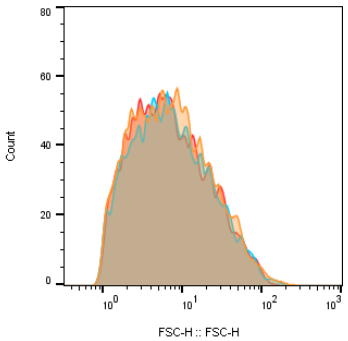
FP 17



FP 17
Chlorophyll Pos, Phycocyanin Neg

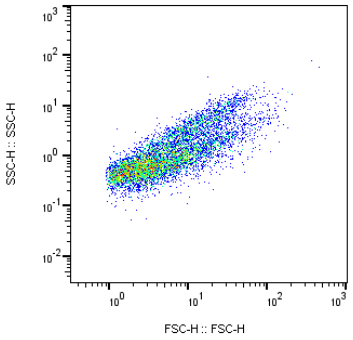


FP 17
Phycocyanin Pos

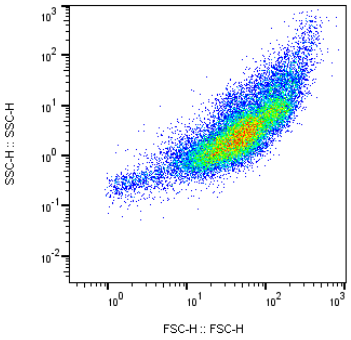


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5504
Chlorophyll Pos, Phycocyanin Neg	5074
Chlorophyll Pos, Phycocyanin Neg	5230

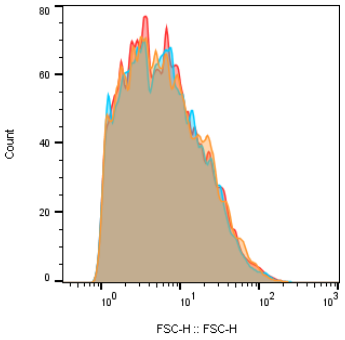
TP 17



TP 17
Chlorophyll Pos, Phycocyanin Neg

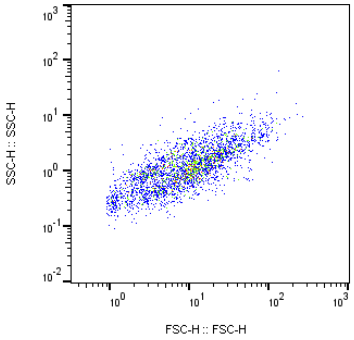


TP 17
Phycocyanin Pos

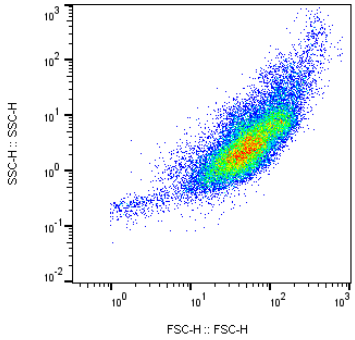


	Subset Name	Count
	Chlorophyll Pos, Phycocyanin Neg	6722
	Chlorophyll Pos, Phycocyanin Neg	6584
	Chlorophyll Pos, Phycocyanin Neg	6795

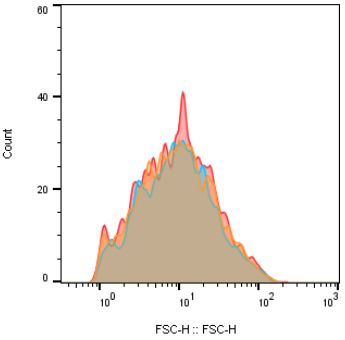
FP 18



FP 18
Chlorophyll Pos, Phycocyanin Neg

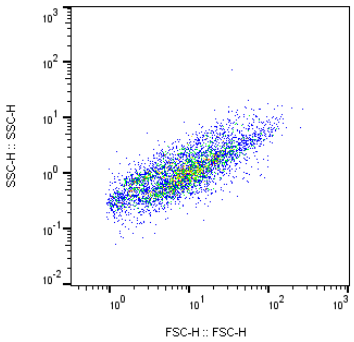


FP 18
Phycocyanin Pos

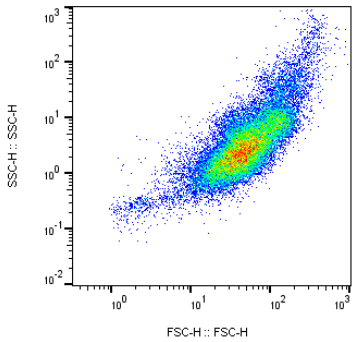


	Subset Name	Count
	Chlorophyll Pos, Phycocyanin Neg	2767
	Chlorophyll Pos, Phycocyanin Neg	2593
	Chlorophyll Pos, Phycocyanin Neg	2982

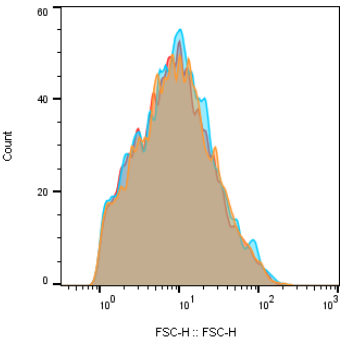
TP 18



TP 18
Chlorophyll Pos, Phycocyanin Neg

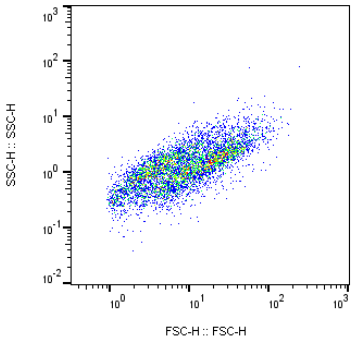


TP 18
Phycocyanin Pos

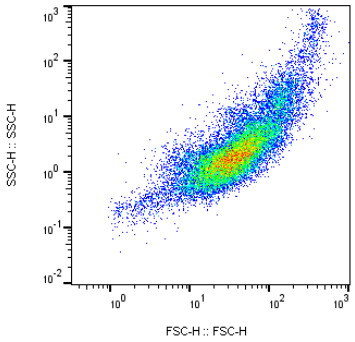


	Subset Name	Count
	Chlorophyll Pos, Phycocyanin Neg	4377
	Chlorophyll Pos, Phycocyanin Neg	4625
	Chlorophyll Pos, Phycocyanin Neg	4380

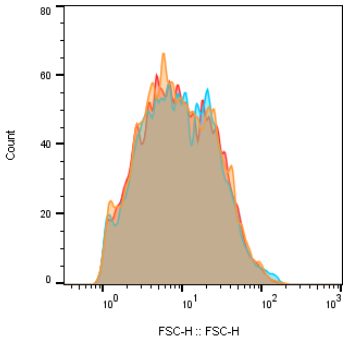
FP 19



FP 19
Chlorophyll Pos, Phycocyanin Neg

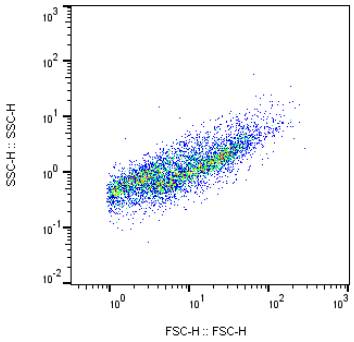


FP 19
Phycocyanin Pos

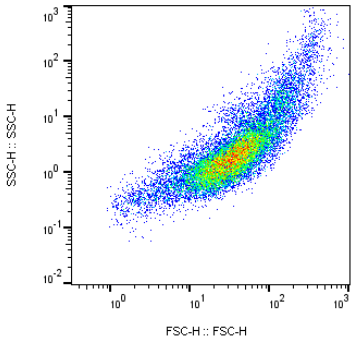


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5850
Chlorophyll Pos, Phycocyanin Neg	5561
Chlorophyll Pos, Phycocyanin Neg	5673

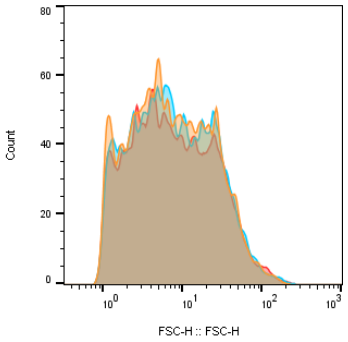
TP 19



TP 19
Chlorophyll Pos, Phycocyanin Neg

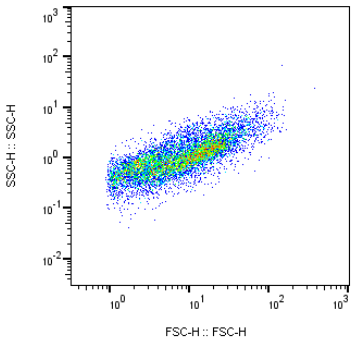


TP 19
Phycocyanin Pos

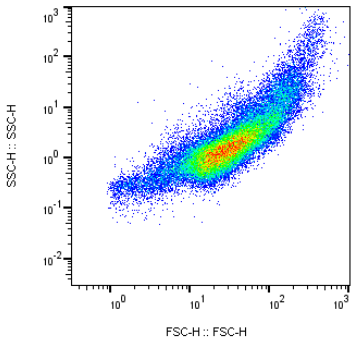


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	6167
Chlorophyll Pos, Phycocyanin Neg	5984
Chlorophyll Pos, Phycocyanin Neg	5572

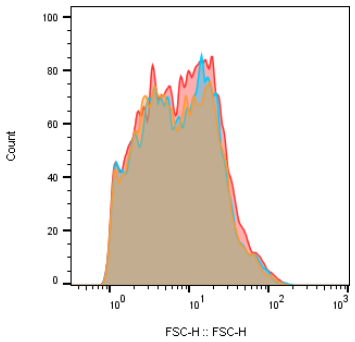
FP 20



FP 20
Chlorophyll Pos, Phycocyanin Neg

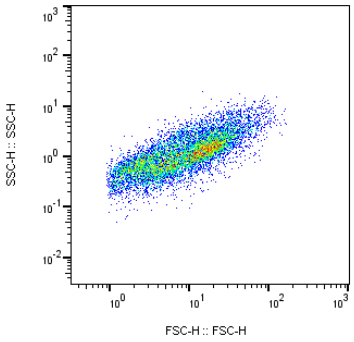


FP 20
Phycocyanin Pos

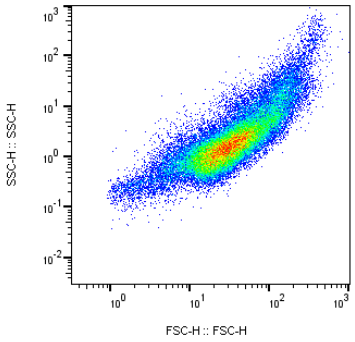


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	7515
Chlorophyll Pos, Phycocyanin Neg	7591
Chlorophyll Pos, Phycocyanin Neg	8449

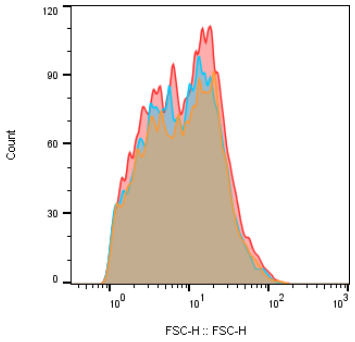
TP 20



TP 20
Chlorophyll Pos, Phycocyanin Neg

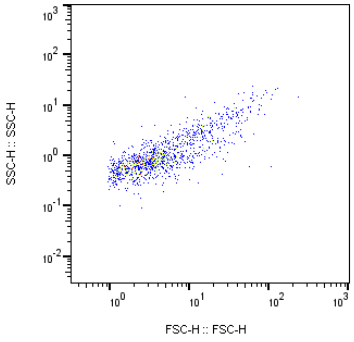


TP 20
Phycocyanin Pos

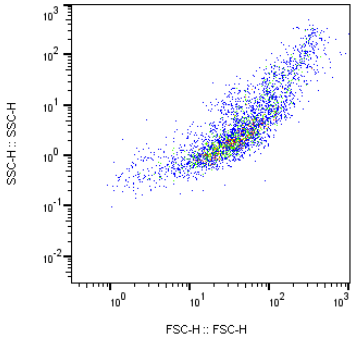


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	7868
Chlorophyll Pos, Phycocyanin Neg	8342
Chlorophyll Pos, Phycocyanin Neg	9575

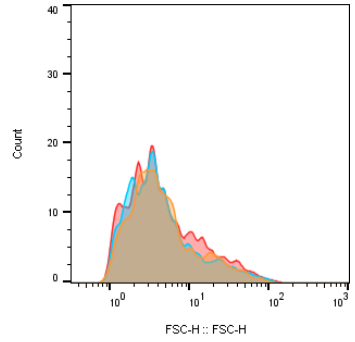
FP 21



FP 21
Chlorophyll Pos, Phycocyanin Neg

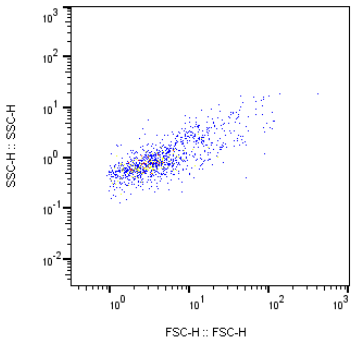


FP 21
Phycocyanin Pos

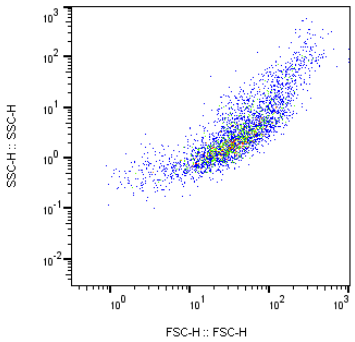


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1034
Chlorophyll Pos, Phycocyanin Neg	1083
Chlorophyll Pos, Phycocyanin Neg	1213

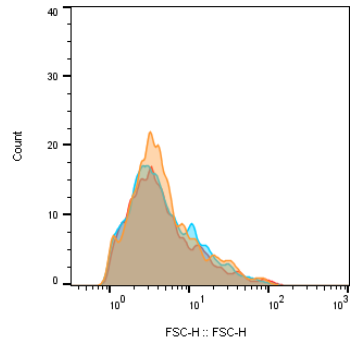
TP 21



TP 21
Chlorophyll Pos, Phycocyanin Neg

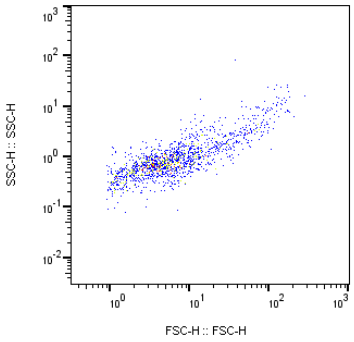


TP 21
Phycocyanin Pos

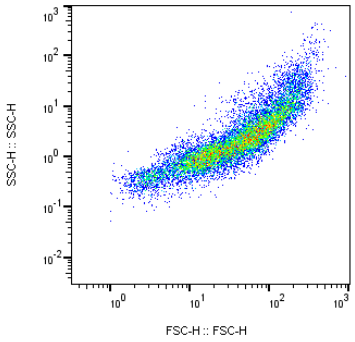


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1253
Chlorophyll Pos, Phycocyanin Neg	1166
Chlorophyll Pos, Phycocyanin Neg	1059

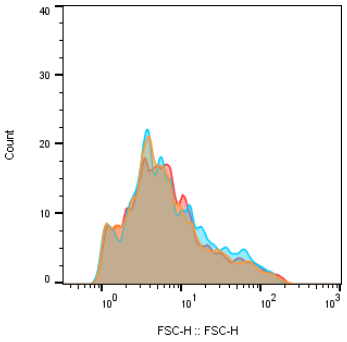
FP 22



FP 22
Chlorophyll Pos, Phycocyanin Neg

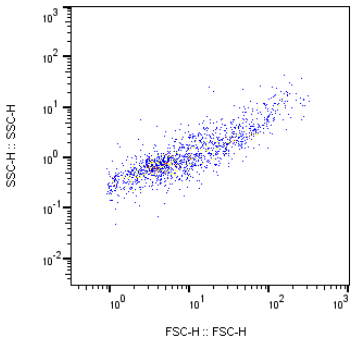


FP 22
Phycocyanin Pos

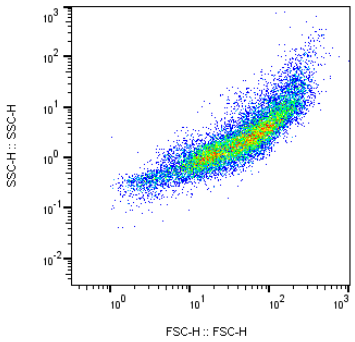


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1433
Chlorophyll Pos, Phycocyanin Neg	1517
Chlorophyll Pos, Phycocyanin Neg	1440

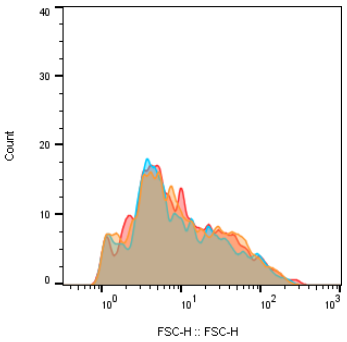
TP 22



TP 22
Chlorophyll Pos, Phycocyanin Neg

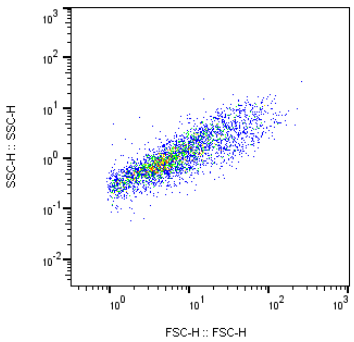


TP 22
Phycocyanin Pos

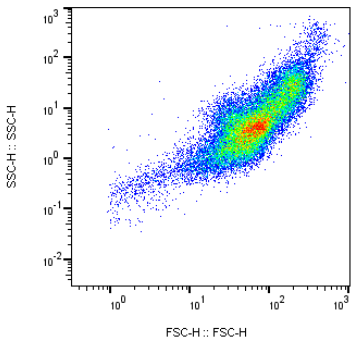


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1483
Chlorophyll Pos, Phycocyanin Neg	1370
Chlorophyll Pos, Phycocyanin Neg	1520

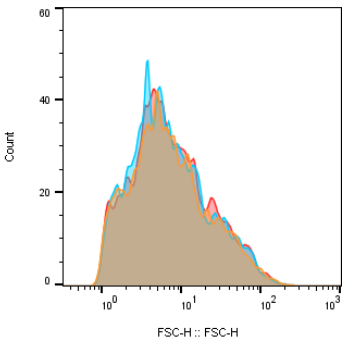
FP 23



FP 23
Chlorophyll Pos, Phycocyanin Neg

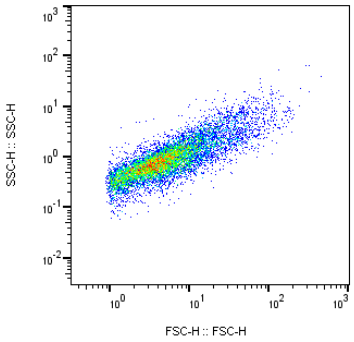


FP 23
Phycocyanin Pos

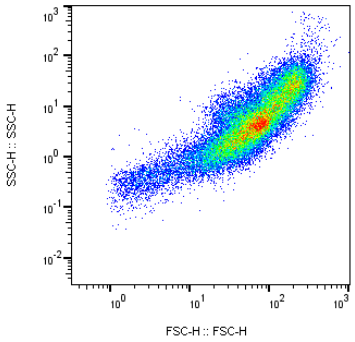


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3186
Chlorophyll Pos, Phycocyanin Neg	3438
Chlorophyll Pos, Phycocyanin Neg	3405

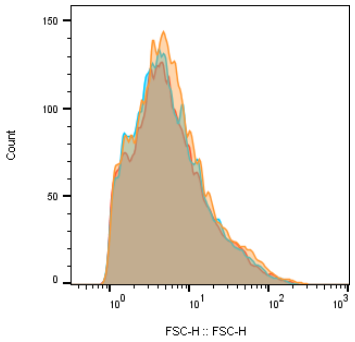
TP 23



TP 23
Chlorophyll Pos, Phycocyanin Neg

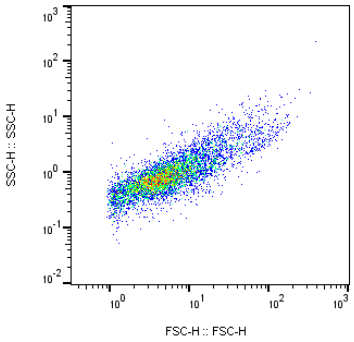


TP 23
Phycocyanin Pos

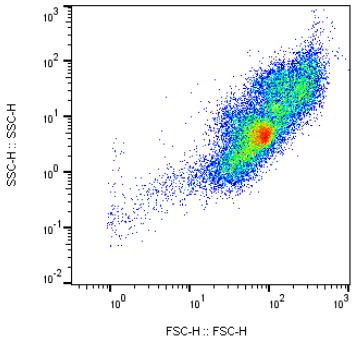


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	10776
Chlorophyll Pos, Phycocyanin Neg	10028
Chlorophyll Pos, Phycocyanin Neg	9535

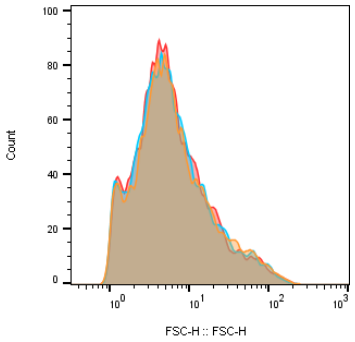
FP 24



FP 24
Chlorophyll Pos, Phycocyanin Neg

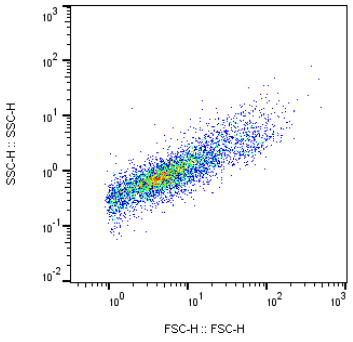


FP 24
Phycocyanin Pos

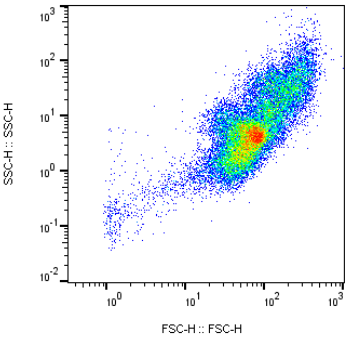


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5825
Chlorophyll Pos, Phycocyanin Neg	5997
Chlorophyll Pos, Phycocyanin Neg	6101

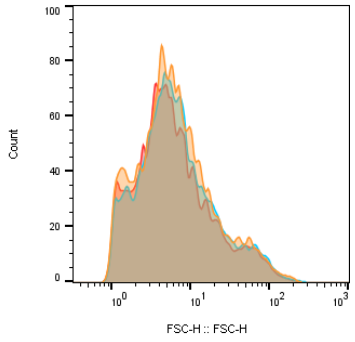
TP 24



TP 24
Chlorophyll Pos, Phycocyanin Neg

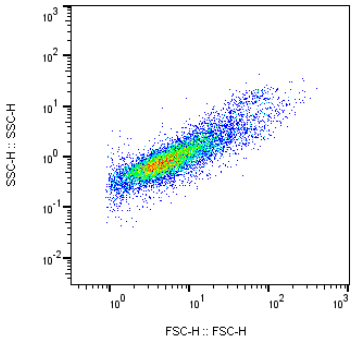


TP 24
Phycocyanin Pos

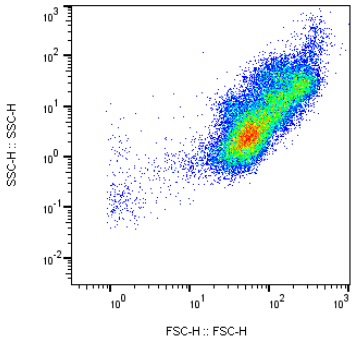


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	6019
Chlorophyll Pos, Phycocyanin Neg	5519
Chlorophyll Pos, Phycocyanin Neg	5298

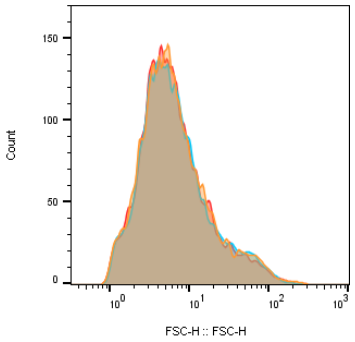
FP 25



FP 25
Chlorophyll Pos, Phycocyanin Neg

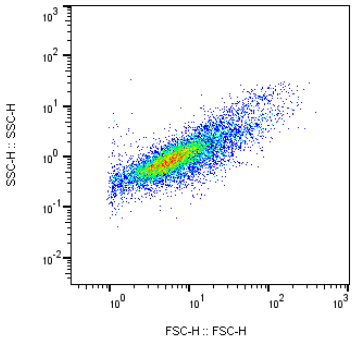


FP 25
Phycocyanin Pos

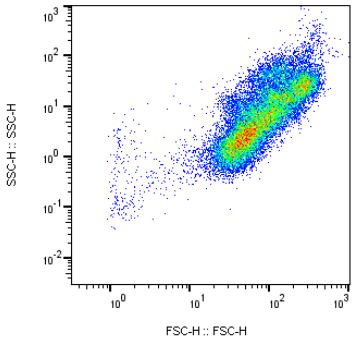


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	9465
Chlorophyll Pos, Phycocyanin Neg	9190
Chlorophyll Pos, Phycocyanin Neg	9344

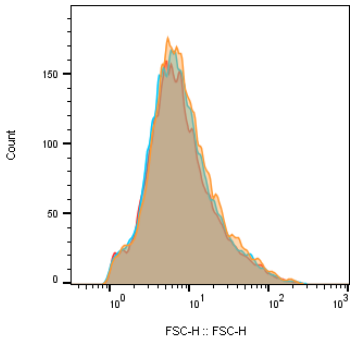
TP 25



TP 25
Chlorophyll Pos, Phycocyanin Neg

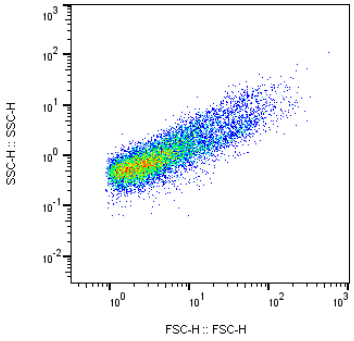


TP 25
Phycocyanin Pos

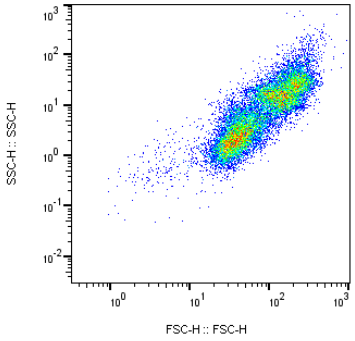


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	11129
Chlorophyll Pos, Phycocyanin Neg	10785
Chlorophyll Pos, Phycocyanin Neg	10077

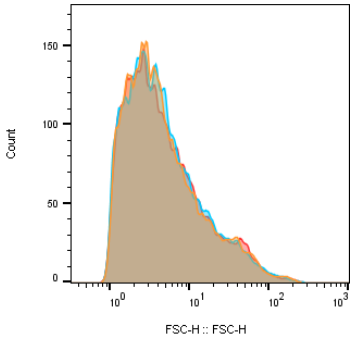
FP 26



FP 26
Chlorophyll Pos, Phycocyanin Neg

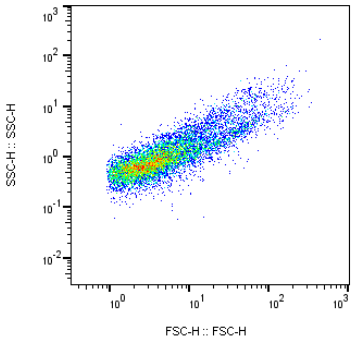


FP 26
Phycocyanin Pos

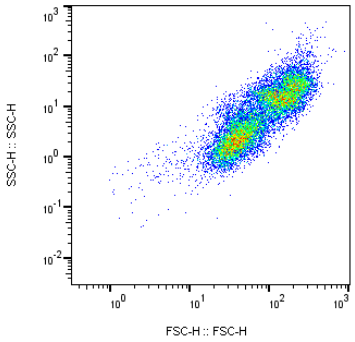


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	10731
Chlorophyll Pos, Phycocyanin Neg	10913
Chlorophyll Pos, Phycocyanin Neg	10596

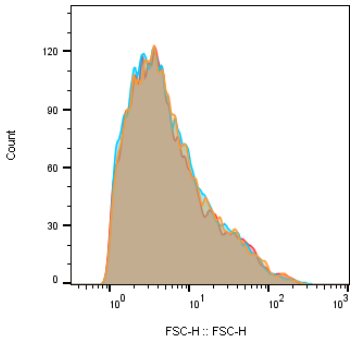
TP 26



TP 26
Chlorophyll Pos, Phycocyanin Neg

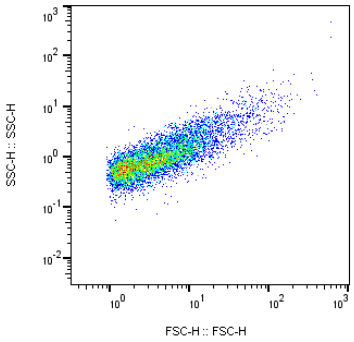


TP 26
Phycocyanin Pos

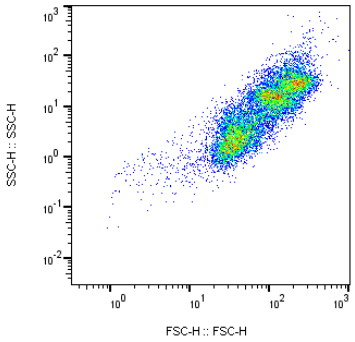


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	9430
Chlorophyll Pos, Phycocyanin Neg	9624
Chlorophyll Pos, Phycocyanin Neg	9252

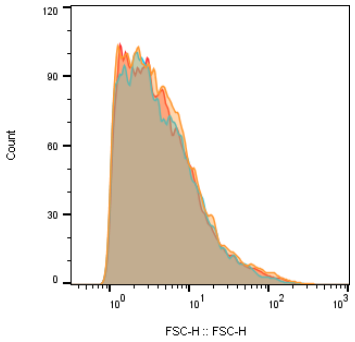
FP 27



FP 27
Chlorophyll Pos, Phycocyanin Neg

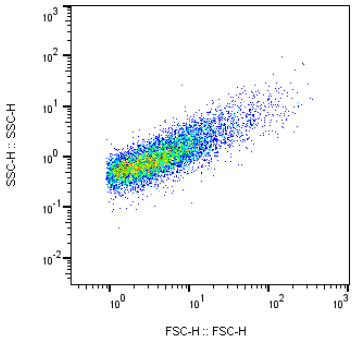


FP 27
Phycocyanin Pos

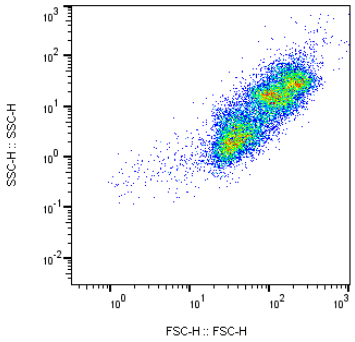


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8335
Chlorophyll Pos, Phycocyanin Neg	7681
Chlorophyll Pos, Phycocyanin Neg	7734

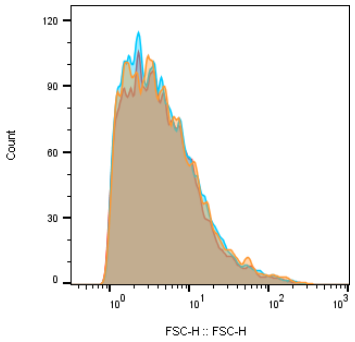
TP 27



TP 27
Chlorophyll Pos, Phycocyanin Neg

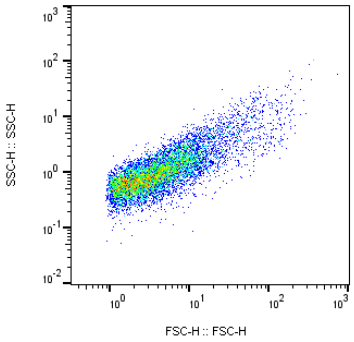


TP 27
Phycocyanin Pos

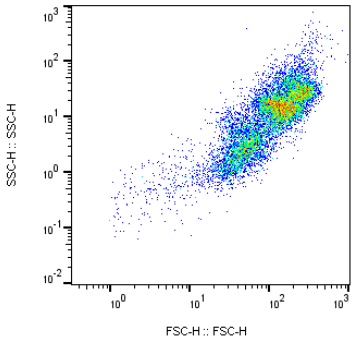


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8283
Chlorophyll Pos, Phycocyanin Neg	8458
Chlorophyll Pos, Phycocyanin Neg	7842

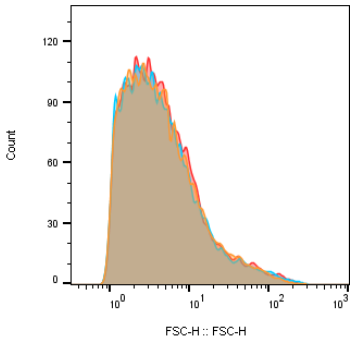
FP 28



FP 28
Chlorophyll Pos, Phycocyanin Neg

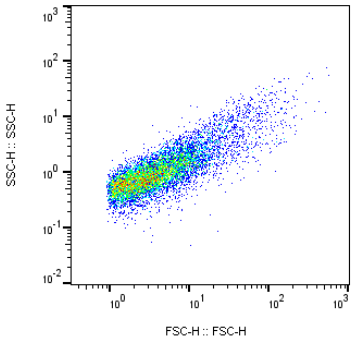


FP 28
Phycocyanin Pos

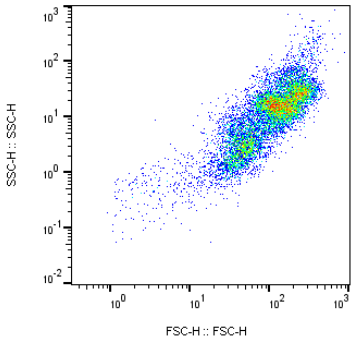


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8460
Chlorophyll Pos, Phycocyanin Neg	8389
Chlorophyll Pos, Phycocyanin Neg	8659

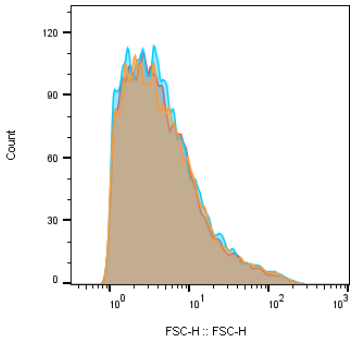
TP 28



TP 28
Chlorophyll Pos, Phycocyanin Neg

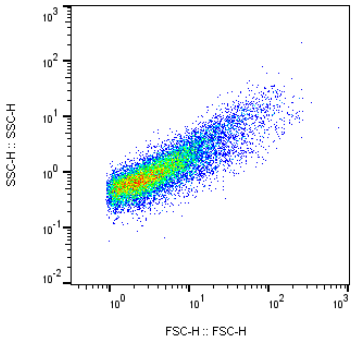


TP 28
Phycocyanin Pos

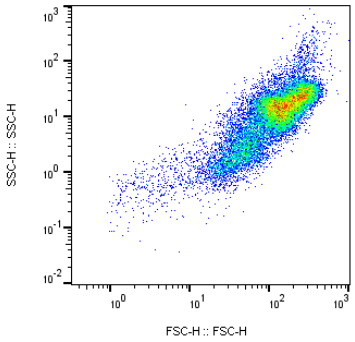


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8371
Chlorophyll Pos, Phycocyanin Neg	8978
Chlorophyll Pos, Phycocyanin Neg	8447

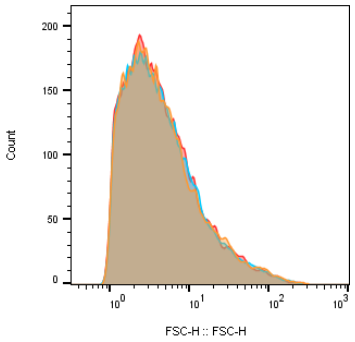
FP 29



FP 29
Chlorophyll Pos, Phycocyanin Neg

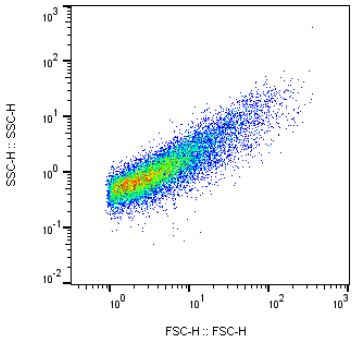


FP 29
Phycocyanin Pos

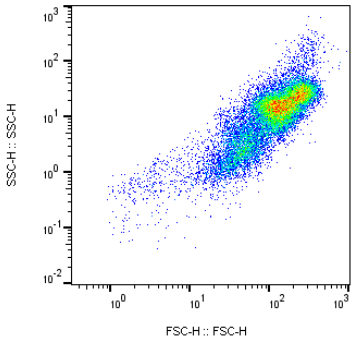


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	13777
Chlorophyll Pos, Phycocyanin Neg	13697
Chlorophyll Pos, Phycocyanin Neg	14015

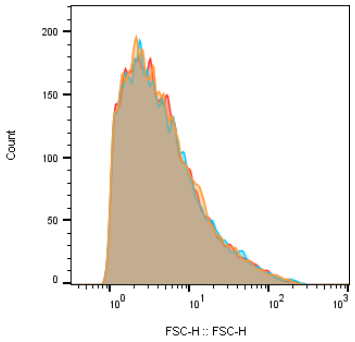
TP 29



TP 29
Chlorophyll Pos, Phycocyanin Neg

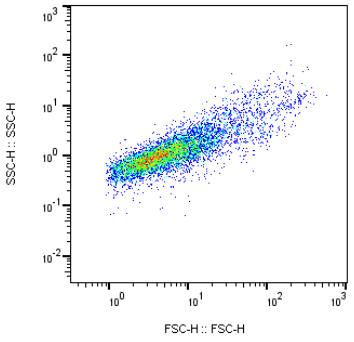


TP 29
Phycocyanin Pos

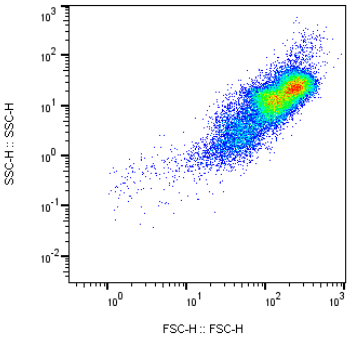


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	14344
Chlorophyll Pos, Phycocyanin Neg	13982
Chlorophyll Pos, Phycocyanin Neg	14047

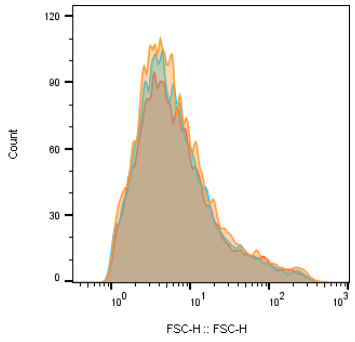
FP 30



FP 30
Chlorophyll Pos, Phycocyanin Neg

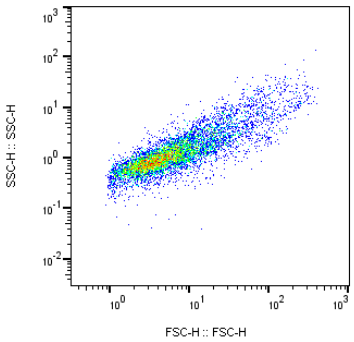


FP 30
Phycocyanin Pos

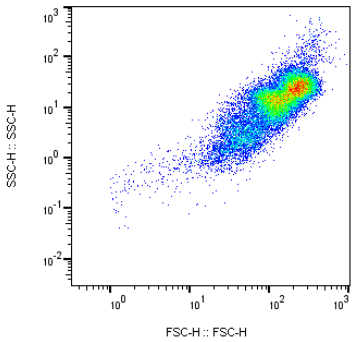


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	8172
Chlorophyll Pos, Phycocyanin Neg	7438
Chlorophyll Pos, Phycocyanin Neg	6972

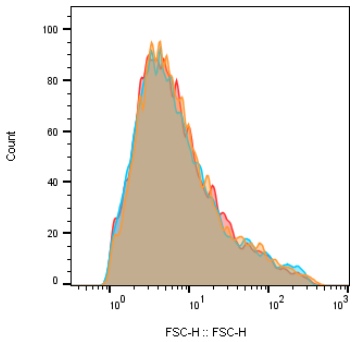
TP 30



TP 30
Chlorophyll Pos, Phycocyanin Neg

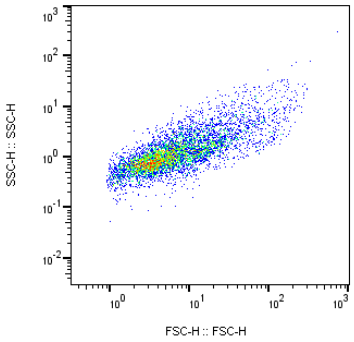


TP 30
Phycocyanin Pos

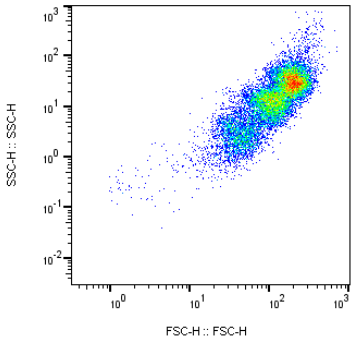


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	7240
Chlorophyll Pos, Phycocyanin Neg	7174
Chlorophyll Pos, Phycocyanin Neg	7280

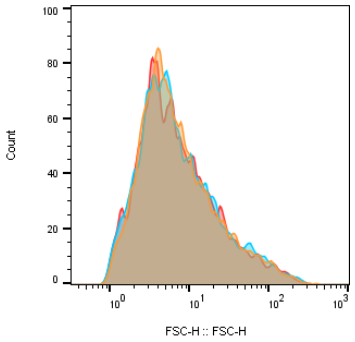
FP 31



FP 31
Chlorophyll Pos, Phycocyanin Neg

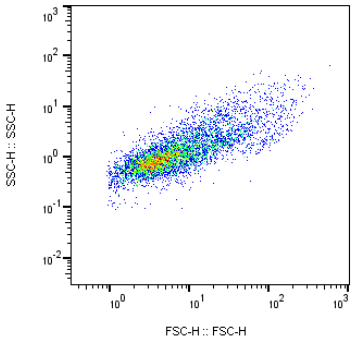


FP 31
Phycocyanin Pos

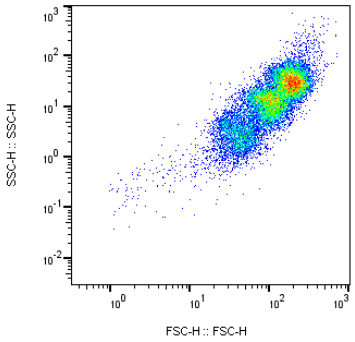


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5718
Chlorophyll Pos, Phycocyanin Neg	5735
Chlorophyll Pos, Phycocyanin Neg	5651

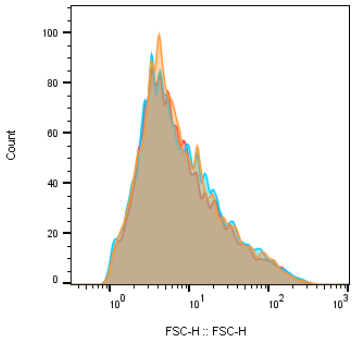
TP 31



TP 31
Chlorophyll Pos, Phycocyanin Neg

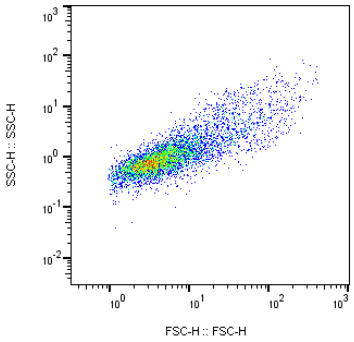


TP 31
Phycocyanin Pos

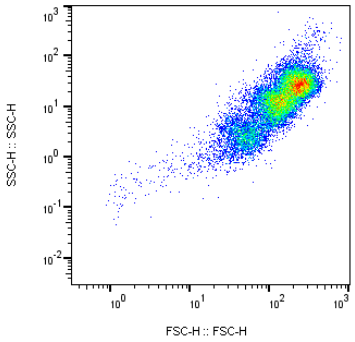


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	6483
Chlorophyll Pos, Phycocyanin Neg	6447
Chlorophyll Pos, Phycocyanin Neg	6109

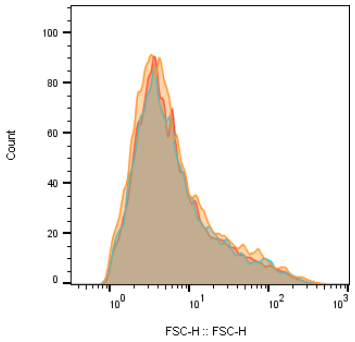
FP 32



FP 32
Chlorophyll Pos, Phycocyanin Neg

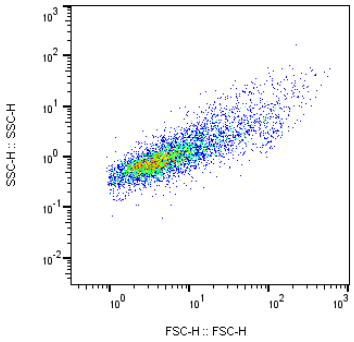


FP 32
Phycocyanin Pos

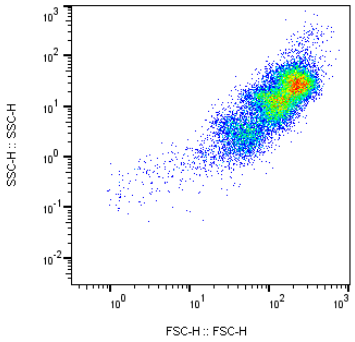


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	6378
Chlorophyll Pos, Phycocyanin Neg	5485
Chlorophyll Pos, Phycocyanin Neg	5536

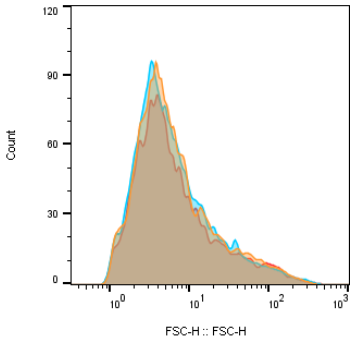
TP 32



TP 32
Chlorophyll Pos, Phycocyanin Neg

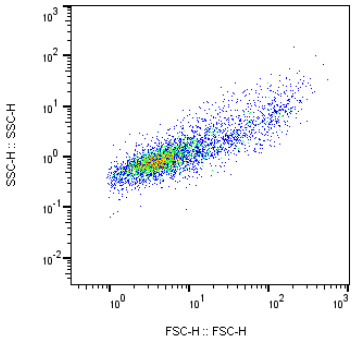


TP 32
Phycocyanin Pos

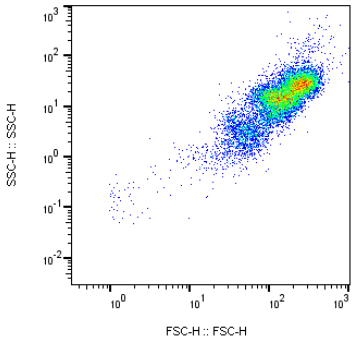


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	6069
Chlorophyll Pos, Phycocyanin Neg	6112
Chlorophyll Pos, Phycocyanin Neg	5349

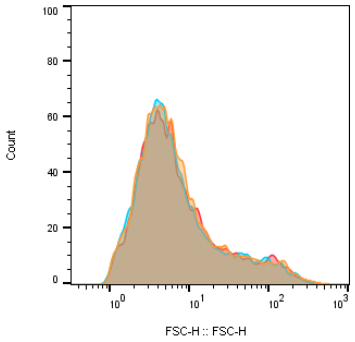
FP 33



FP 33
Chlorophyll Pos, Phycocyanin Neg

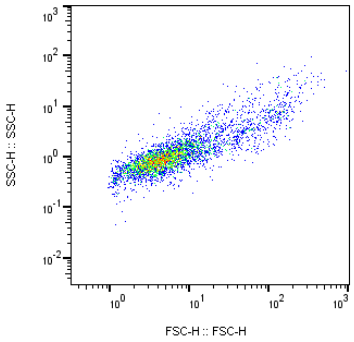


FP 33
Phycocyanin Pos

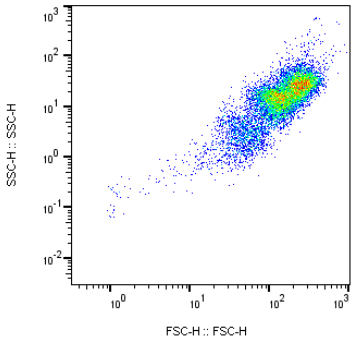


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4539
Chlorophyll Pos, Phycocyanin Neg	4348
Chlorophyll Pos, Phycocyanin Neg	4299

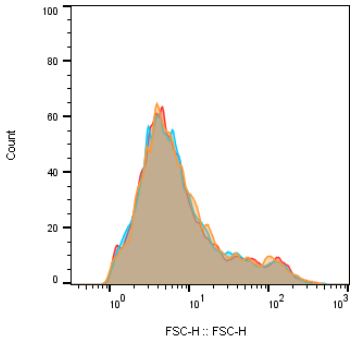
TP 33



TP 33
Chlorophyll Pos, Phycocyanin Neg

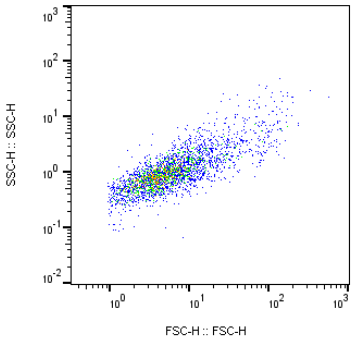


TP 33
Phycocyanin Pos

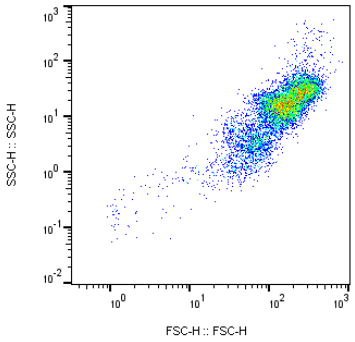


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4159
Chlorophyll Pos, Phycocyanin Neg	4116
Chlorophyll Pos, Phycocyanin Neg	4109

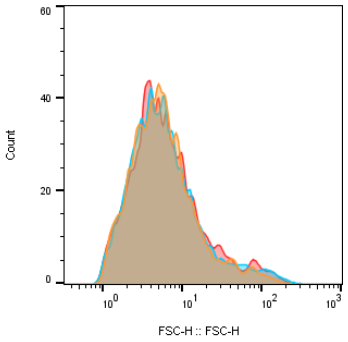
FP 34



FP 34
Chlorophyll Pos, Phycocyanin Neg

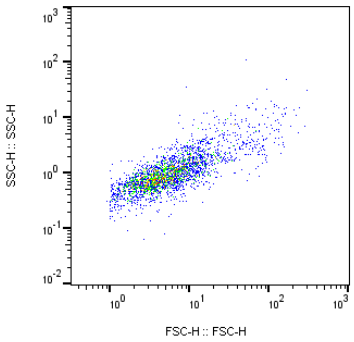


FP 34
Phycocyanin Pos

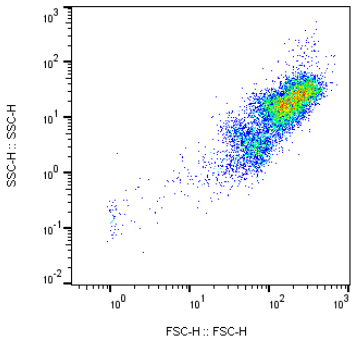


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2766
Chlorophyll Pos, Phycocyanin Neg	2779
Chlorophyll Pos, Phycocyanin Neg	2836

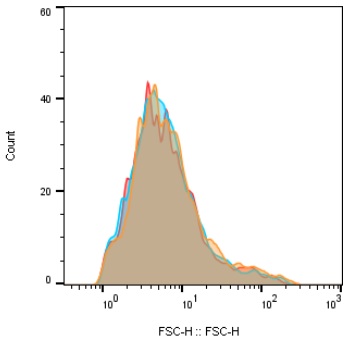
TP 34



TP 34
Chlorophyll Pos, Phycocyanin Neg

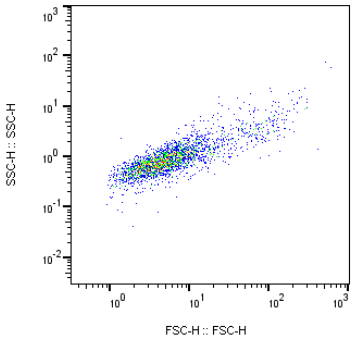


TP 34
Phycocyanin Pos

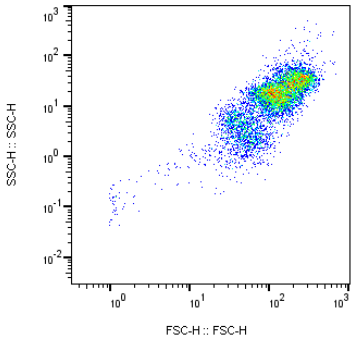


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2756
Chlorophyll Pos, Phycocyanin Neg	2784
Chlorophyll Pos, Phycocyanin Neg	2654

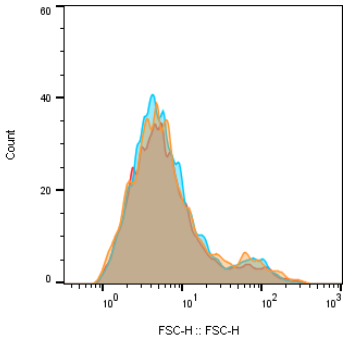
FP 35



FP 35
Chlorophyll Pos, Phycocyanin Neg

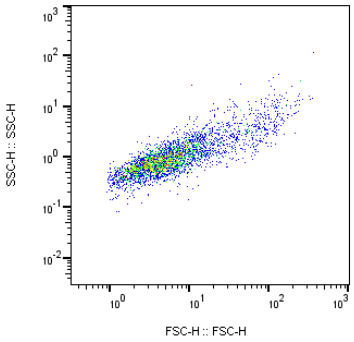


FP 35
Phycocyanin Pos

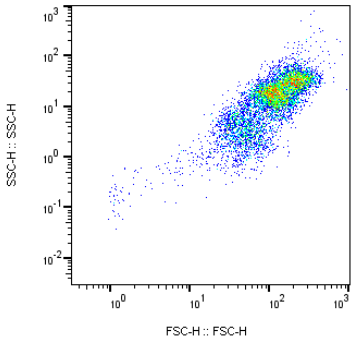


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2499
Chlorophyll Pos, Phycocyanin Neg	2530
Chlorophyll Pos, Phycocyanin Neg	2256

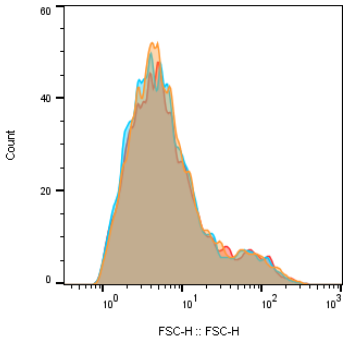
TP 35



TP 35
Chlorophyll Pos, Phycocyanin Neg

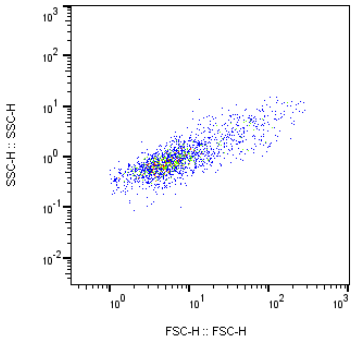


TP 35
Phycocyanin Pos

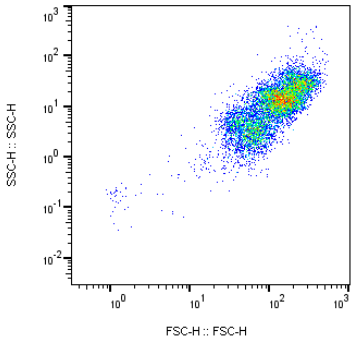


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3526
Chlorophyll Pos, Phycocyanin Neg	3509
Chlorophyll Pos, Phycocyanin Neg	3331

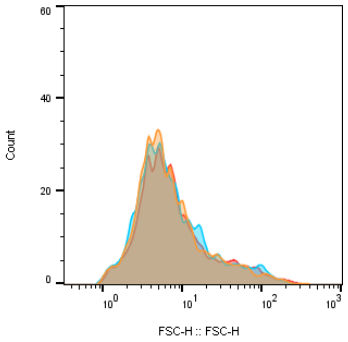
FP 36



FP 36
Chlorophyll Pos, Phycocyanin Neg

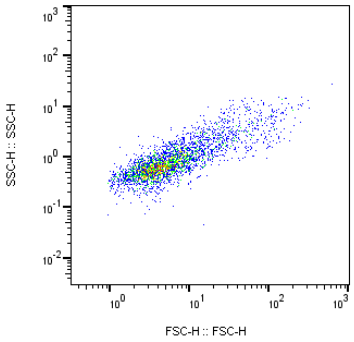


FP 36
Phycocyanin Pos

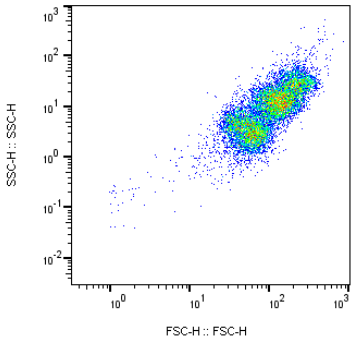


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1891
Chlorophyll Pos, Phycocyanin Neg	1920
Chlorophyll Pos, Phycocyanin Neg	1762

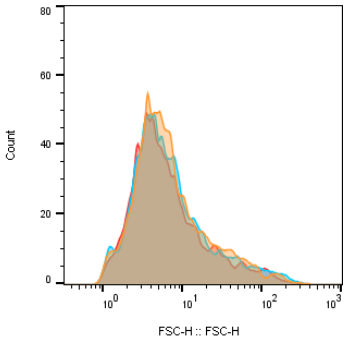
TP 36



TP 36
Chlorophyll Pos, Phycocyanin Neg

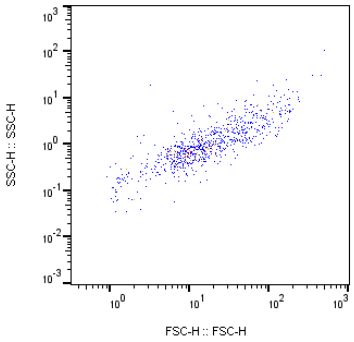


TP 36
Phycocyanin Pos

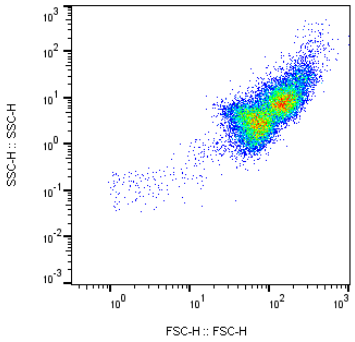


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3303
Chlorophyll Pos, Phycocyanin Neg	3201
Chlorophyll Pos, Phycocyanin Neg	2997

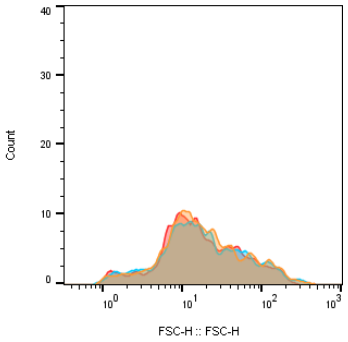
FP 37



FP 37
Chlorophyll Pos, Phycocyanin Neg

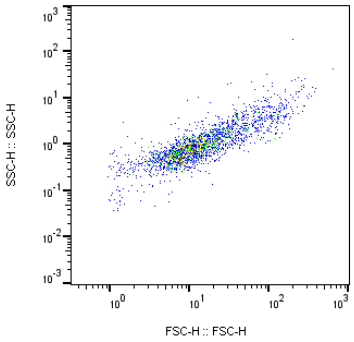


FP 37
Phycocyanin Pos

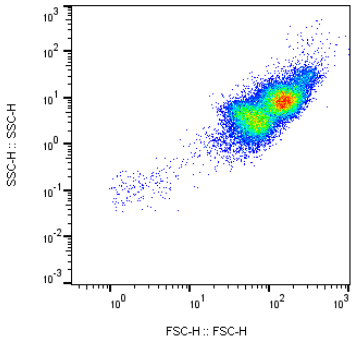


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	843
Chlorophyll Pos, Phycocyanin Neg	793
Chlorophyll Pos, Phycocyanin Neg	802

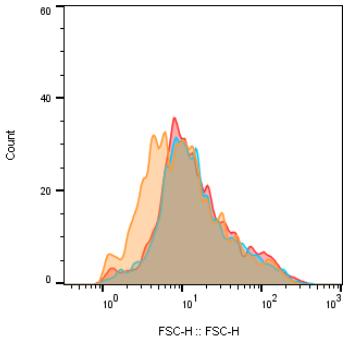
TP 37



TP 37
Chlorophyll Pos, Phycocyanin Neg

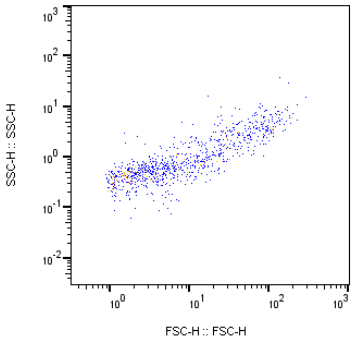


TP 37
Phycocyanin Pos

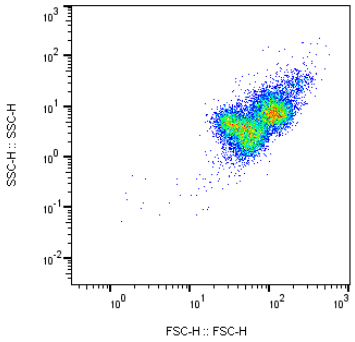


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2735
Chlorophyll Pos, Phycocyanin Neg	2109
Chlorophyll Pos, Phycocyanin Neg	2284

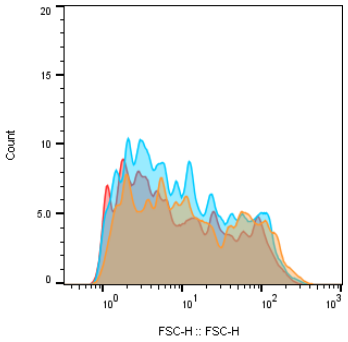
FP 38



FP 38
Chlorophyll Pos, Phycocyanin Neg

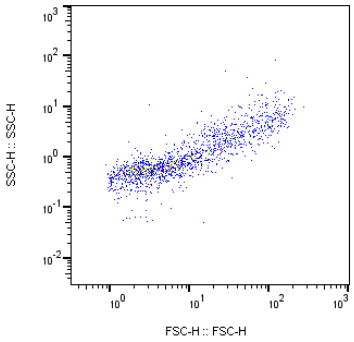


FP 38
Phycocyanin Pos

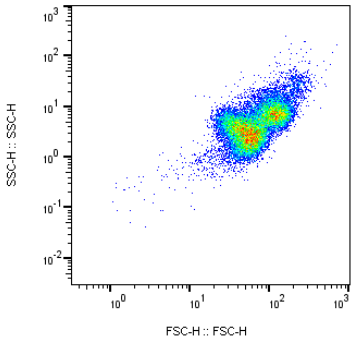


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	882
Chlorophyll Pos, Phycocyanin Neg	1136
Chlorophyll Pos, Phycocyanin Neg	890

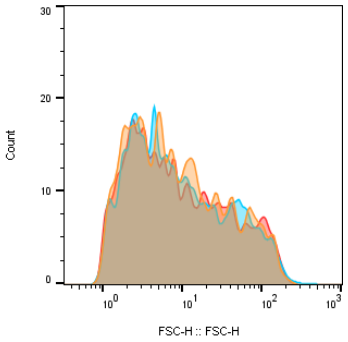
TP 38



TP 38
Chlorophyll Pos, Phycocyanin Neg

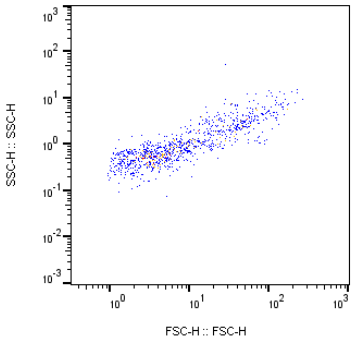


TP 38
Phycocyanin Pos

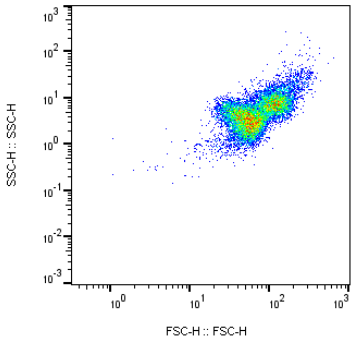


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1881
Chlorophyll Pos, Phycocyanin Neg	1782
Chlorophyll Pos, Phycocyanin Neg	1744

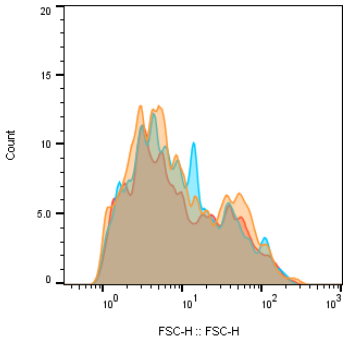
FP 39



FP 39
Chlorophyll Pos, Phycocyanin Neg

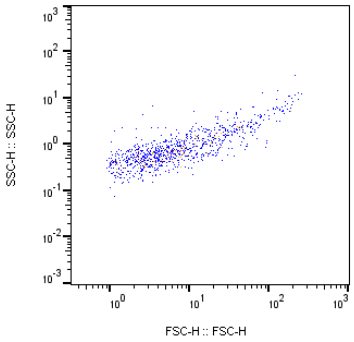


FP 39
Phycocyanin Pos

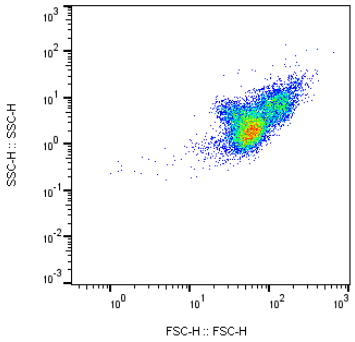


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1193
Chlorophyll Pos, Phycocyanin Neg	1088
Chlorophyll Pos, Phycocyanin Neg	970

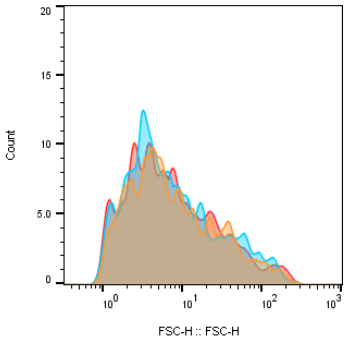
TP 39



TP 39
Chlorophyll Pos, Phycocyanin Neg

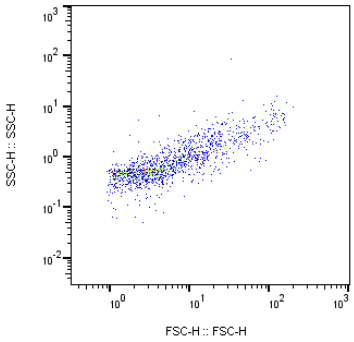


TP 39
Phycocyanin Pos

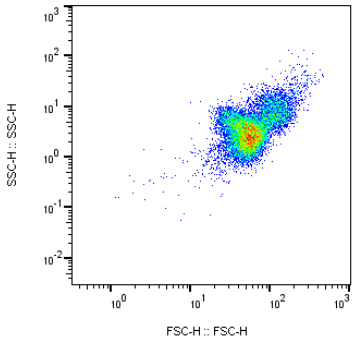


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	846
Chlorophyll Pos, Phycocyanin Neg	951
Chlorophyll Pos, Phycocyanin Neg	914

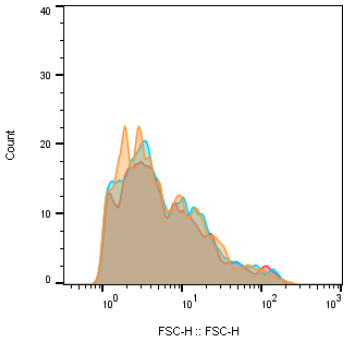
FP 40



FP 40
Chlorophyll Pos, Phycocyanin Neg

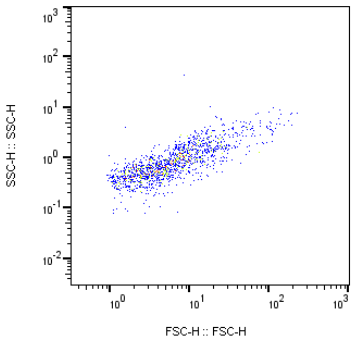


FP 40
Phycocyanin Pos

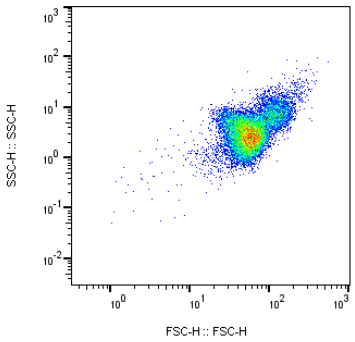


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1706
Chlorophyll Pos, Phycocyanin Neg	1660
Chlorophyll Pos, Phycocyanin Neg	1498

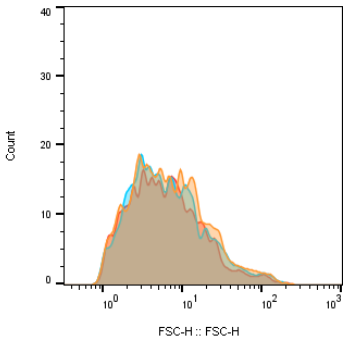
TP 40



TP 40
Chlorophyll Pos, Phycocyanin Neg

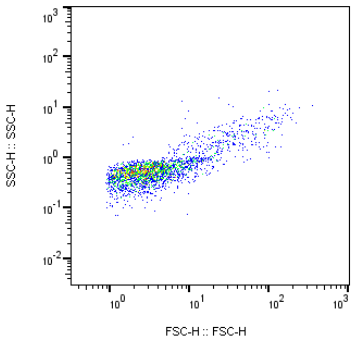


TP 40
Phycocyanin Pos

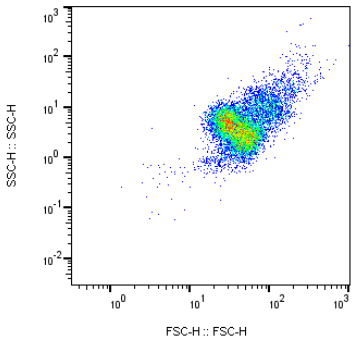


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1600
Chlorophyll Pos, Phycocyanin Neg	1483
Chlorophyll Pos, Phycocyanin Neg	1372

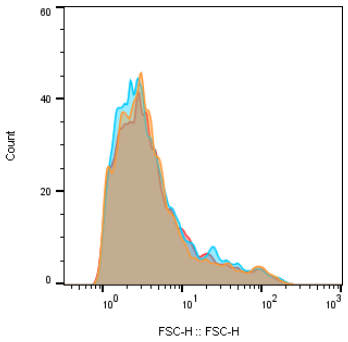
FP 41



FP 41
Chlorophyll Pos, Phycocyanin Neg

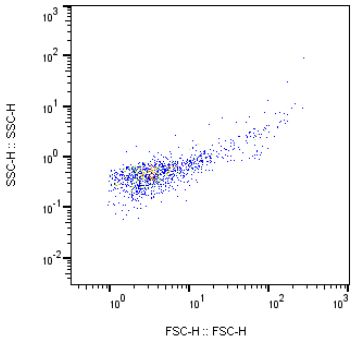


FP 41
Phycocyanin Pos

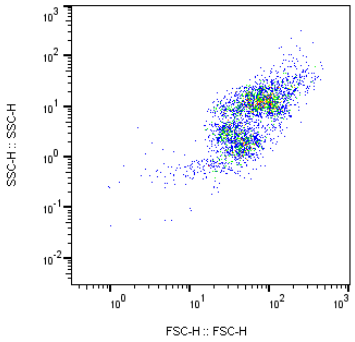


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2629
Chlorophyll Pos, Phycocyanin Neg	2778
Chlorophyll Pos, Phycocyanin Neg	2564

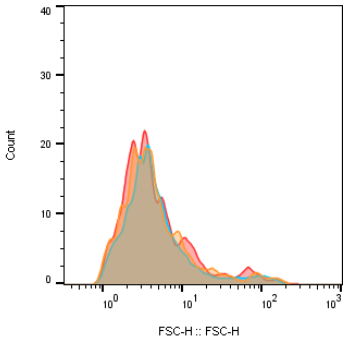
TP 41



TP 41
Chlorophyll Pos, Phycocyanin Neg

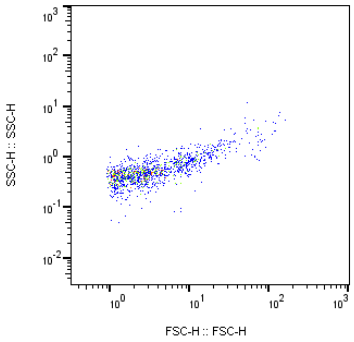


TP 41
Phycocyanin Pos

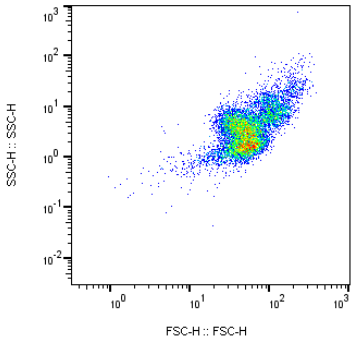


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1094
Chlorophyll Pos, Phycocyanin Neg	963
Chlorophyll Pos, Phycocyanin Neg	1170

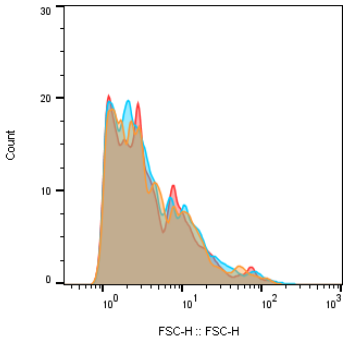
FP 42



FP 42
Chlorophyll Pos, Phycocyanin Neg

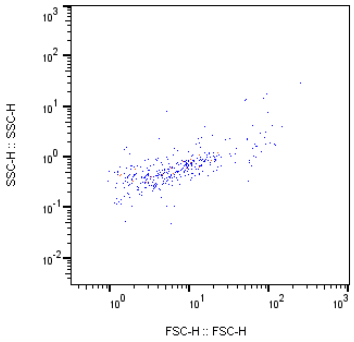


FP 42
Phycocyanin Pos

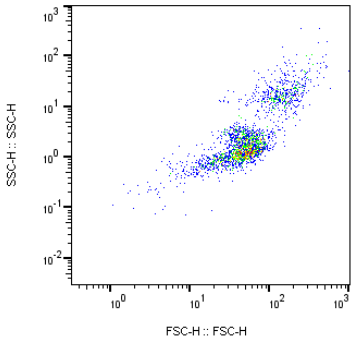


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1264
Chlorophyll Pos, Phycocyanin Neg	1345
Chlorophyll Pos, Phycocyanin Neg	1242

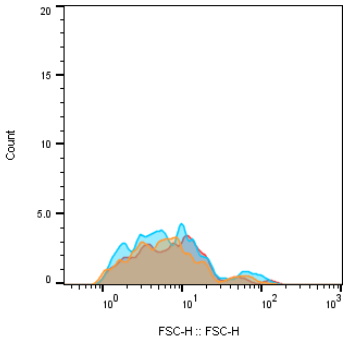
TP 42



TP 42
Chlorophyll Pos, Phycocyanin Neg

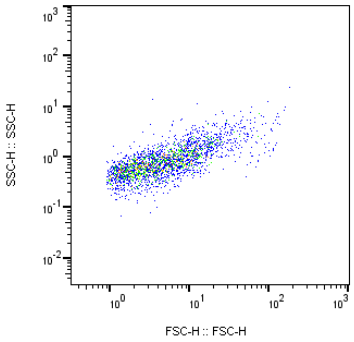


TP 42
Phycocyanin Pos

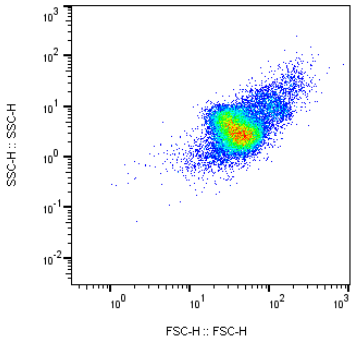


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	265
Chlorophyll Pos, Phycocyanin Neg	349
Chlorophyll Pos, Phycocyanin Neg	271

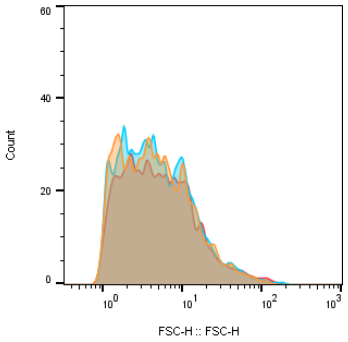
FP 43



FP 43
Chlorophyll Pos, Phycocyanin Neg

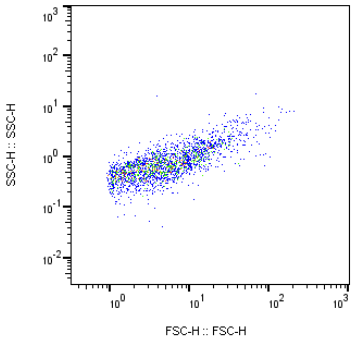


FP 43
Phycocyanin Pos

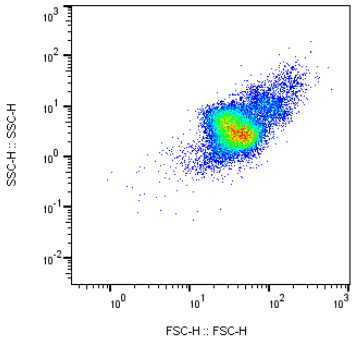


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2669
Chlorophyll Pos, Phycocyanin Neg	2754
Chlorophyll Pos, Phycocyanin Neg	2399

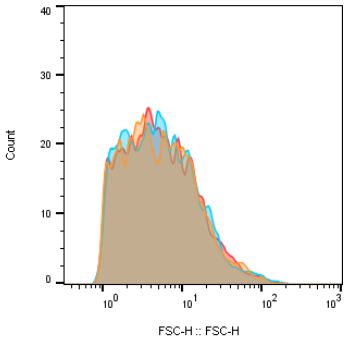
TP 43



TP 43
Chlorophyll Pos, Phycocyanin Neg

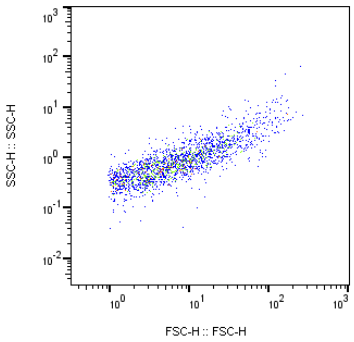


TP 43
Phycocyanin Pos

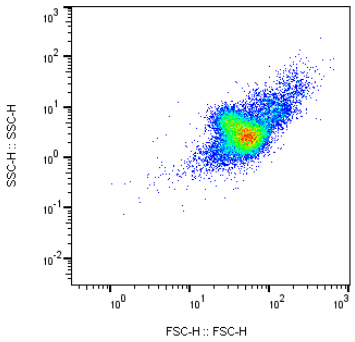


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2123
Chlorophyll Pos, Phycocyanin Neg	2179
Chlorophyll Pos, Phycocyanin Neg	2133

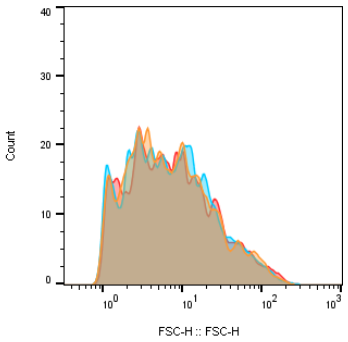
FP 44



FP 44
Chlorophyll Pos, Phycocyanin Neg

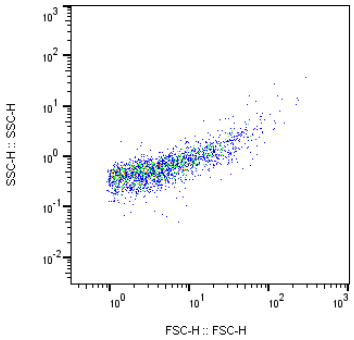


FP 44
Phycocyanin Pos

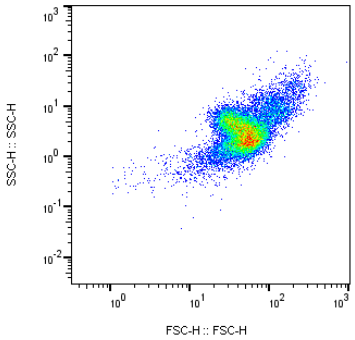


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2133
Chlorophyll Pos, Phycocyanin Neg	2154
Chlorophyll Pos, Phycocyanin Neg	2066

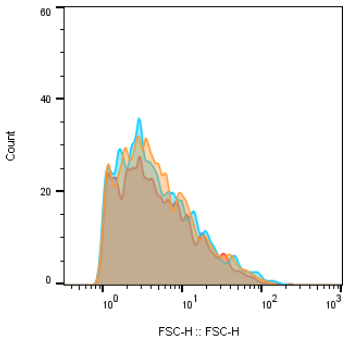
TP 44



TP 44
Chlorophyll Pos, Phycocyanin Neg

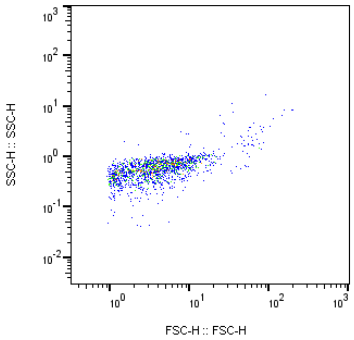


TP 44
Phycocyanin Pos

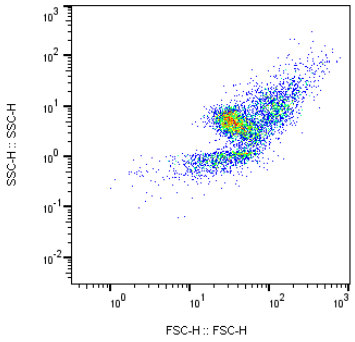


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2546
Chlorophyll Pos, Phycocyanin Neg	2572
Chlorophyll Pos, Phycocyanin Neg	2167

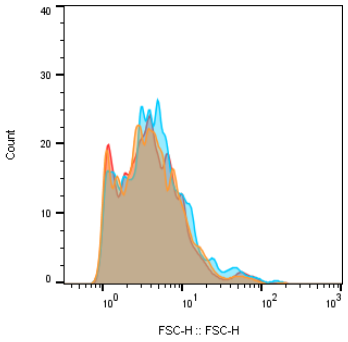
FP 45



FP 45
Chlorophyll Pos, Phycocyanin Neg

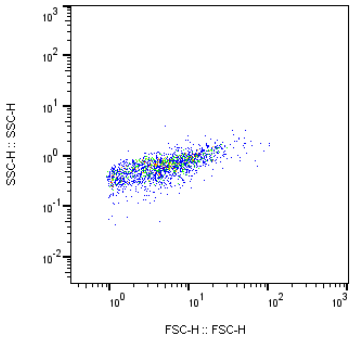


FP 45
Phycocyanin Pos

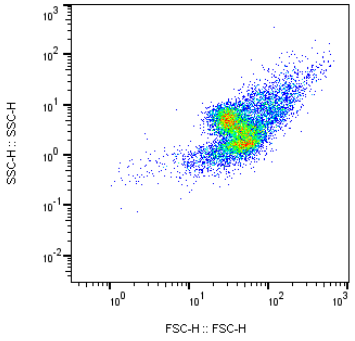


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1590
Chlorophyll Pos, Phycocyanin Neg	1728
Chlorophyll Pos, Phycocyanin Neg	1566

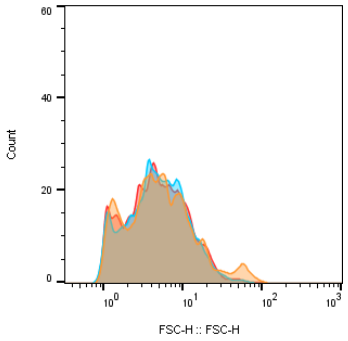
TP 45



TP 45
Chlorophyll Pos, Phycocyanin Neg

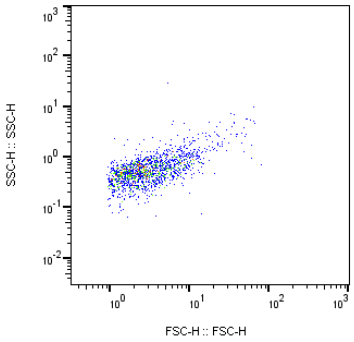


TP 45
Phycocyanin Pos

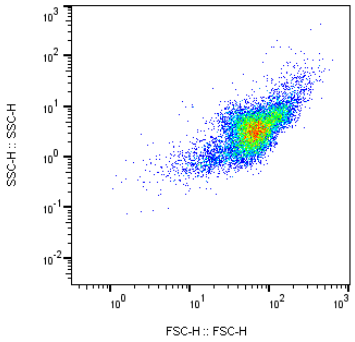


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1814
Chlorophyll Pos, Phycocyanin Neg	1773
Chlorophyll Pos, Phycocyanin Neg	1803

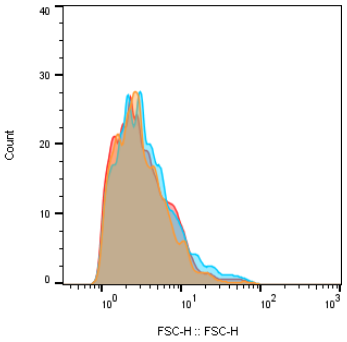
FP 46



FP 46
Chlorophyll Pos, Phycocyanin Neg

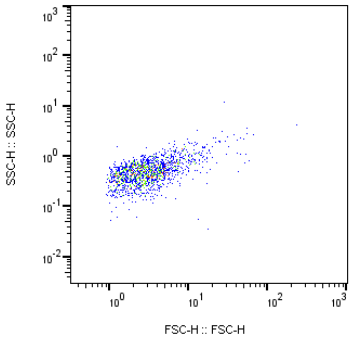


FP 46
Phycocyanin Pos

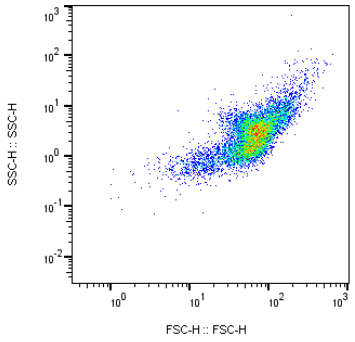


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1425
Chlorophyll Pos, Phycocyanin Neg	1580
Chlorophyll Pos, Phycocyanin Neg	1509

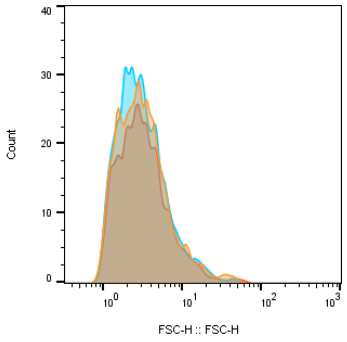
TP 46



TP 46
Chlorophyll Pos, Phycocyanin Neg

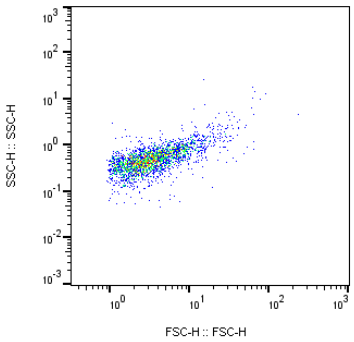


TP 46
Phycocyanin Pos

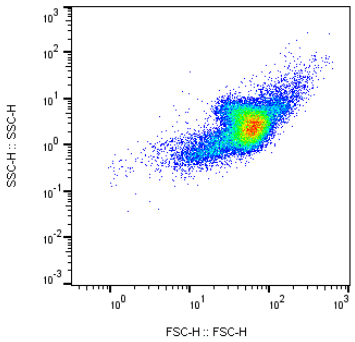


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1607
Chlorophyll Pos, Phycocyanin Neg	1687
Chlorophyll Pos, Phycocyanin Neg	1393

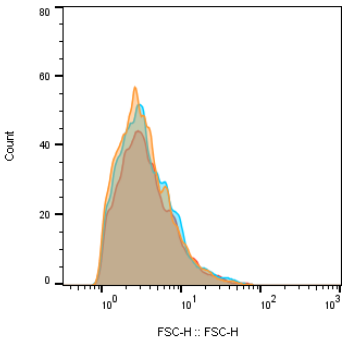
FP 47



FP 47
Chlorophyll Pos, Phycocyanin Neg

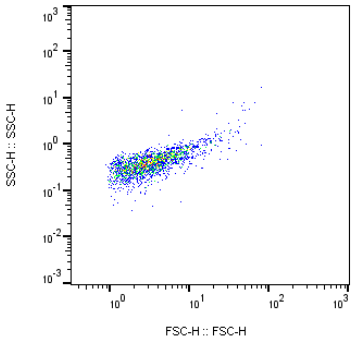


FP 47
Phycocyanin Pos

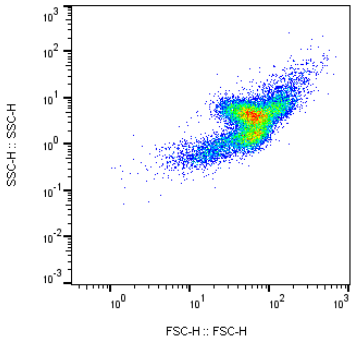


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3024
Chlorophyll Pos, Phycocyanin Neg	2914
Chlorophyll Pos, Phycocyanin Neg	2528

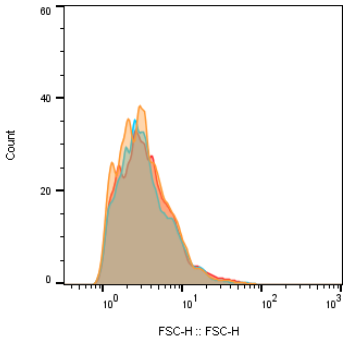
TP 47



TP 47
Chlorophyll Pos, Phycocyanin Neg

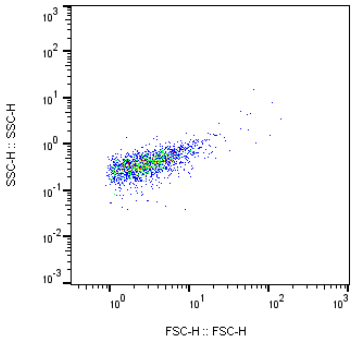


TP 47
Phycocyanin Pos

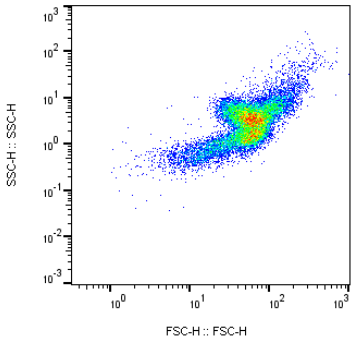


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2098
Chlorophyll Pos, Phycocyanin Neg	1819
Chlorophyll Pos, Phycocyanin Neg	1854

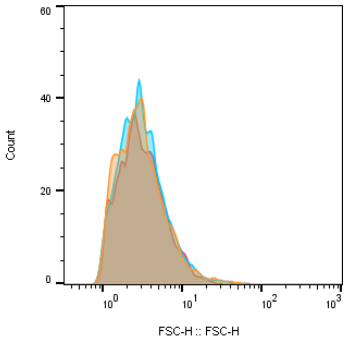
FP 48



FP 48
Chlorophyll Pos, Phycocyanin Neg

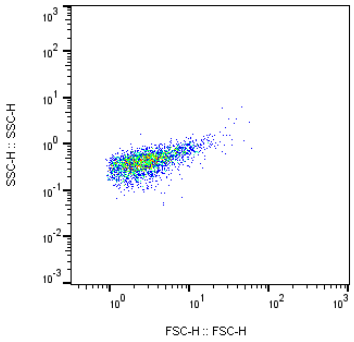


FP 48
Phycocyanin Pos

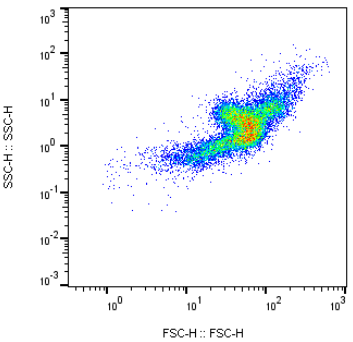


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1983
Chlorophyll Pos, Phycocyanin Neg	2047
Chlorophyll Pos, Phycocyanin Neg	1798

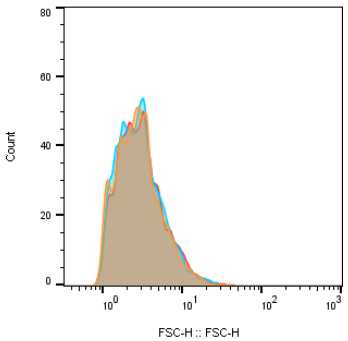
TP 48



TP 48
Chlorophyll Pos, Phycocyanin Neg

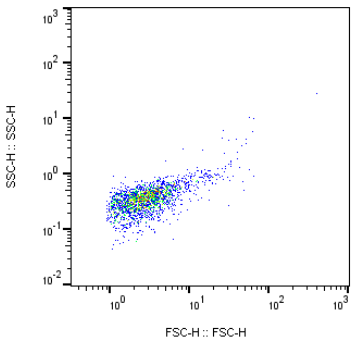


TP 48
Phycocyanin Pos

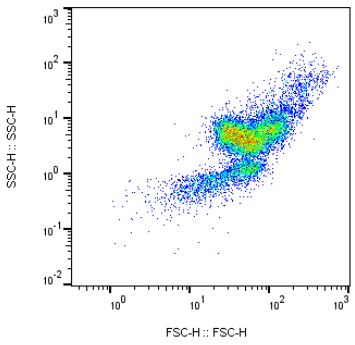


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2534
Chlorophyll Pos, Phycocyanin Neg	2626
Chlorophyll Pos, Phycocyanin Neg	2519

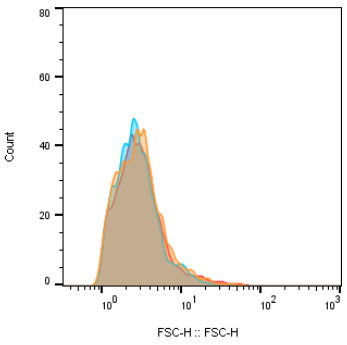
FP 49



FP 49
Chlorophyll Pos, Phycocyanin Neg

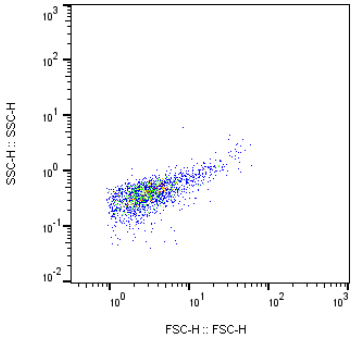


FP 49
Phycocyanin Pos

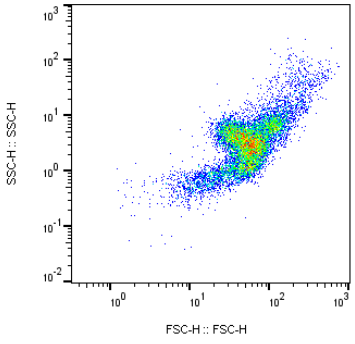


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2235
Chlorophyll Pos, Phycocyanin Neg	2113
Chlorophyll Pos, Phycocyanin Neg	2074

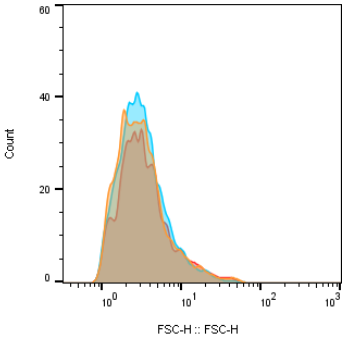
TP 49



TP 49
Chlorophyll Pos, Phycocyanin Neg

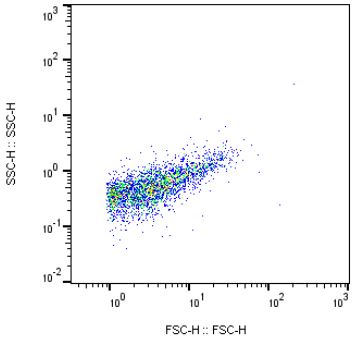


TP 49
Phycocyanin Pos

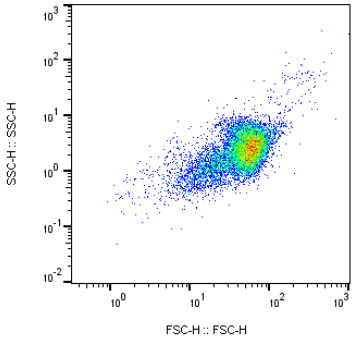


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1932
Chlorophyll Pos, Phycocyanin Neg	2011
Chlorophyll Pos, Phycocyanin Neg	1672

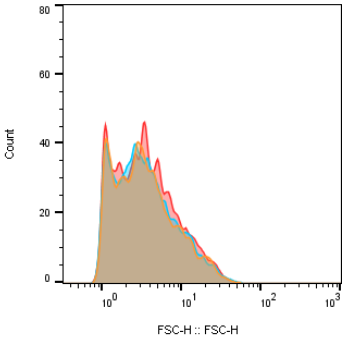
FP 50



FP 50
Chlorophyll Pos, Phycocyanin Neg

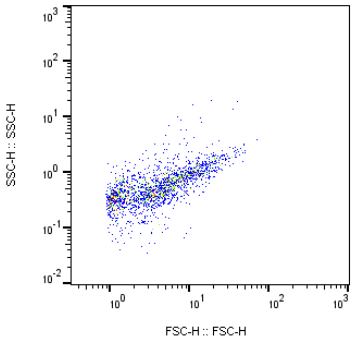


FP 50
Phycocyanin Pos

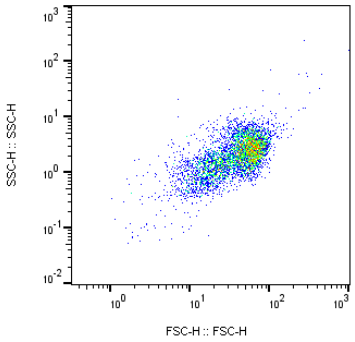


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2660
Chlorophyll Pos, Phycocyanin Neg	2725
Chlorophyll Pos, Phycocyanin Neg	2953

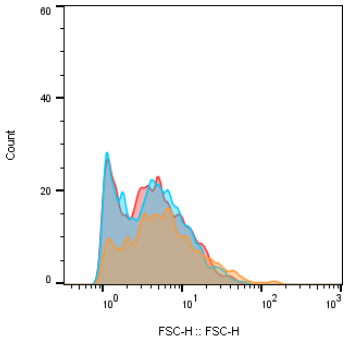
TP 50



TP 50
Chlorophyll Pos, Phycocyanin Neg

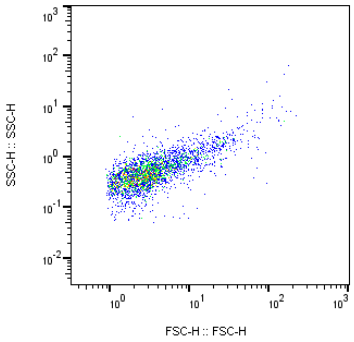


TP 50
Phycocyanin Pos

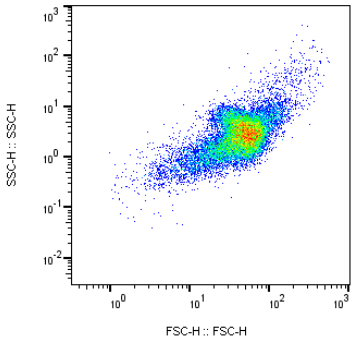


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1271
Chlorophyll Pos, Phycocyanin Neg	1833
Chlorophyll Pos, Phycocyanin Neg	1846

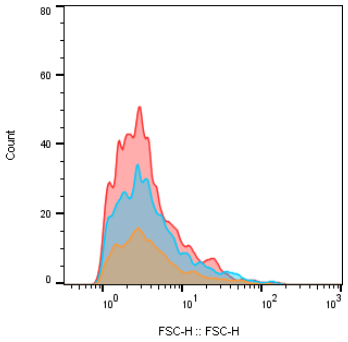
FP 51



FP 51
Chlorophyll Pos, Phycocyanin Neg

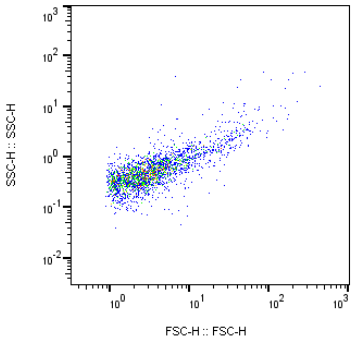


FP 51
Phycocyanin Pos

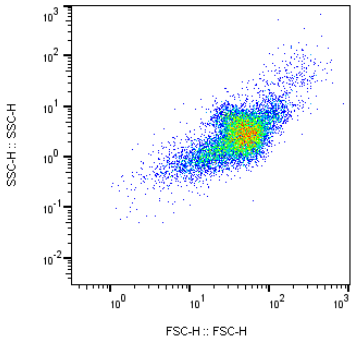


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	962
Chlorophyll Pos, Phycocyanin Neg	2024
Chlorophyll Pos, Phycocyanin Neg	2826

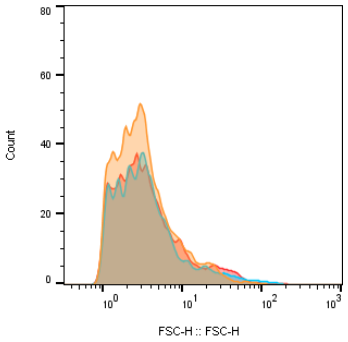
TP 51



TP 51
Chlorophyll Pos, Phycocyanin Neg

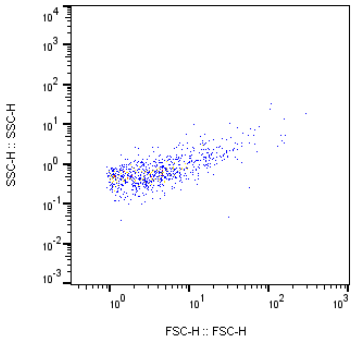


TP 51
Phycocyanin Pos

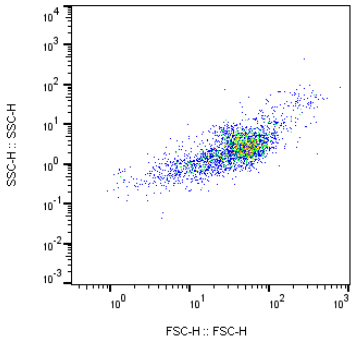


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2873
Chlorophyll Pos, Phycocyanin Neg	2216
Chlorophyll Pos, Phycocyanin Neg	2355

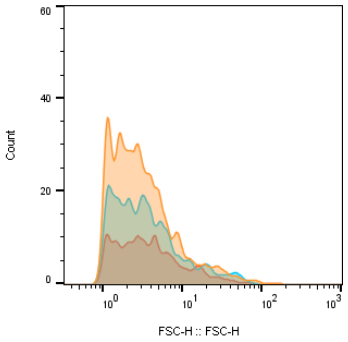
FP 52



FP 52
Chlorophyll Pos, Phycocyanin Neg

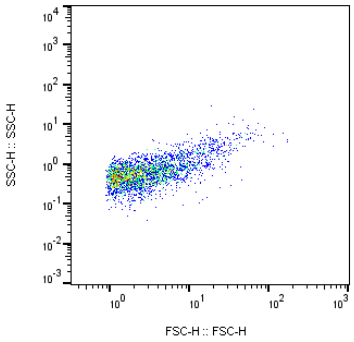


FP 52
Phycocyanin Pos

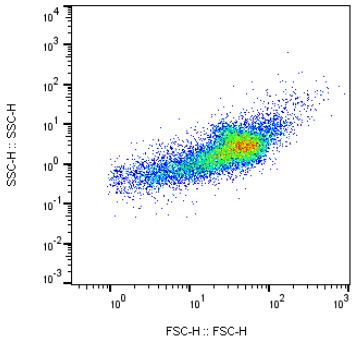


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	2075
Chlorophyll Pos, Phycocyanin Neg	1385
Chlorophyll Pos, Phycocyanin Neg	804

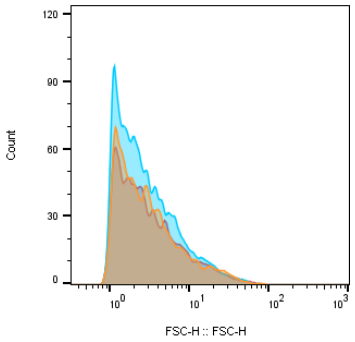
TP 52



TP 52
Chlorophyll Pos, Phycocyanin Neg

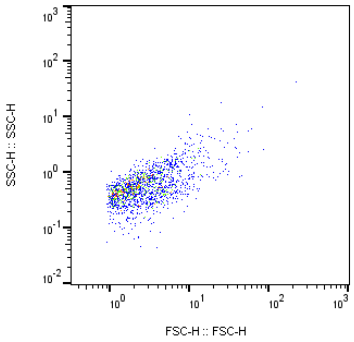


TP 52
Phycocyanin Pos

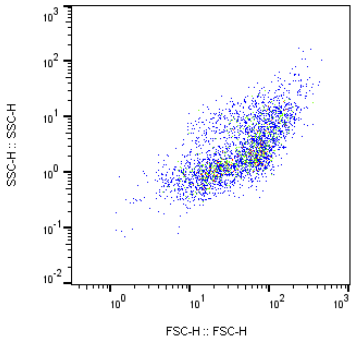


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3239
Chlorophyll Pos, Phycocyanin Neg	4287
Chlorophyll Pos, Phycocyanin Neg	3086

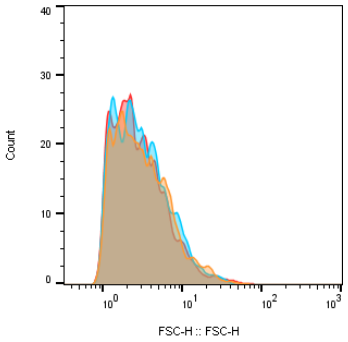
FP 53



FP 53
Chlorophyll Pos, Phycocyanin Neg

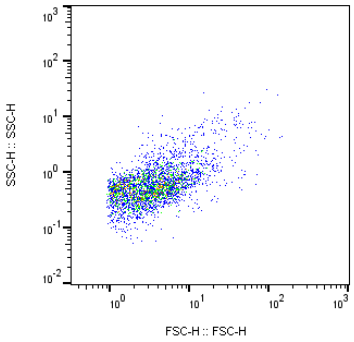


FP 53
Phycocyanin Pos

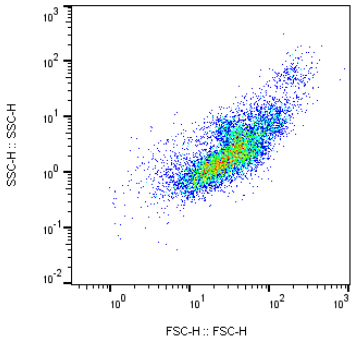


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1470
Chlorophyll Pos, Phycocyanin Neg	1594
Chlorophyll Pos, Phycocyanin Neg	1535

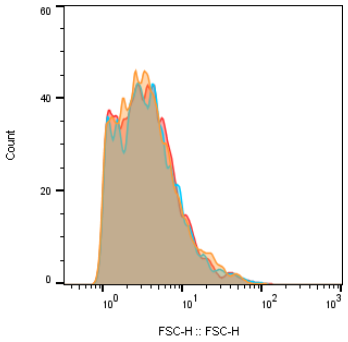
TP 53



TP 53
Chlorophyll Pos, Phycocyanin Neg

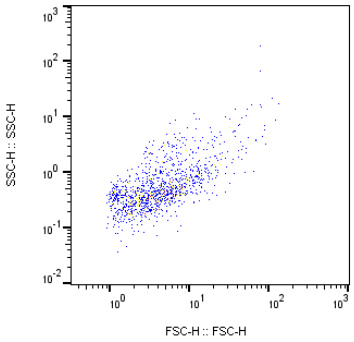


TP 53
Phycocyanin Pos

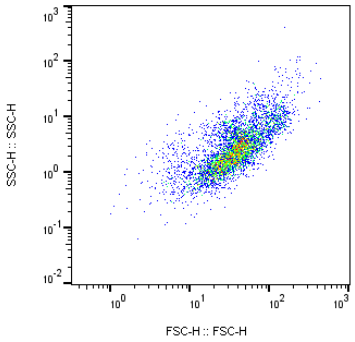


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3109
Chlorophyll Pos, Phycocyanin Neg	2933
Chlorophyll Pos, Phycocyanin Neg	3050

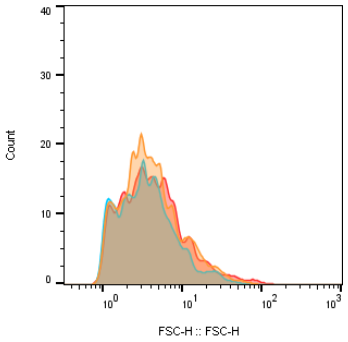
FP 54



FP 54
Chlorophyll Pos, Phycocyanin Neg

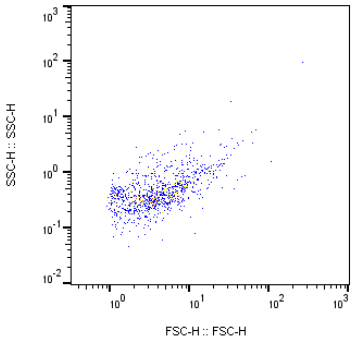


FP 54
Phycocyanin Pos

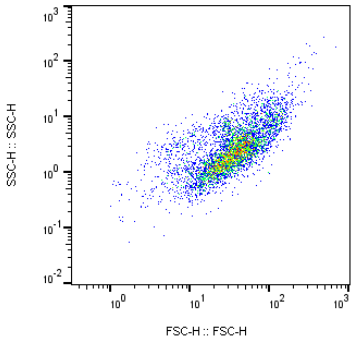


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1278
Chlorophyll Pos, Phycocyanin Neg	1043
Chlorophyll Pos, Phycocyanin Neg	1185

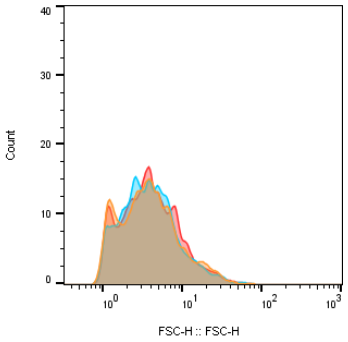
TP 54



TP 54
Chlorophyll Pos, Phycocyanin Neg

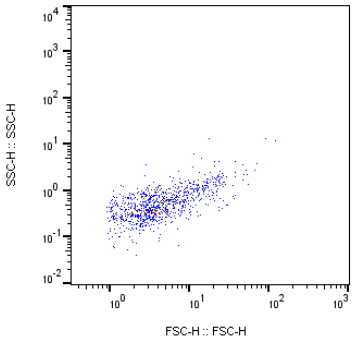


TP 54
Phycocyanin Pos

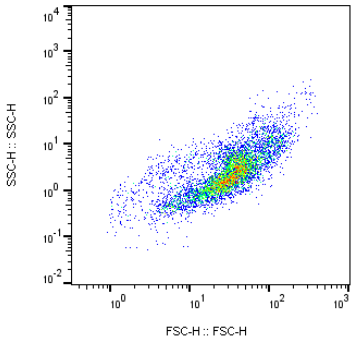


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	995
Chlorophyll Pos, Phycocyanin Neg	1000
Chlorophyll Pos, Phycocyanin Neg	1028

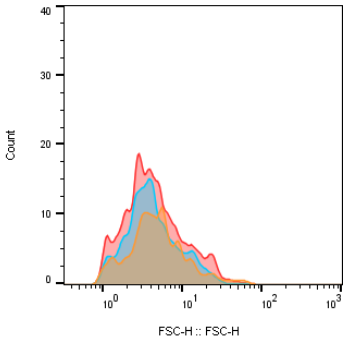
FP 55



FP 55
Chlorophyll Pos, Phycocyanin Neg

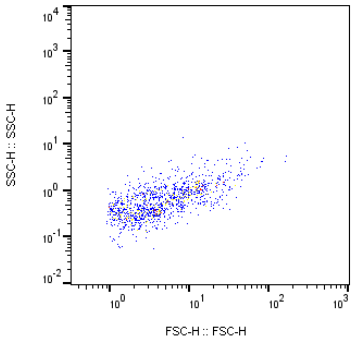


FP 55
Phycocyanin Pos

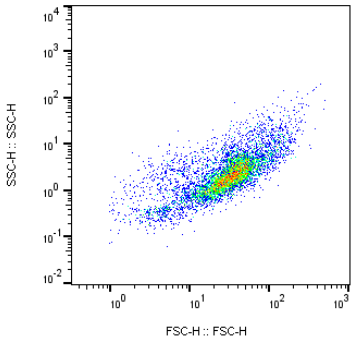


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	616
Chlorophyll Pos, Phycocyanin Neg	777
Chlorophyll Pos, Phycocyanin Neg	1054

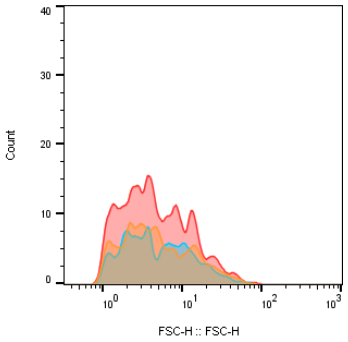
TP 55



TP 55
Chlorophyll Pos, Phycocyanin Neg

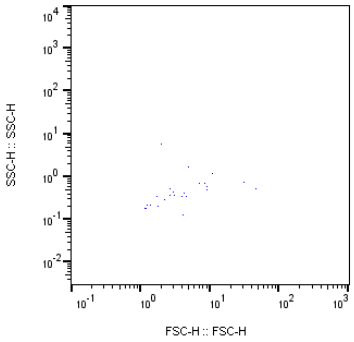


TP 55
Phycocyanin Pos

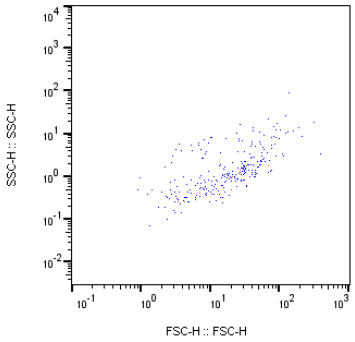


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	694
Chlorophyll Pos, Phycocyanin Neg	609
Chlorophyll Pos, Phycocyanin Neg	1174

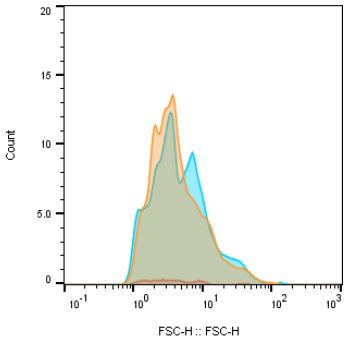
FP 56



FP 56
Chlorophyll Pos, Phycocyanin Neg

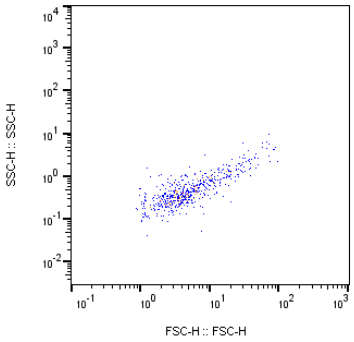


FP 56
Phycocyanin Pos

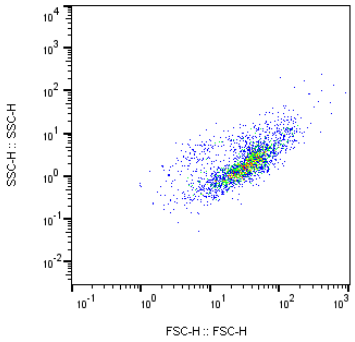


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	678
Chlorophyll Pos, Phycocyanin Neg	667
Chlorophyll Pos, Phycocyanin Neg	24.0

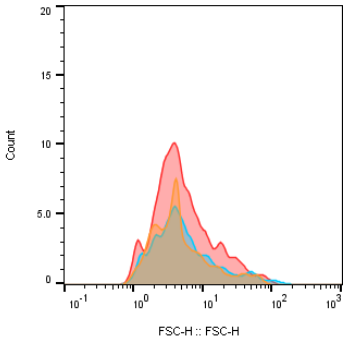
TP 56



TP 56
Chlorophyll Pos, Phycocyanin Neg

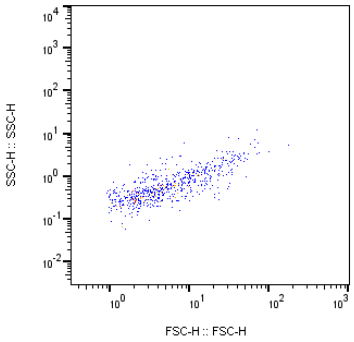


TP 56
Phycocyanin Pos

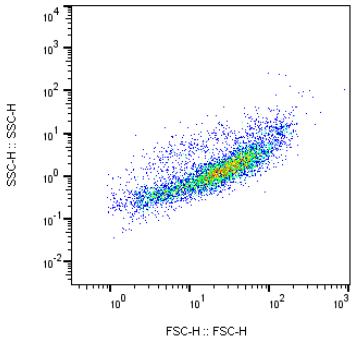


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	297
Chlorophyll Pos, Phycocyanin Neg	299
Chlorophyll Pos, Phycocyanin Neg	538

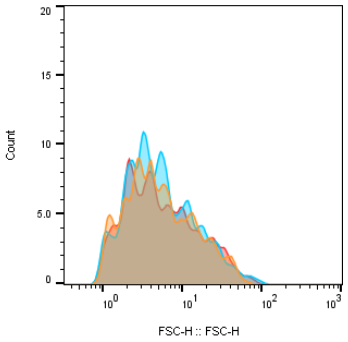
FP 57



FP 57
Chlorophyll Pos, Phycocyanin Neg

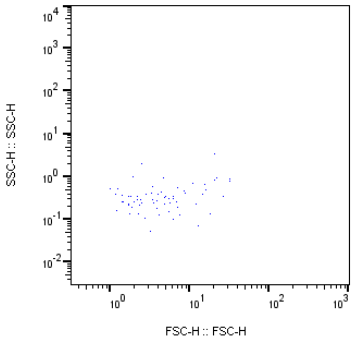


FP 57
Phycocyanin Pos

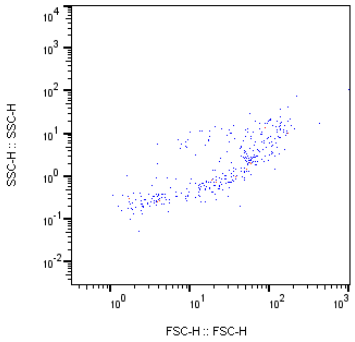


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	665
Chlorophyll Pos, Phycocyanin Neg	742
Chlorophyll Pos, Phycocyanin Neg	648

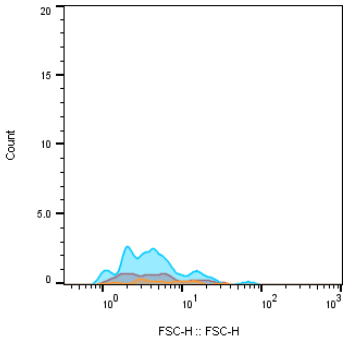
TP 57



TP 57
Chlorophyll Pos, Phycocyanin Neg

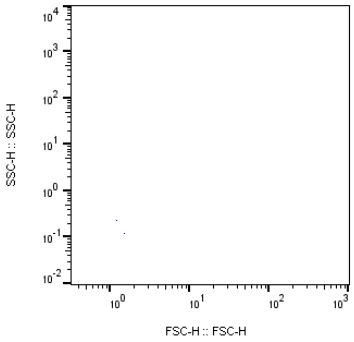


TP 57
Phycocyanin Pos

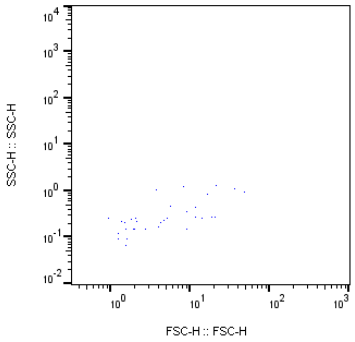


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	26.0
Chlorophyll Pos, Phycocyanin Neg	169
Chlorophyll Pos, Phycocyanin Neg	63.0

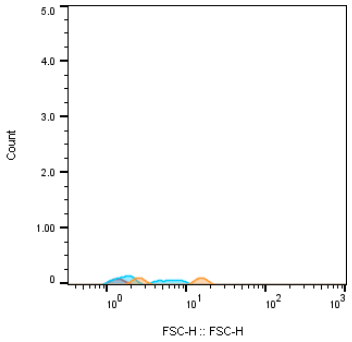
FP 58



FP 58
Chlorophyll Pos, Phycocyanin Neg

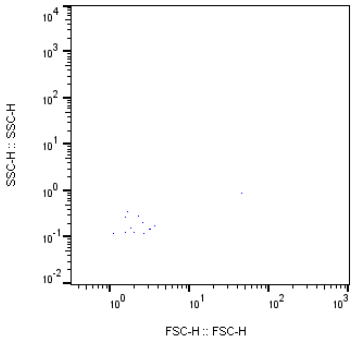


FP 58
Phycocyanin Pos

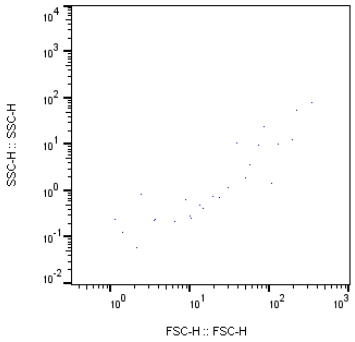


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4.00
Chlorophyll Pos, Phycocyanin Neg	7.00
Chlorophyll Pos, Phycocyanin Neg	2.00

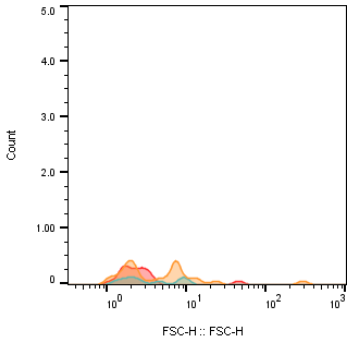
TP 58



TP 58
Chlorophyll Pos, Phycocyanin Neg

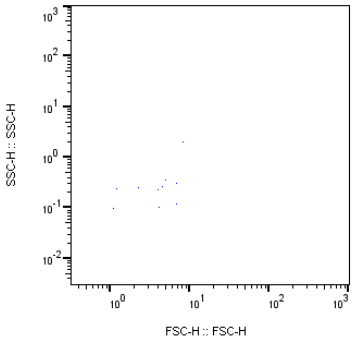


TP 58
Phycocyanin Pos

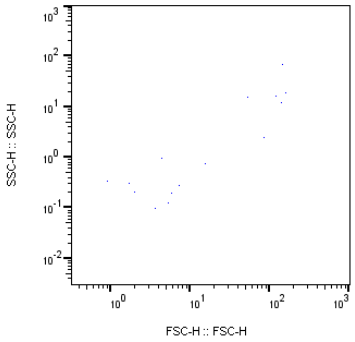


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	22.0
Chlorophyll Pos, Phycocyanin Neg	7.00
Chlorophyll Pos, Phycocyanin Neg	13.0

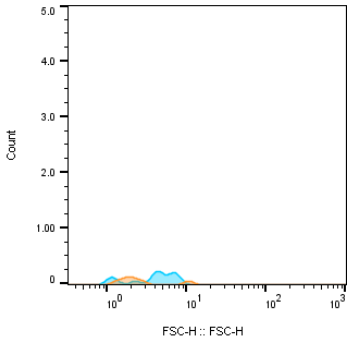
FP 59



FP 59
Chlorophyll Pos, Phycocyanin Neg

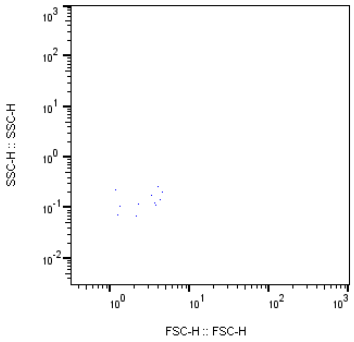


FP 59
Phycocyanin Pos

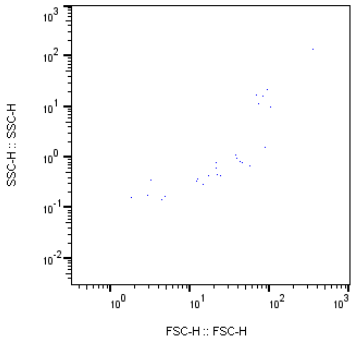


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5.00
Chlorophyll Pos, Phycocyanin Neg	10.0
Chlorophyll Pos, Phycocyanin Neg	0

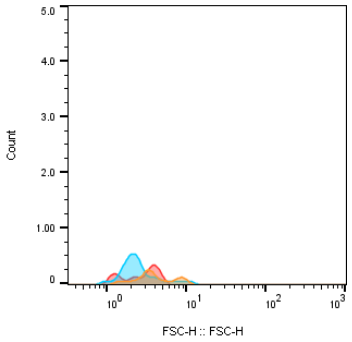
TP 59



TP 59
Chlorophyll Pos, Phycocyanin Neg

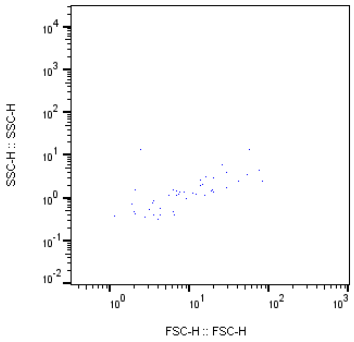


TP 59
Phycocyanin Pos

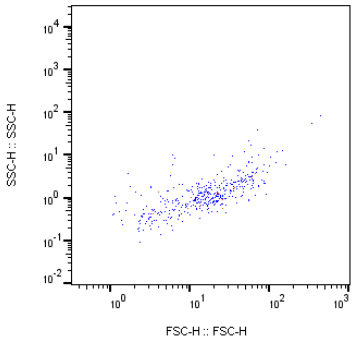


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	9.00
Chlorophyll Pos, Phycocyanin Neg	17.0
Chlorophyll Pos, Phycocyanin Neg	11.0

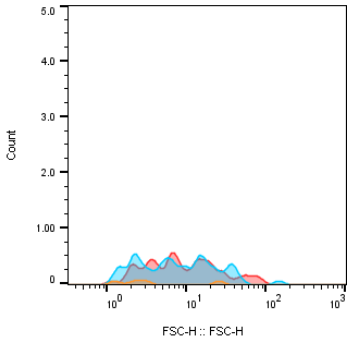
FP 60



FP 60
Chlorophyll Pos, Phycocyanin Neg

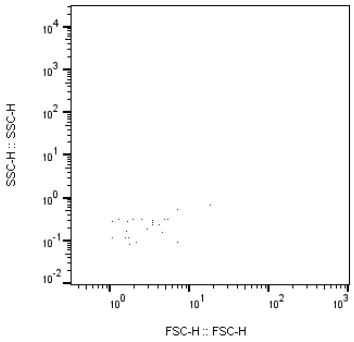


FP 60
Phycocyanin Pos

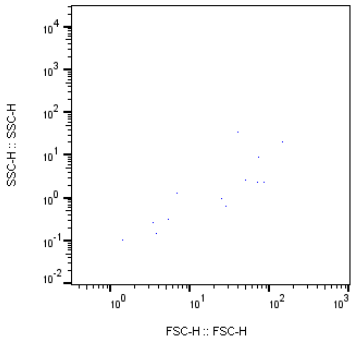


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4.00
Chlorophyll Pos, Phycocyanin Neg	47.0
Chlorophyll Pos, Phycocyanin Neg	43.0

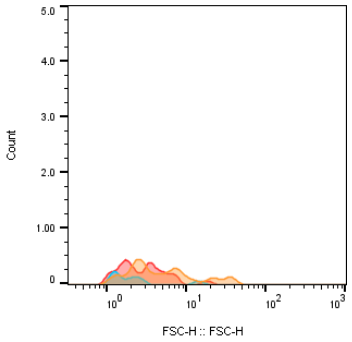
TP 60



TP 60
Chlorophyll Pos, Phycocyanin Neg

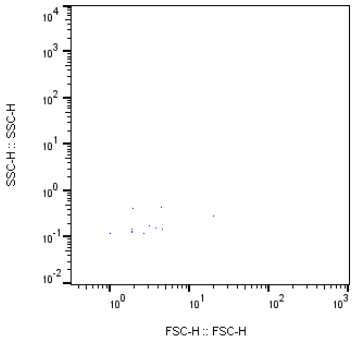


TP 60
Phycocyanin Pos

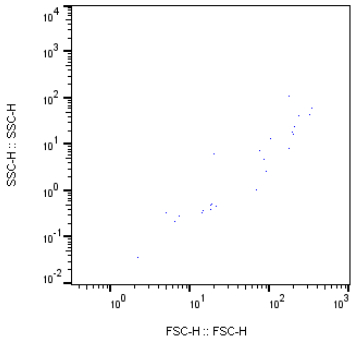


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	25.0
Chlorophyll Pos, Phycocyanin Neg	7.00
Chlorophyll Pos, Phycocyanin Neg	22.0

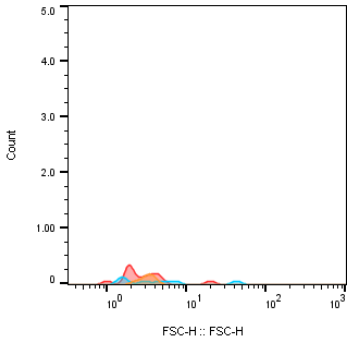
FP 61



FP 61
Chlorophyll Pos, Phycocyanin Neg

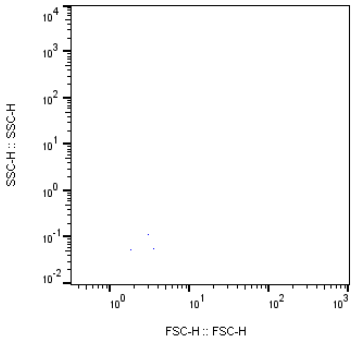


FP 61
Phycocyanin Pos

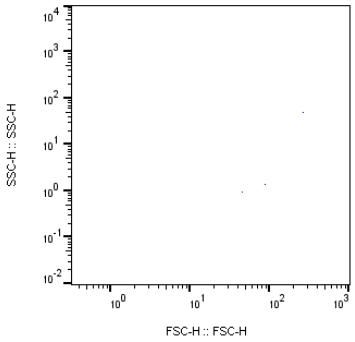


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	4.00
Chlorophyll Pos, Phycocyanin Neg	6.00
Chlorophyll Pos, Phycocyanin Neg	11.0

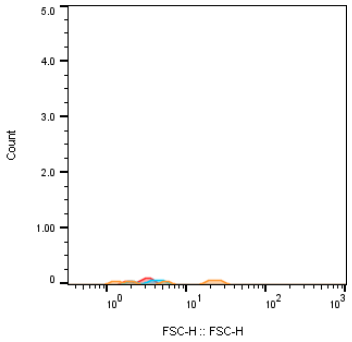
TP 61



TP 61
Chlorophyll Pos, Phycocyanin Neg

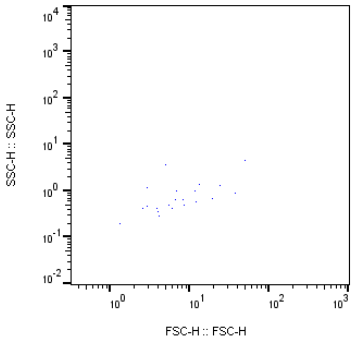


TP 61
Phycocyanin Pos

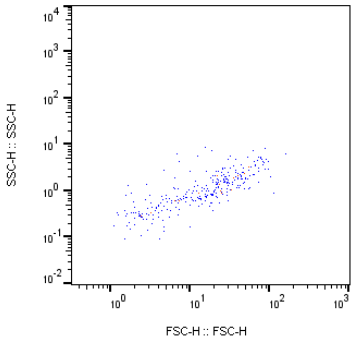


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	5.00
Chlorophyll Pos, Phycocyanin Neg	3.00
Chlorophyll Pos, Phycocyanin Neg	3.00

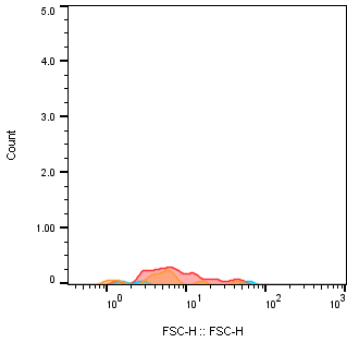
FP 62



FP 62
Chlorophyll Pos, Phycocyanin Neg

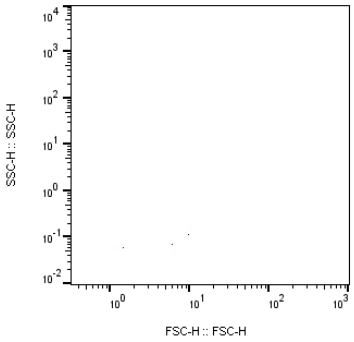


FP 62
Phycocyanin Pos

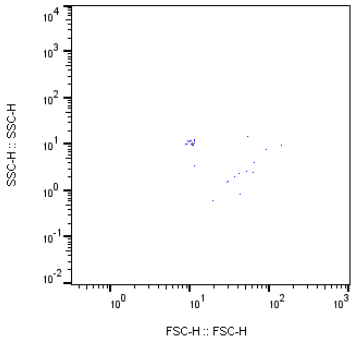


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	11.0
Chlorophyll Pos, Phycocyanin Neg	3.00
Chlorophyll Pos, Phycocyanin Neg	21.0

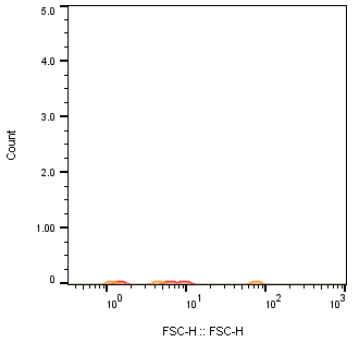
TP 62



TP 62
Chlorophyll Pos, Phycocyanin Neg

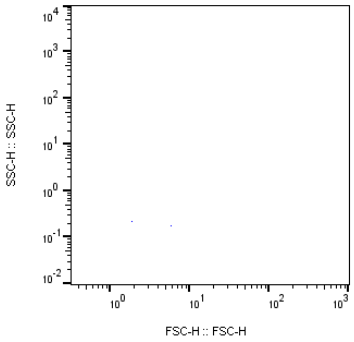


TP 62
Phycocyanin Pos

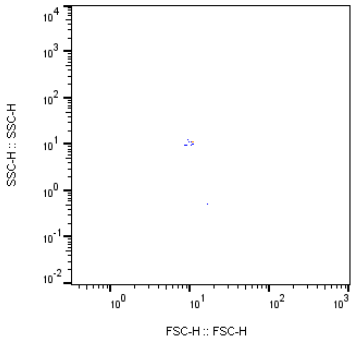


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	3.00
Chlorophyll Pos, Phycocyanin Neg	0
Chlorophyll Pos, Phycocyanin Neg	3.00

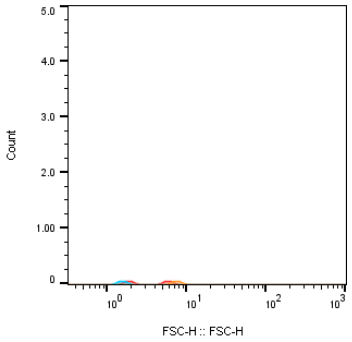
FP 63



FP 63
Chlorophyll Pos, Phycocyanin Neg

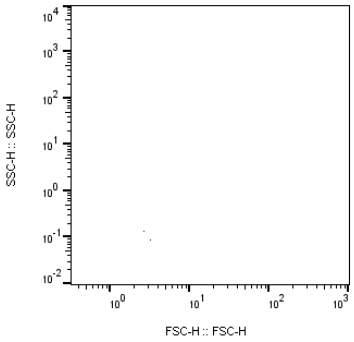


FP 63
Phycocyanin Pos

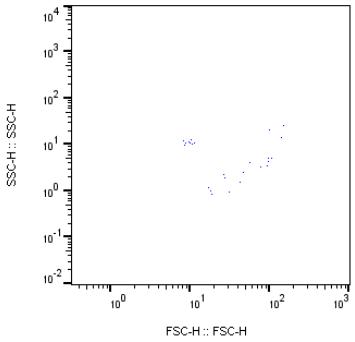


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1.00
Chlorophyll Pos, Phycocyanin Neg	1.00
Chlorophyll Pos, Phycocyanin Neg	2.00

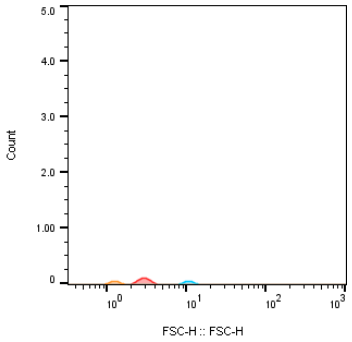
TP 63



TP 63
Chlorophyll Pos, Phycocyanin Neg

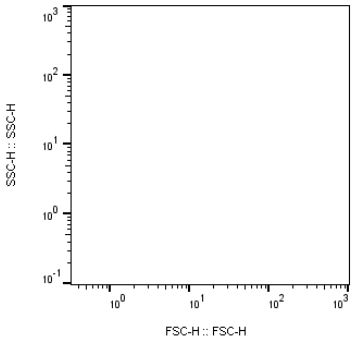


TP 63
Phycocyanin Pos

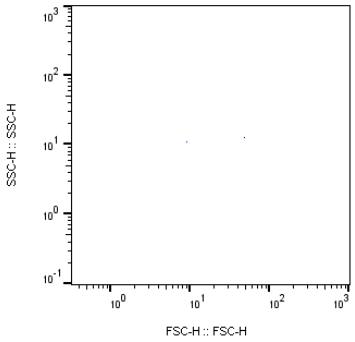


Subset Name	Count
Chlorophyll Pos, Phycocyanin Neg	1.00
Chlorophyll Pos, Phycocyanin Neg	1.00
Chlorophyll Pos, Phycocyanin Neg	2.00

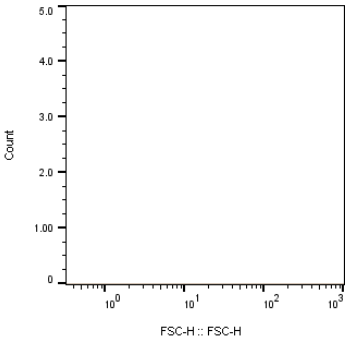
FP 64



FP 64
Chlorophyll Pos, Phycocyanin Neg

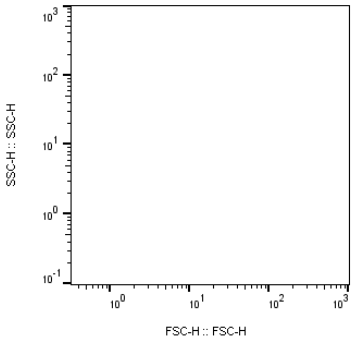


FP 64
Phycocyanin Pos

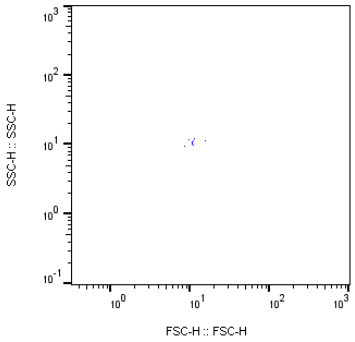


	Subset Name	Count
	Chlorophyll Pos, Phycocyanin Neg	0
	Chlorophyll Pos, Phycocyanin Neg	0
	Chlorophyll Pos, Phycocyanin Neg	0

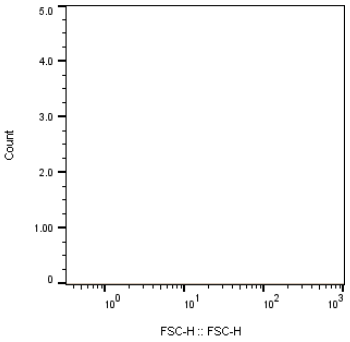
TP 64



TP 64
Chlorophyll Pos, Phycocyanin Neg



TP 64
Phycocyanin Pos



	Subset Name	Count
	Chlorophyll Pos, Phycocyanin Neg	0
	Chlorophyll Pos, Phycocyanin Neg	0
	Chlorophyll Pos, Phycocyanin Neg	0

Cosine Values of each Parameter Analysed using PCA Analysis for Selection of the most Representative Correlation Circle

FP

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
Nitrate	**0.397**	0.110	0.178	0.015	0.016	0.092	0.003	0.020	0.006	0.125	0.003	0.020	0.010	0.003	0.001	0.000
Nitrite	0.098	**0.405**	0.177	0.146	0.043	0.001	0.000	0.008	0.020	0.020	0.030	0.000	0.044	0.007	0.000	0.000
Ammonium	0.000	**0.393**	0.023	0.042	0.084	0.294	0.009	0.125	0.023	0.002	0.000	0.003	0.003	0.000	0.001	0.000
Phosphate	0.090	**0.447**	0.194	0.011	0.026	0.021	0.000	0.047	0.096	0.008	0.008	0.044	0.008	0.000	0.000	0.000
Temperature	0.301	0.054	**0.432**	0.049	0.016	0.007	0.099	0.000	0.004	0.003	0.006	0.001	0.000	0.009	0.019	0.000
pH	0.203	**0.535**	0.047	0.000	0.015	0.064	0.017	0.016	0.021	0.034	0.001	0.002	0.003	0.041	0.001	0.000
Oxygen	0.099	0.033	**0.487**	0.110	0.054	0.074	0.012	0.036	0.029	0.014	0.007	0.012	0.026	0.005	0.002	0.000
Turbidity	0.176	**0.376**	0.035	0.117	0.047	0.029	0.007	0.140	0.024	0.000	0.025	0.005	0.013	0.003	0.001	0.000
Hardness	0.018	0.041	0.246	**0.267**	0.164	0.138	0.055	0.026	0.002	0.015	0.011	0.000	0.014	0.003	0.000	0.000
Feeding Rate	0.039	0.112	0.237	0.202	0.001	0.000	**0.382**	0.007	0.002	0.013	0.000	0.001	0.003	0.000	0.000	0.000
Bacteria	0.184	0.044	**0.238**	0.153	0.219	0.045	0.005	0.008	0.009	0.055	0.000	0.031	0.002	0.007	0.001	0.000
Phytoplankton	**0.540**	0.001	0.001	0.038	0.231	0.000	0.005	0.004	0.109	0.018	0.026	0.025	0.001	0.000	0.001	0.000
Chl-Ctn Cells	**0.565**	0.156	0.142	0.001	0.062	0.031	0.005	0.015	0.002	0.003	0.011	0.001	0.005	0.001	0.000	0.000
Cyano Cells	**0.467**	0.177	0.164	0.000	0.114	0.033	0.004	0.015	0.009	0.001	0.008	0.003	0.005	0.001	0.001	0.000
Chl (Torch)	**0.765**	0.008	0.032	0.065	0.019	0.001	0.018	0.014	0.005	0.013	0.003	0.026	0.007	0.005	0.019	0.000
Cyano (Torch)	**0.711**	0.077	0.000	0.055	0.000	0.001	0.000	0.002	0.025	0.005	0.083	0.009	0.012	0.018	0.000	0.000

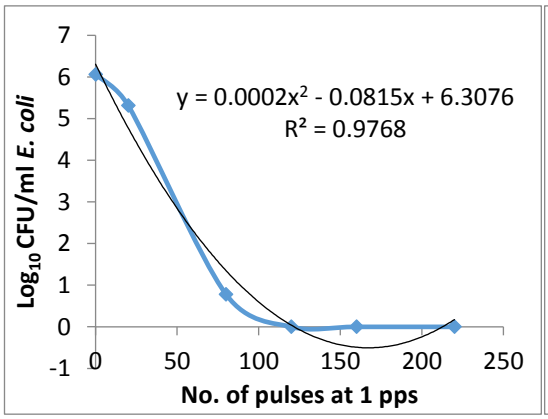
Values in bold correspond for each variable to the factor for which the squared cosine is the largest

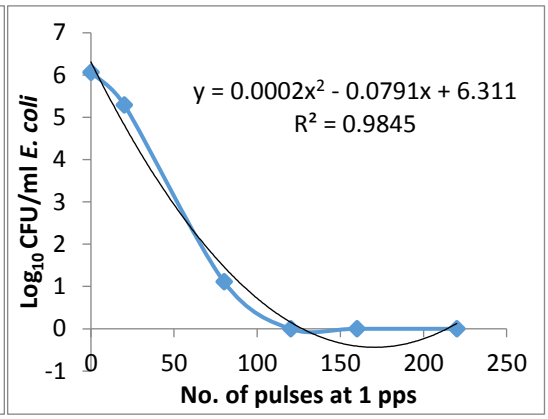
TP

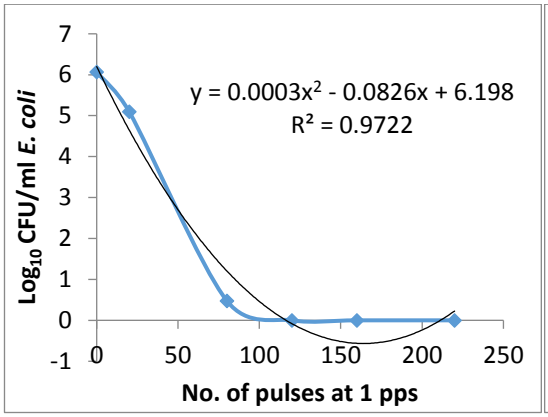
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
Nitrate	**0.454**	0.191	0.134	0.016	0.004	0.051	0.012	0.015	0.024	0.039	0.007	0.002	0.043	0.010	0.000	0.000
Nitrite	0.068	**0.512**	0.060	0.062	0.088	0.090	0.007	0.000	0.013	0.001	0.092	0.002	0.002	0.004	0.000	0.000
Ammonium	0.000	**0.463**	0.001	0.003	0.018	0.358	0.002	0.087	0.025	0.026	0.005	0.001	0.010	0.001	0.000	0.000
Phosphate	0.022	**0.530**	0.194	0.007	0.032	0.008	0.042	0.045	0.012	0.033	0.052	0.005	0.015	0.002	0.000	0.000
Temperature	0.351	0.008	**0.449**	0.040	0.014	0.005	0.065	0.024	0.000	0.012	0.000	0.005	0.000	0.006	0.021	0.000
pH	0.227	**0.424**	0.106	0.002	0.077	0.048	0.023	0.004	0.029	0.006	0.005	0.001	0.014	0.032	0.002	0.000
Oxygen	0.080	0.077	**0.452**	0.200	0.000	0.047	0.002	0.061	0.022	0.001	0.002	0.049	0.000	0.006	0.002	0.000
Turbidity	0.154	**0.328**	0.095	0.052	0.053	0.041	0.034	0.174	0.023	0.018	0.005	0.012	0.006	0.004	0.001	0.000
Hardness	0.015	0.232	0.006	0.044	**0.498**	0.092	0.043	0.012	0.044	0.000	0.011	0.001	0.000	0.001	0.000	0.000
Feeding Rate	0.069	0.040	0.284	0.153	0.002	0.022	**0.311**	0.079	0.021	0.004	0.010	0.001	0.003	0.000	0.000	0.000
Bacteria	0.211	0.008	0.220	**0.286**	0.104	0.002	0.058	0.002	0.073	0.016	0.001	0.001	0.013	0.003	0.001	0.000
Phytoplankton	**0.582**	0.048	0.000	0.078	0.067	0.035	0.036	0.025	0.002	0.102	0.004	0.001	0.018	0.000	0.002	0.000
Chl-Ctn Cells	**0.576**	0.103	0.183	0.027	0.005	0.033	0.044	0.016	0.002	0.000	0.007	0.001	0.002	0.001	0.000	0.000
Cyano Cells	**0.472**	0.101	0.227	0.055	0.015	0.058	0.038	0.012	0.002	0.004	0.007	0.002	0.006	0.001	0.001	0.000
Chl (Torch)	**0.774**	0.003	0.030	0.060	0.001	0.003	0.040	0.003	0.032	0.001	0.002	0.016	0.002	0.016	0.017	0.000
Cyano (Torch)	**0.670**	0.061	0.001	0.086	0.008	0.021	0.006	0.011	0.025	0.013	0.000	0.087	0.001	0.011	0.000	0.000

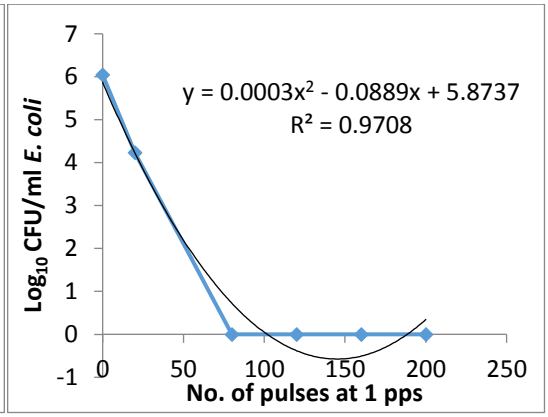
Values in bold correspond for each variable to the factor for which the squared cosine is the largest

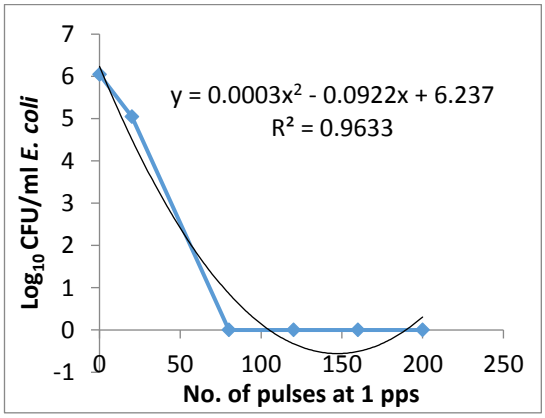
Kill curves for *E. coli* following PUV inactivation, displaying the equation of the line in order to calculate death values



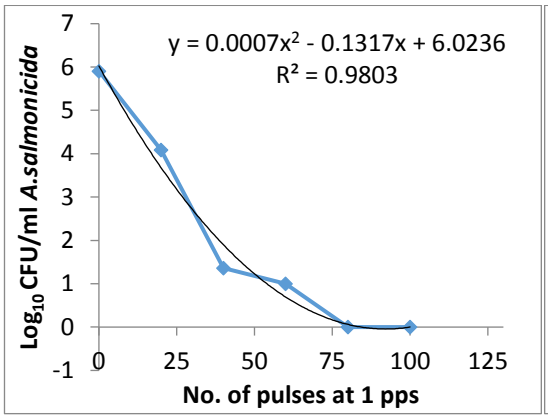


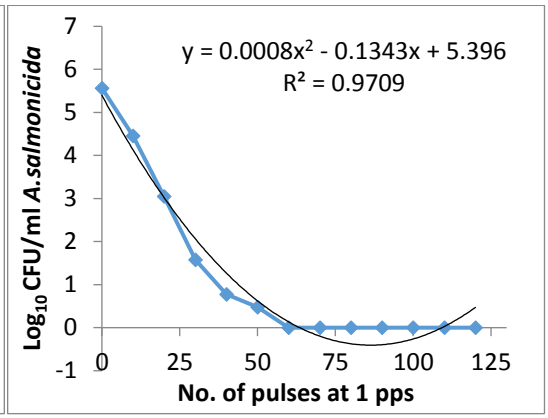


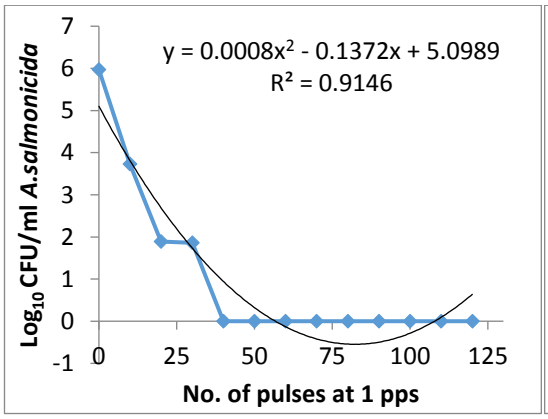


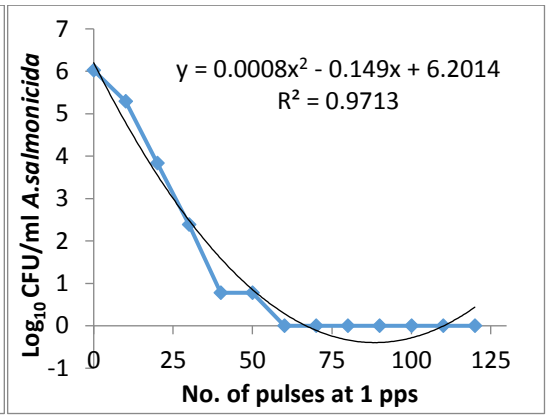


Kill curves for *A. salmonicida* following PUV inactivation, displaying the equation of the line in order to calculate death values

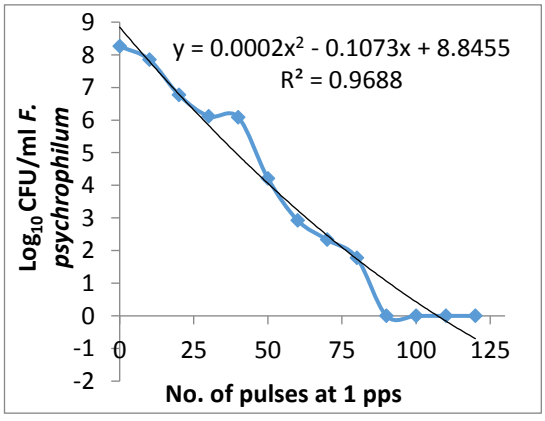


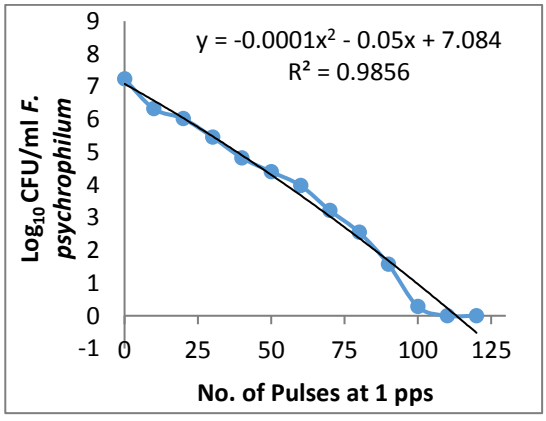




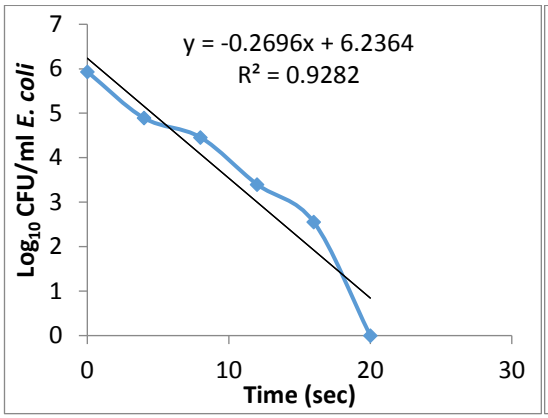


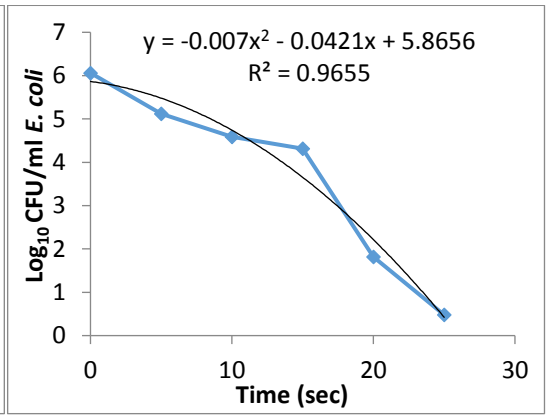
Kill curves for *A. salmonicida* following PUV inactivation, displaying the equation of the line in order to calculate death values

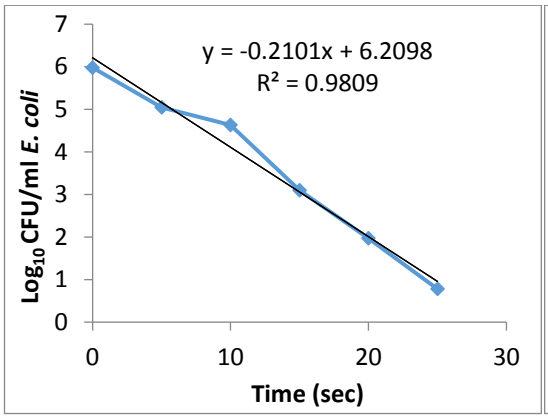


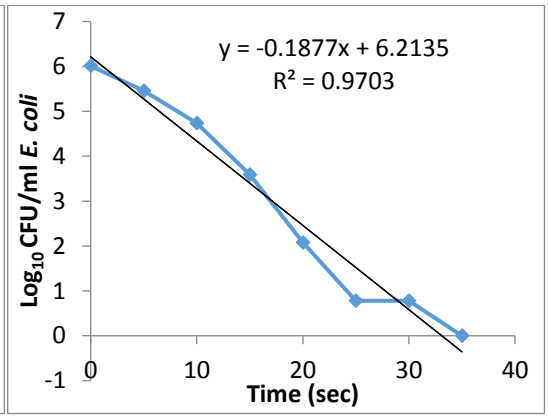


Kill curves for *E. coli* following static UV inactivation, displaying the equation of the line in order to calculate death values

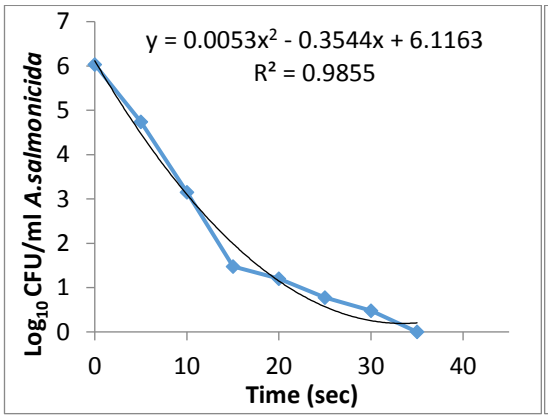


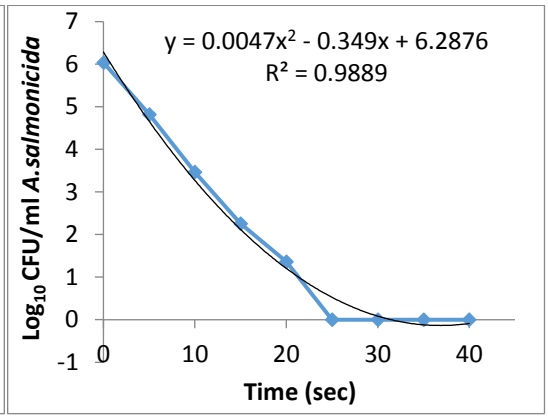


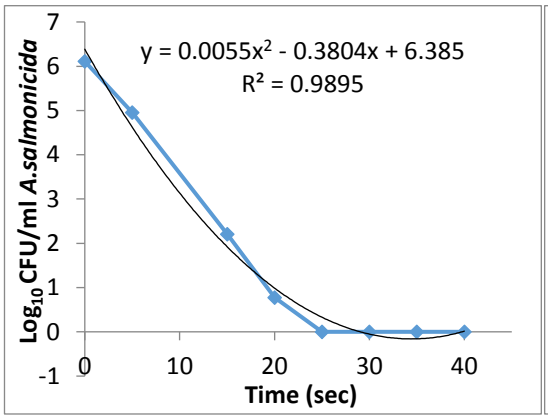


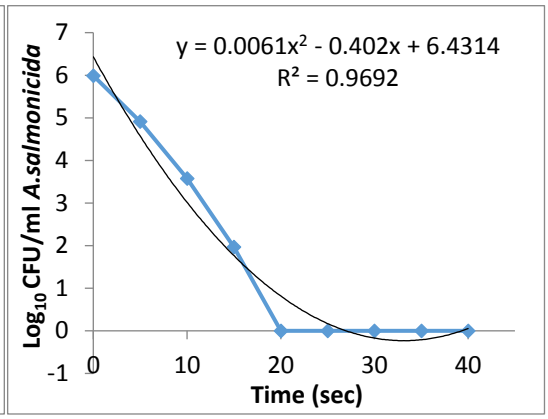


Kill curves for *A. salmonicida* following static UV inactivation, displaying the equation of the line in order to calculate death values









Appendix B

Novel use of real-time flow cytometry and in-field AlgaeTorch® technologies to evaluate key parameters influencing the diversity and wastewater treatment role of microalgae in a freshwater aquaculture recirculation system: A case study from the Republic of Ireland

Sarah Naughton[1*], Siobhán Kavanagh[1,2], Mark Lynch[1], Neil J Rowan[1,3]

[1]Bioscience Research Institute, Athlone Institute of Technology, Ireland
[2]Department of Life and Physical Science, Athlone Institute of Technology, Ireland
[3]Centre for Disinfection, Sterilisation and Biosecurity, Athlone Institute of Technology, Ireland

*Corresponding author: s.naughton@research.ait.ie

Highlights

- Flow-cytometry correlated with in-field AlgaeTorch for analysing microalgae in aquaculture
- Microalgae and Cyanobacteria were dominant in rearing and treatment ponds
- PCA analysis reveal nitrates and temperature as main parameters influencing microalgae
- Drought conditions did not affect microalgae occurrence in freshwater aquaculture
- *Chlorophyta, Bacillariophyta* and *Cryptophyta* were the most dominant algal phyla

Abstract

Traditional aquaculture produces large amounts of wastewater threatening global sustainability. There has been growing interest in exploiting microalgae as a natural process for low cost wastewater treatment and for water quality control in aquaculture. This constitutes the first study to report on a strong relationship

between use of sophisticated flow cytometry and in-field AlgaeTorch technologies for determining microalgae and bacteria in a freshwater pill-pond aquaculture farm over a 10-month monitoring period producing Eurasian Perch, *Perca fluviatilis*. Nitrate levels and temperature were the most significant factors influencing microalgae numbers in rearing and treatment ponds as determined by Principle Component Analysis (PCA). Drought conditions did not affect microalgae or microbial numbers. *Chlorophyta, Bacillariophyta* and *Cryptophyta* were the most dominant algal divisions observed in this recirculation aquaculture system. Determining baseline microalgal profile in rearing water, followed by elucidating physicochemical parameters governing wastewater treatment performance, can inform future intensification and diversification of freshwater aquaculture by exploiting and replicating knowledge of favourable algal-microbial ecosystems.

Keywords

Microalgae, freshwater aquaculture, waste treatment, resource utilization, sustainability

1. Introduction

Aquaculture has become the fastest-growing food sector in the world (FAO, 2018a). The growing human population, the exhaustion of ocean fisheries stocks, and the increased consumption rates *per capita* are incentives for the aquaculture industry to expand its growth (Crab *et al*., 2007; Zhang *et al*., 2011; FAO, 2016). According to FAO (2018b), the aquaculture industry is achieving a remarkable growth in fish production to meet this rapidly increasing demand for human consumption. During the period 2010 to 2030, global aquaculture production needs to increase threefold in order to meet the demands for fish and food (DAFM, 2015). In the aquaculture industry, water quality needs to be closely monitored in order to maintain, as closely as possible, the optimal growth conditions for a given cultured fish, and consequently to ensure optimal production (European Food Safety Authority (EFSA), 2008a). Commensurately, water pollution has become a concern, posing threats to environmental protection that will retard intensive sustainability of aquaculture globally. To overcome these challenges, significant effort has been devoted to control wastewater pollution and to improve water quality control in aquaculture (Han *et al*., 2019). However, traditional environmental remediation approaches, such as aeration, filtration and other physical technologies require high energy consumption or substantial add to the investment that increases total cost and financial burden of the industry (Longo *et al*., 2016; Tahar *et al*., 2019). These traditional technologies are often unable to fully utilize and recycle resources such as nutrients (including

nitrogen, phosphorous and carbon) along with producing large amounts of CO_2 and sludge that cause secondary environmental pollution (Lu *et al*., 2019a). Moreover, antibiotics and medicines are frequently used in aquaculture to mitigate against disease and to reduce risk of outbreaks, which adds to growing concerns over antibiotic-resistance crisis globally (Muziasari *et al*., 2016). Consequently, there has been growing interest in the development of alternative or complementary environmental-friendly and economically feasible solutions to advance aquaculture.

In aquaculture, fish are reared at high densities for increased productivity, which can lead to the build-up of in-organic nutrients, excreted waste and feed remnants that can lead to unwanted eutrophication in the receiving aquatic environment (Bentzon-Tilia *et al*., 2016). When conditions are optimal, namely high nutrient loads, high temperature and sunlight, algae can grow to an excessive number forming unwanted blooms in the rearing water that can have a detrimental effect on fish health (Drikas *et al*., 2001). With high density rearing practices, harmful pathogens also have a greater chance of rapid circulation and persistence resulting in the potential to cause a disease outbreak. However, microalgae are capable of assimilating nutrients in eutrophic water bodies (Leng *et al*., 2018) along with wastewater remediation from many sources including food industry, agriculture and municipal effluents (Han *et al*., 2019). *Chlorella* species and *Scenedesmus* species have been previously reported to positively contribute to the natural treatment of wastewater due to their efficiency at nutrient, antibiotic and heavy metal removal (Nurdogan and Oswald, 1995; Min *et al.*, 2011; Chen *et al.*, 2012; Choi and Lee, 2012; Godos *et al.*, 2012; Delgadillo-Mirquez *et al.*, 2016; Delrue *et al.*, 2016). In addition to treating wastewater, microalgae can also synthesize value-added components such as proteins, lipids and natural pigments (Han *et al*., 2019). Previous researchers have reported on the value-added biomass derived from microalgae activities that could contribute to aquaculture feed along with augmenting immunity of farmed fish (Sirakov *et al*., 2015; Ansari *et al*., 2017). Microalgae-assisted aquaculture generates oxygen as a natural aeration process that can also influence and adjust microbial communities, which if effectively controlled, could be applied to negate oxygen depletion and unwanted algal blooms (Han *et al*., 2019; Lu *et al*, 2019b).

Thus, a biotic balance should be achieved to ensure that the algal and bacterial numbers and populations are having this positive influence on productivity, rather than negatively influencing rearing water quality and consequently the production and efficiency of an aquaculture farm. However, there is a lack of knowledge regarding microbial interactions, and ecology of these systems, that prevents the utilization of microbial communities in the assessment, improvement and control of aquaculture farms (Bentzon-Tilia, Sonnenschien & Gram, 2016). There is increasing evidence to suggest that co-dependent relationship exists between phytoplankton and nutrients in rearing water, as phytoplankton abundance depends on nutrient

availability and nutrient cycling depends majorly on the presence of phytoplankton (Xuemei *et al*., 2011). This highlights how important the microalga balance is in rearing water, as not only do the algae produce oxygen during daylight (Moriarty, 1997), but also recycle metabolites that would otherwise build up in the water. Moriarty (1997) also highlights the importance of nitrogen and phosphorus on microalgae productivity. However, there is a gap in the knowledge of real-time and simultaneous determinations of algal and microbial communities and putative relationship with physicochemical and environmental parameters to inform sustainability in aquaculture.

The aim of this timely study was to gain a comprehensive understanding of the role of microalga species in freshwater aquaculture over a 10-month monitoring period using both in-field and real-time measurements to define and enhance a freshwater aquaculture process.

2. Materials and Methods

2.1 Study Site and Sampling

Samples were collected from a freshwater fish farm located in Boyle, Co. Sligo, the Republic of Ireland that produces Eurasian perch (*Perca fluviatilis*). It contains broodstock tanks, a hatchery for eggs and larvae and a nursery for juvenile growth. All of these indoor systems are based in tanks and operate under RAS. There are three grow out-ponds for the larger fish. These are earthen pill-pond systems based on low surface flow water sourced from a local stream. This study focused on one of the grow out ponds, designated Pond 1 that is denoted by the red perimeter shown in Fig. 1. Pond 1 is divided into the fish pond (FP) that is stocked with adult perch, which is connected to a treatment pond (TP) devoid of fish. Flow is circulated in and out of the FP region using paddle wheels guided by walls to aid aeration. The TP supports the growth of microalgae that also provides oxygenation and wastewater remediation. A schematic is also inserted into Fig. 1 to help contextualize the operational concept. Sampling occurred in triplicate from March 2018 to November 2018 in order to capture seasonality as briefly outlined in Table 1. Samples were transported in a cooler box to the laboratory for analysis. Preserved samples were stored at 4°C further analysis was carried out.

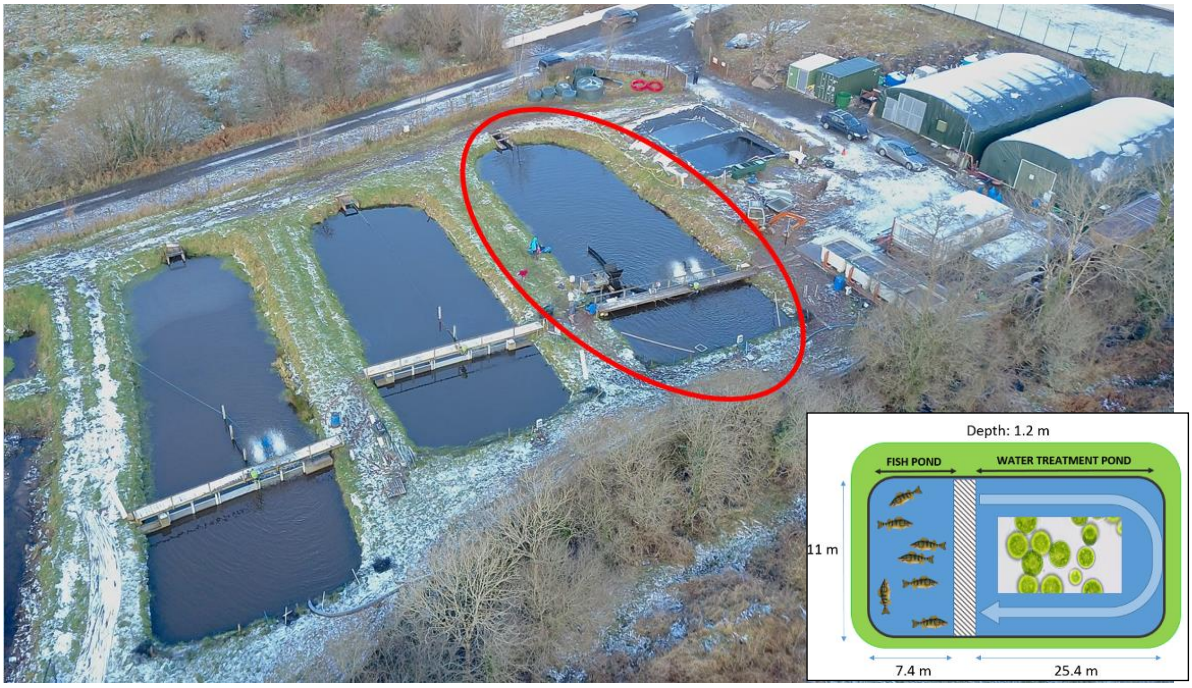


Fig. 1. Perch farm flow through ponds - aerial view, with insert schematic of Pond 1 layout.

Table 1. Sampling regime for obtaining rearing water profile at the perch farm.

Sample Type	Volume	Application	Frequency	Preservative
Algal	500 ml	Enumeration, Identification & Profile Development	Biweekly	1% Lugol's Iodine
Bacterial	500 ml	Enumeration	Biweekly	1% Formaldehyde
Physicochemical	500 ml	Parameter Measurement	Biweekly	Sulphuric Acid

2.2 Physicochemical Parameter Measurement

Physicochemical parameters that were measured in the laboratory included nitrates, nitrites, ammonium and phosphorus concentrations, pH and carbonate hardness. Whilst nitrates, nitrites, ammonia, phosphate, pH, oxygen and turbidity were measured *in situ* on the farm by standard methods. All parameters measured in the lab were carried so using individual test kits for each specific parameter as outlined in Table 2. After initial preservation with sulphuric acid, the pH was increased to between 6 to 7 that was required for all tests. Each test was carried out as per the individual kit instruction manual in duplicate. A Jenway UV-Vis Spectrophotometer was used for the spectrophotometric tests.

Table 2. Test kits used for the physicochemical analysis of rearing water samples.

Parameter	Test	Method Basis
Nitrates	Spectroquant® Nitrate Test – 1.09713	Photometric

Nitrites	Spectroquant® Nitrite Test – 1.14776	Photometric
Ammonium	Spectroquant® Ammonium Test – 1.14752	Photometric
Phosphate	Spectroquant® Phosphate Test – 1.14848	Photometric
pH	pH 1100 L Meter - VWR	pH electrode
Carbonate Hardness	MColortest™ Carbonate Hardness Test – 1.08048	Titration

2.3 Use of Flow Cytometry to Enumerate Microalgae

The MACSQuant® Analyzer 10 (Miltenyi Biotec), flow cytometry (FCM) system was used for this study. Samples were centrifuged at 3,500 x *g* for 20 mins, thereafter the cell pellet was resuspended in running buffer (PBS with 1 mM EDTA, 0.2% Tween, 0.1% sodium azide, 0.2 µm filtered). Samples were stained with nucleic acid dye SYBR Green (1:10,000X), to separate the DNA-containing cells from cellular debris and sedimentation present in the pond. SYBR Green fluorescence was detected on the B1 channel. An unstained algal control was required to eliminate natural auto-fluorescence in the B1 channel caused by excitation by the blue laser. Relative Cell Size and granularity was determined by forward scatter (FSC) and side scatter (SSC) channels. The blue laser stimulates chlorophyll (Chl) fluorescence on the B3 channel, and red laser stimulates phycocyanin (PC) fluorescence (R1 channel). Each sample was analysed in triplicate.

The gating method to obtain and enumerate the desired population, i.e. algae excluding cyanobacteria, was adapted from Moorhouse *et al*. (2018), Haynes *et al*. (2015) and Read *et al*. (2014) for phytoplankton and plankton analysis. This process is explained in Fig. 2 and involved running an unstained representative for each sample for the elimination of auto-fluorescence. This gate was used to determine DNA-containing cells in the SYBR Green-stained samples, without natural autofluorescence interference. Fig. 2(B) illustrates the distribution of cells present with all auto-fluorescent cells or unwanted material falling outside of the area/gate of interest. The Chl^+ population was isolated by acquiring the cells that fluoresced in the B3 channel. Fig. 2(C) illustrates the Chl^+ population with FSC on the x-axis relating to cell size and SSC on the y-axis relating to cell granularity and complexity. Cyanobacteria were present in this population as they also possess chlorophyll. This group was eliminated by graphing B3 against R1, and gating around the Chl^+/PC^- population. This is illustrated in Fig. 2(D) The final population depicted in Fig. 2(E) is the population of interest viewed under FSC and SSC. The isolated cyanobacterial populations from each sample were also analysed and enumerated to determine the trends over the duration of the study. All flow cytometry data gating was carried out using FlowJo software package.

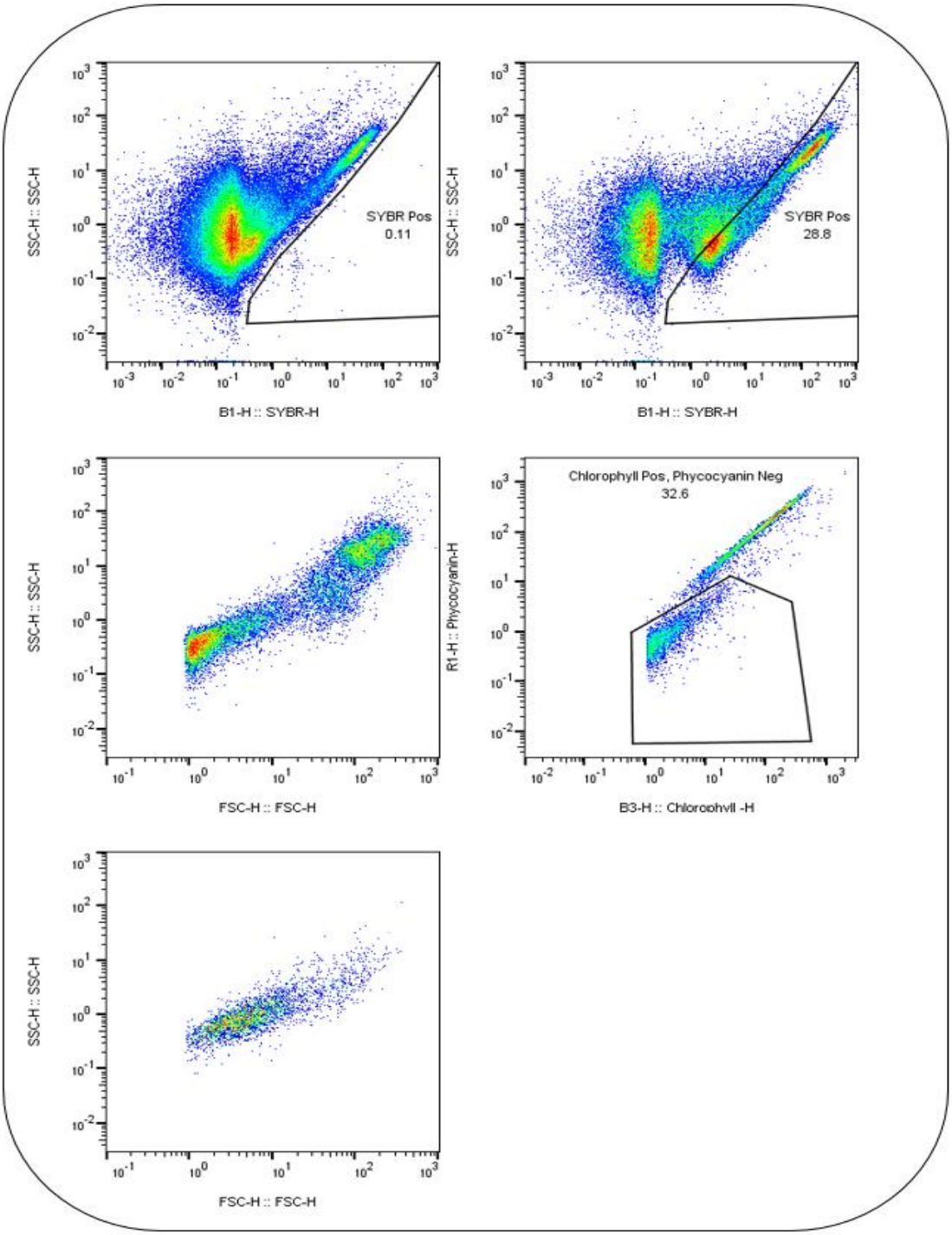


Fig. 2. Flow cytometry algal gating process involving **(A)** Gating for autofluorescence in the unstained sample, **(B)** Gating for relevant living organisms in the SYBR Green stained sample, **(C)** Chlorophyll positive population of the living cells viewed under FSC vs SSC, **(D)** Gating to eliminate cyanobacterial population and **(E)** Algal population of interest for enumeration viewed under FSC vs. SSC.

2.4 Microalga Profile Development

Biomass analysis tends to be an insufficient method for speciation of phytoplankton that lack distinctive features (Xuemei *et al*., 2011), which highlights the importance

of traditional microscopic analysis, which complements the emerging techniques, such flow cytometry, in aquaculture research. There are many ways of counting phytoplankton through the traditional slide methods such as the Sedgewick-Rafter slide, the Palmer-Maloney slide, the standard haemocytometer and the Petroff-Hausser slide for cells of bacterial dimension (Guillard, 1978). The most common method for phytoplankton enumeration, especially for multispecies communities, is the Utermöhl method, however, this requires an inverted microscopic with sophisticated optics in order to ensure reliable results (Vuorio *et al.*, 2007) which was not available for this project. There is also a lot of intervariation between operators of the method, and variation between microscopes used (Vuorio *et al.*, 2007). Therefore, identification using a standard inverted microscope for morphological analysis and photographic identification keys were used in conjunction with flow cytometry for enumeration to maximise the information obtained.

2.5 In-field AlgaeTorch Monitoring

In addition to FCM analysis of microalgal populations, the chlorophyll and Cyanobacteria populations were also measured *in situ* using the AlgaeTorch (bbe Moldaenke). The AlgaeTorch is based on real-time *in vivo* fluorescent measurement upon excitation of the microalgal cells in response to six LEDs of three different wavelengths, 470, 525 and 610nm. The measurement analysis carried out is in accordance with ISO 10260:1992 Water quality – Measurement of biochemical parameters – Spectrometric determination of the chlorophyll-a concentration and DIN 38412/16:1985 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 (L 16). Both chlorophyll and cyanobacterial content were measured in µg chl-a/l (bbe Moldaenke, 2017).

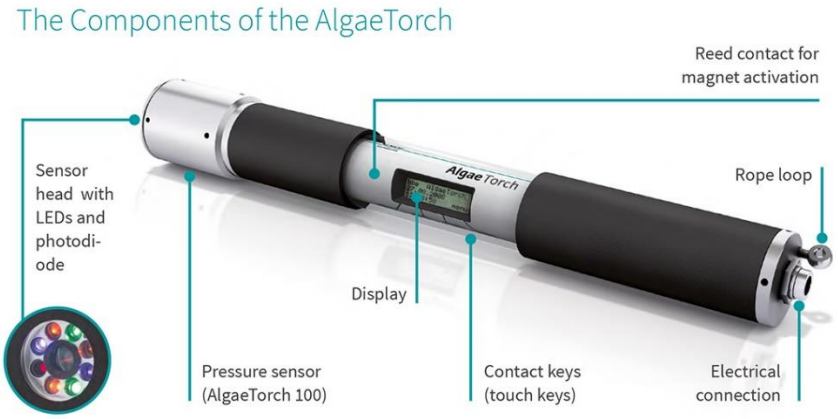


Fig. 3. AlgaeTorch diagram outlining the main components for operation (Source: bbe Moldaenke).

2.6 Bacterial Enumeration using Epifluorescent Microscopy

Sample preparation for bacterial enumeration was carried out as per Bloem and Vos (2004). SYBR Gold was used to stain the microbial cells. Bacteria were filtered using Sartorius filtration apparatus onto 0.22µm isopore membrane filters and a support Whatman™ filter was used to enhance vacuum distribution filtered onto the membrane. Residual background staining was removed using distilled deionised water. The filter was placed on a glass slide and counted immediately to avoid fading of the stain (Kumaravel *et al.*, 2009).

For each filter, a selection of random fields was counted until a total of at least 300 cells were counted over a minimum five fields. Counting was facilitated *via* the use of an epifluorescent microscope under oil immersion (100x objective lens). The samples were observed under a blue optical filter on which has an excitation from 465 to 505 nm, 510 nm cut-off; emission from 515 to 565 nm as adopted from Shibata *et al*. (2006). Bacterial cells were visualised as green dots or lines. Once a count was obtained, the following formula was used for enumeration of bacterial cells per millilitre:

$$\frac{Count\ x\ 176}{Dilution\ factor\ x\ No.of\ fields\ counts\ x\ 0.186}$$

where 176 was the area of the field of view on the microscope and 0.186 was the filter area. Each sample was counted in triplicate and where numbers exceeded 100 cells per field of view, a minimum of 4 counts was obtained or a further dilution was made.

2.7 Statistical Analysis

All statistical analysis for physicochemical measurements, algal enumeration and bacterial enumeration was carried out using GraphPad Prism 8. A D'Agostino-Pearson normality test was carried out to test the normality of all data. Results from this informed the use of a parametric unpaired t-test or a nonparametric Mann-Whitney test for comparison of results between the FP and the TP. A value of $p \leq 0.05$ indicated a significant difference at the 95% level of confidence. FlowJo software package was used for analysing flow cytometry data and for the generation of cytograms in order to establish an algal cell count. XLSTAT was used to generate the Principle Component Analysis (PCA) using Pearson correlation matrix scores to compare all parameters analysed. The closer the score to 1 or (-) 1 the greater the positive or negative correlation between two parameters, respectively. In the case where correlations existed between parameters, yellow scores denote a moderately strong correlation, red scores denote a strong correlation and blue scores denote a very strong correlation.

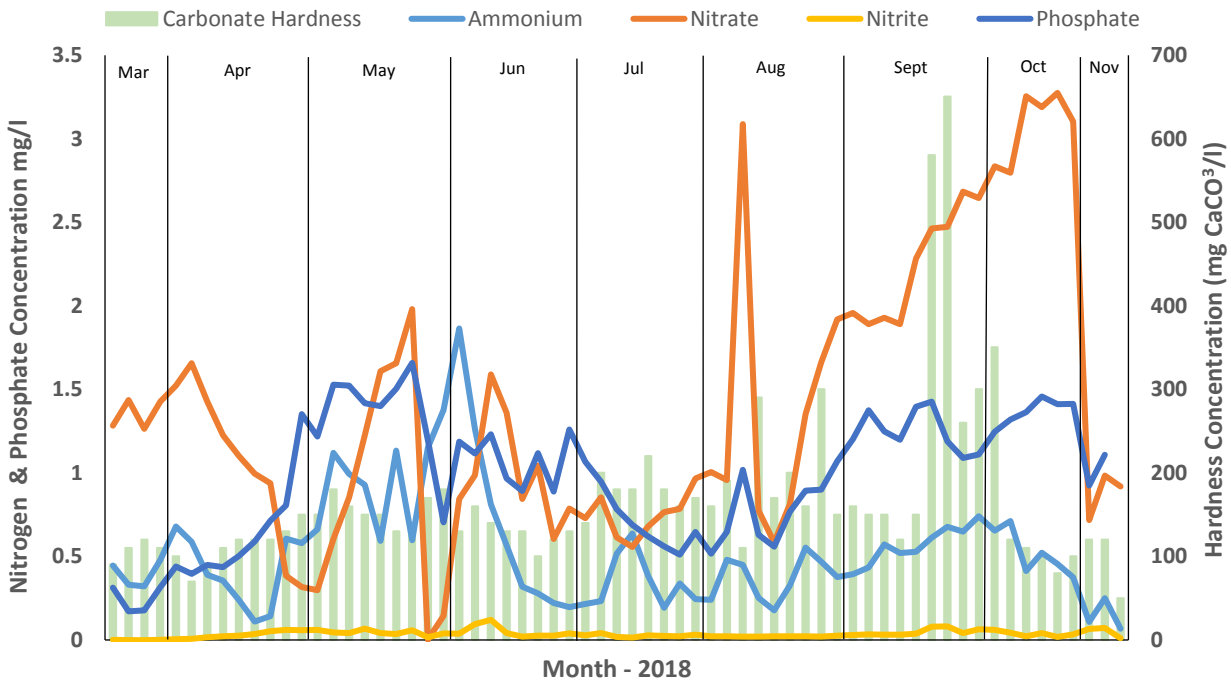
3. Results

3.1 Physicochemical Parameter Analysis

All physicochemical measurements analysed in the lab are displayed in Table 3. Fig. 4 displays the physicochemical parameter trends for the FP and the TP. Parameter levels were assessed according to Boyd (1998) and SI No. 77 of 2019 – European Union Environmental Objectives (Surface Waters) (Amendment) Regulations 2019. Nitrates, nitrites, ammonium and phosphates results were expressed in mg/l, with water hardness expressed in the most common form as calcium carbonate hardness ($CaCO_3$) as per Wurts and Durborow (1992). In all cases an R^2 value of > 0.99 was achieved for each standard curve, indicating the reliability of the test method with very little variation. The nitrate concentration peaked in October reaching levels of 3.27 mg/l and 3.25 mg/l for the FP and TP. The levels were lowest at the end of May with levels below the limit of detection recorded for both the FP and TP (Fig. 4). Nitrite levels ranged from below the level of detection in March to 0.118 mg/l and 0.103 mg/l in June, for the FP and TP respectively (Fig. 4). The ammonium trends were lowest at levels of 0.12 and 0.08 mg/l for the FP and TP in April, respectively. The highest ammonium concentration levels were in June for both ponds, with a concentration of 1.89 mg/l in the FP and 1.69 mg/l in the TP (Fig. 4). The highest phosphate concentrations were measured at 1.66 mg/l in the FP and 2.20 mg/l in the TP (Fig. 4). Water hardness levels ranged from 70.06 to 650.57 mg $CaCO_3$/l in the FP and 50.04 to 630.55 mg $CaCO_3$/l in the TP (Fig 4). The water hardness was highest in September, with average hardness levels reaching 157.95 mg $CaCO_3$/l in FP and 129.53 mg $CaCO_3$/l in TP (Fig. 4), when the two high outliers reached in September were excluded.

Table 3 Physicochemical parameter analysis at the perch farm from March to November 2018 – methods, results and acceptable limits.

Parameter	Measurement	R^2 Coefficient	Result Ranges (mg/l)		MAC (mg/l)	
			FP	TP	SI No. 77 (2019)	Boyd (1998)
Nitrates	Photometric	0.998	< 0.01 – 3.27	< 0.01 – 3.25	50	0.2 – 10.0
Nitrites	Photometric	1	< 0.01 – 0.12	< 0.01 – 0.10	0.03	< 0.3
Ammonium	Photometric	0.998	0.12 – 1.89	0.08 – 1.69	-	0.2 – 2.0
Ammonia	Calculation	-	< 0.01	< 0.01	< 0.03	< 0.1
Phosphates	Photometric	0.999	0.17 – 1.66	0.10 – 2.20	0.025	0.005 – 0.2
Carbonate Hardness	Titration	-	70.06 – 650.57	50.04 – 630.55	-	50 – 200



281

282 **Fig. 4.** Trends for nitrogen, phosphate and carbonate hardness for the FP at the perch farm, 2018.

283 There were no statistically significant differences between the data obtained for both
284 sections of the pond with $p > 0.05$ for nitrate, nitrite, ammonium, phosphate and
285 carbonate hardness, regardless of the test used for analysis, with the p values
286 displayed in Table 4.

287 **Table 4** P values for determining significance between the data obtained for the FP and TP in the perch
288 farm for nitrate, nitrite, ammonium, phosphate and carbonate hardness concentrations.

Parameter	P Value (where p < 0.05 = significant)
Nitrate	0.9445
Nitrite	0.9347
Ammonium	0.7567
Phosphate	0.9215
Hardness	0.0619

289 ### 3.2 Microalgal Enumeration

290 The algal numbers from March until November of 2018 in the perch farm are
291 displayed in Figure 5 expressed as algal cells per ml. As stated in the gating
292 methodology for algal enumeration, the desired population included the chlorophyll
293 population excluding cells positive for PC, therefore the graphs below do not include
294 the majority of the Cyanobacteria numbers. The algal population in terms of
295 enumeration remained steady for March and April and then increased in May when

the light levels and temperatures increased to highs of 19°C and 21°C in May and July. Algal numbers peaked in late June, with counts of 1.54 x 10^5 cells/ml and 1.57 x 10^5 cells/ml observed for the FP and TP respectively. Lowest numbers were detected in the winter months *via* flow cytometry analysis, of less than 100 cells/ml. There was a slight decrease in the algal numbers in August in the TP compared with the FP a trend that was reversed in September, when a higher algal count was observed for the TP. A p-value of 0.821, following a Mann-Whitney test, indicated that there was no significant difference between the values obtained for both the FP and TP in terms of enumeration.

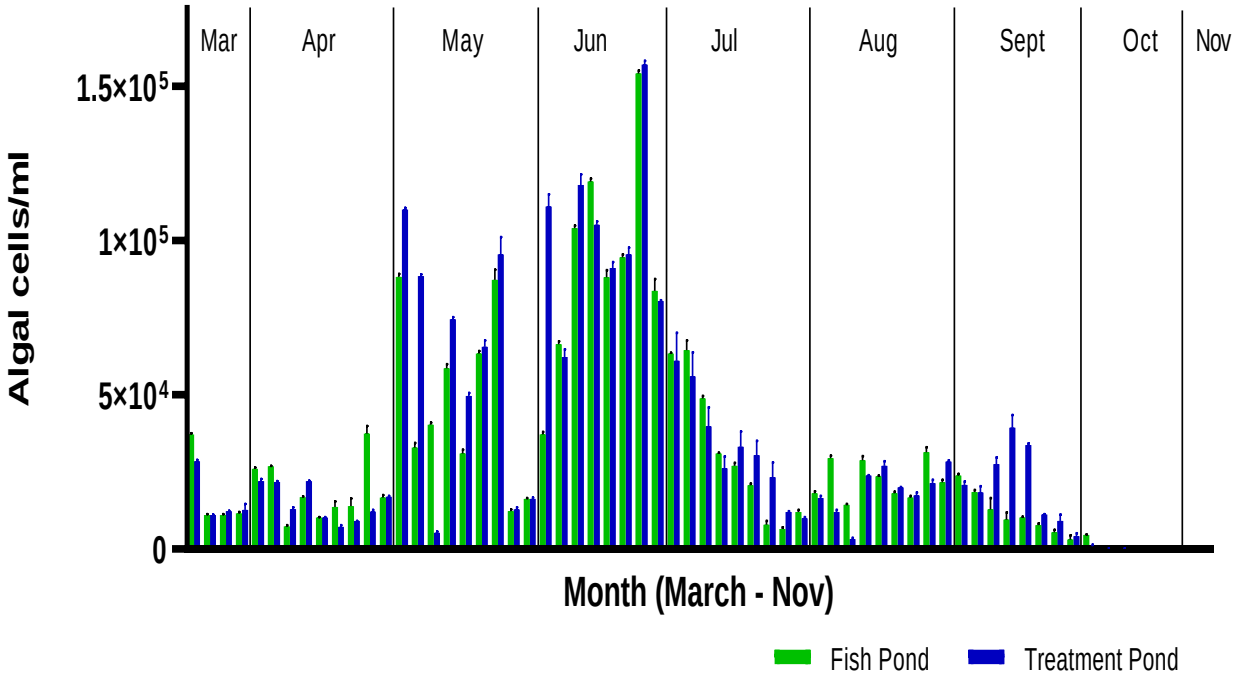
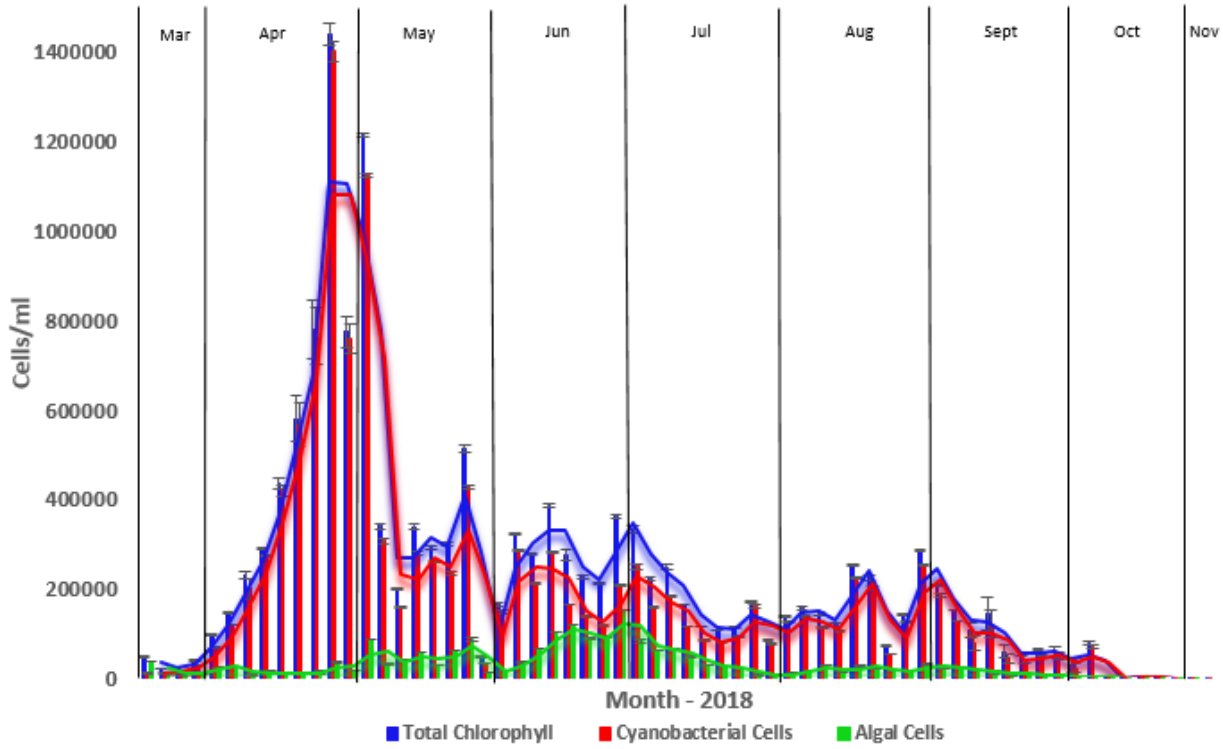


Fig. 5. Algae cells/ml in from March to November 2018 in Pond 1 at the perch farm.

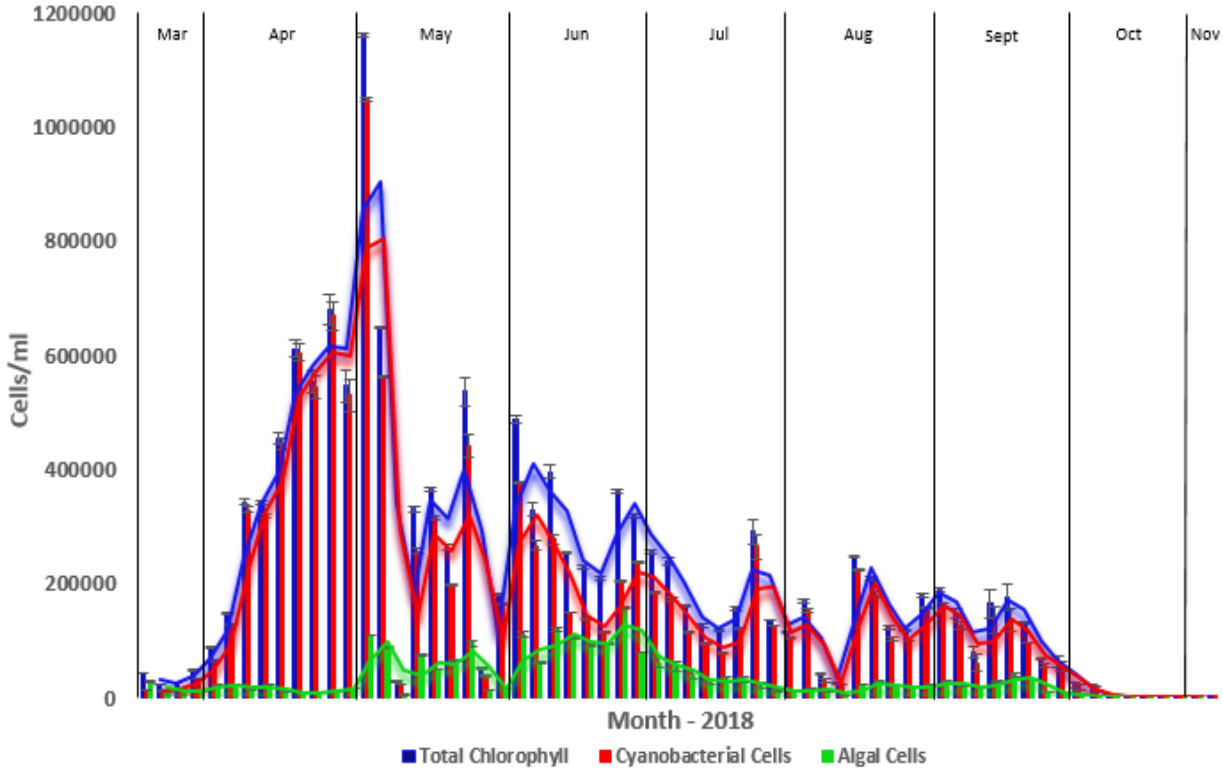
3.3 Cyanobacterial Population Enumeration

The total number of chlorophyll-containing cells was determined *via* flow cytometry. The PC^+ population was assumed to closely represent the cyanobacterial population in this study. This data was used to establish the cyanobacterial population from the total number of chlorophyll positive cells. Figures 6 and 7 illustrate the total chlorophyll positive population and the number of cells in this population that represent algal cells compared to cyanobacterial cells for the FP and TP, respectively. On average Cyanobacteria accounted for 80% of the chlorophyll-containing cells present in the rearing water, whereas the algal cells only accounted for 20% of the chlorophyll-containing cells.



317

318 **Fig. 6** Enumeration of total chorophyll-containing cells consisting of cyanobacterial and algal cells in
319 the FP at the perch farm, 2018.



320

321 **Fig. 7** Enumeration of total chorophyll-containing cells consisting of cyanobacterial and algal cells in
322 the TP at the perch farm, 2018.

3.4 Microalgal Community Profiling

Through the use of the inverted light microscope, the microalgal profile and species diversity for each month were determined. The dominant species for each month are presented in Table 5 for the FP and Table 6 for the TP. The most numerous species are listed with the dominating phylum for each month shaded in pink. Over 40 different algal species were observed, with the majority identified to species level using photographic identification keys. Chlorophyta was the most dominant phylum for the FP and the TP overall, with the most commonly occuring species including *Chlamydomonas* sp., *Chlorella* sp., *Dictyosphaerium* sp., *Monoraphidium* sp., *Pandorina* sp., *Scenedesmus* sp., *Selenastrum* sp., *Tetraspora* sp. and *Westella* sp. In the FP the most dominant phylum for June and September was Bacillariophyta (diatoms). In June this was mainly *Stephanodiscus* sp. and *Cyclotella* sp. In September when the algal number decreased in the FP compared to the TP, the dominant species in the FP were *Aulodiscus* sp., *Hyalodiscus* sp. and *Cyclotella* sp. Whereas the TP was completely dominated by *Cryptomonas* sp., of the Cryptophyta phylum.

Table 5 Dominant algal species from March to November 2018 in the FP of Pond 1 at the perch farm.

	Most to least dominant in each group	* Shading indicates the dominant group	
Month	**Genus**		
March	*Chlorella, Monoraphidium* - **Chlorophyta**		
April	*Chlamydomonas, Chlorella, Scenedesmus, Monoraphidium* - **Chlorophyta**	*Chroomonas, Cryptomonas* - **Cryptophyta**	*Trachelomas* - **Euglenophyta**
May	*Dictyosphaerium, Pandorina, Chlamydomonas, Tetraspora, Westella,* - **Chlorophyta**		
June	*Dictyospaherium, Westella, Chlamydomonas, Chlorella, Tetraspora* - **Chlorophyta**	*Stephanodiscus, Cyclotella* - **Bacillariophyta**	
July	*Dictyosphaerium, Westella, Chlamydomonas, Chlorella, Sphaerocystis* - **Chlorophyta**		
August	*Westella, Dictyosphaerium, Chlamydomonas, Scenedesmus* - **Chlorophyta**	*Cryptomonas* - **Cryptophyta**	
September	*Dictyosphaerium, Selenastrum* - **Chlorophyta**	*Aulodiscus, Hyalodiscus, Cyclotella* - **Bacillariophyta**	
October	*Selenastrum, Dictyosphaerium, Chlorella* - **Chlorophyta**		
November	*Chlorella, Dictyosphaerium, Chlamydomonas* - **Chlorophyta**	*Cryptomonas* - **Cryptophyta**	*Chroococcus* - **Cyanophyta**

Table 6 Dominant algal species from March to November 2018 in the TP of Pond 1 of the perch farm.

	Most to least dominant in each group	* Shading indicates the dominant group	
Month	**Genus**		
March	*Chlorella, Monoraphidium* - **Chlorophyta**		
April	*Chlorella, Monoraphidium, Dictyosphaerium, Scenedesmus, Chlamydomonas* - **Chlorophyta**	*Trachelomas* - **Euglenophyta**	*Aphanocapsa* - **Cyanophyta**
May	*Chlamydomonas, Chlorella, Pandorina, Westella, Tetraspora* - **Chlorophyta**	*Stephanodiscus, Cyclotella* - **Bacillariophyta**	*Cryptomonas* - **Cryptophyta**
June	*Dictyosphaerium, Westella, Chlorella, Chlamydomonas, Tetraspora* - **Chlorophyta**	*Cyclotella* - **Bacillariophyta**	
July	*Dictyosphaerium, Westella, Chlamydomonas* - **Chlorophyta**	*Snowella* - **Cyanophyta**	
August	*Westella, Chlamydomonas, Tetraspora* - **Chlorophyta**		
September	*Chlorella* - **Chlorophyta**	*Cryptomonas* - **Cryptophyta**	
October	*Dictyosphaerium, Selenastrum, Chlorella* - **Chlorophyta**	*Aphanocapsa* - **Cyanophyta**	
November	*Dictyosphaerium, Westella, Chlamydomonas* - **Chlorophyta**		

3.5 Bacterial Enumeration

Bacterial counts were conducted in order to provide supplementary data to algae enumeration and profiling for correlation purposes. The overall average count of total bacteria for the pond was 6.33×10^6 cell/ml. The trend for this analysis from March to November 2018 is displayed in Fig. 8 expressed as $\log_{10}$ cells/ml.

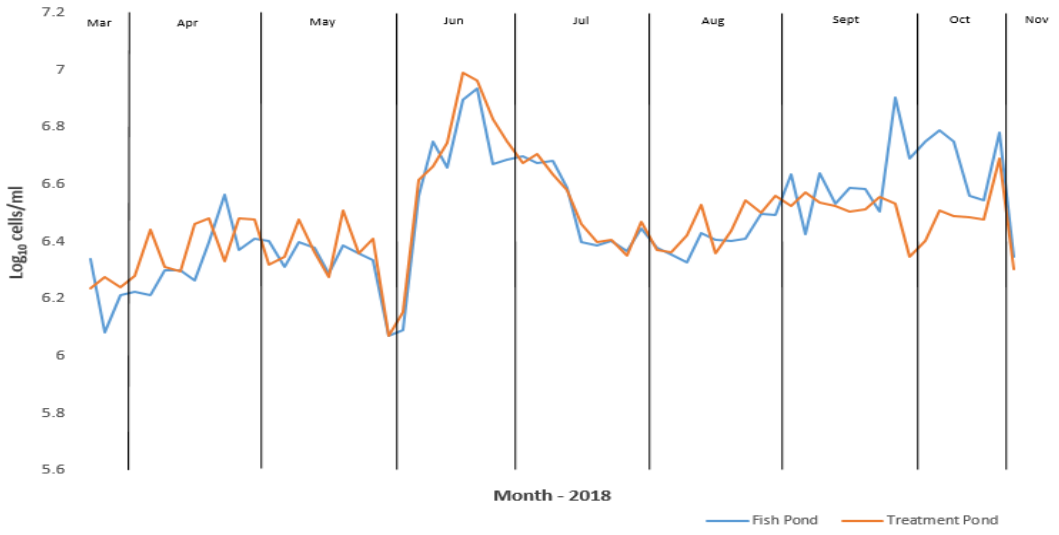


Fig. 8 Bacterial enumeration (log10 cells/ml) from March to November 2018 in Pond 1 at the perch farm.

3.6 Principle Component Analysis

In order to analyse the large volume of data, PCA analysis was conducted in Excel using the XLSTAT software to observe any correlations and or variability between parameters, which can be difficult to ascertain from individual and even overlaid parameters. This type of analysis provides tables and graphs through which observations on the relationships between parameters were made. The parameters analysed included a combination of variables measured in the laboratory and measure on site. The lab parameters analysed were nitrate concentration, nitrite concentration, ammonium concentration, phosphate concentration, water hardness (mg $CaCO_3$/l), bacterial numbers, algal numbers, chlorophyll containing cells and cyanobacterial cells, with the latter four parameters measured in cells/ml. The parameters analysed that were recorded on site included pH, oxygen (mg/l), turbidity (FTU), feeding rate and total chlorophyll and Cyanobacteria measured using an algal torch, with results in µg/l. Tables 7 and 8 display the correlation matrix scores obtained for the parameters analysed in this study.

Table 7 Correlation coefficient scores for PCA analysis carried out on TP parameters at the perch farm using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano =

Variables	Nitrate	Nitrite	Ammonium	Phosphate	Temperature	pH	Oxygen	Turbidity	Hardness	Feeding Rate	Bacteria	Algae	Chl-Ctn Cells	Cyano Cells	Chl (Torch)	Cyano (Torch)
Nitrate	1															
Nitrite	0.200	1														
Ammonium	0.118	0.342	1													
Phosphate	0.261	0.550	0.426	1												
Temperature	-0.173	0.249	0.026	0.268	1											
pH	-0.433	-0.203	-0.331	-0.251	0.616	1										
Oxygen	-0.483	-0.234	-0.120	-0.292	-0.338	0.195	1									
Turbidity	-0.167	0.268	0.421	0.493	-0.001	-0.267	0.161	1								
Hardness	0.240	0.512	0.099	0.171	0.108	0.005	-0.220	-0.145	1							
Feeding Rate	-0.093	-0.091	-0.076	0.068	0.420	0.367	-0.292	-0.060	0.030	1						
Bacteria	-0.030	0.216	-0.080	0.257	0.402	0.304	-0.002	-0.121	0.100	0.235	1					
Algae	-0.369	0.230	0.043	0.190	0.329	0.264	0.336	0.319	-0.160	0.108	0.555	1				
Chl-Ctn Cells	-0.463	0.366	0.065	0.284	0.069	-0.043	0.327	0.484	-0.151	-0.115	0.004	0.441	1			
Cyano Cells	-0.435	0.350	0.063	0.270	0.023	-0.084	0.296	0.463	-0.137	-0.137	-0.082	0.314	0.990	1		
Chl (Torch)	-0.539	0.211	-0.073	0.286	0.731	0.490	0.057	0.357	-0.131	0.308	0.339	0.511	0.555	0.508	1	
Cyano(Torch)	-0.568	0.175	-0.206	0.060	0.445	0.580	0.377	0.100	-0.103	0.151	0.489	0.604	0.527	0.466	0.652	1

0 = No Relationship
> 0.2 = Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

Table 8 Correlation coefficient scores for PCA analysis carried out on TP parameters at the perch farm using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano =

Nitrate	1															
Nitrite	0.282	1														
Ammonium	0.150	0.296	1													
Phosphate	0.398	0.624	0.438	1												
Temperature	-0.261	0.234	0.029	0.276	1											
pH	-0.500	-0.215	-0.338	-0.263	0.616	1										
Oxygen	-0.494	-0.211	-0.150	-0.354	-0.338	0.195	1									
Turbidity	-0.155	0.264	0.391	0.399	-0.001	-0.267	0.161	1								
Hardness	0.246	0.424	0.325	0.209	-0.065	-0.138	-0.079	0.087	1							
Feeding Rate	-0.061	-0.105	-0.081	0.048	0.420	0.367	-0.292	-0.060	-0.052	1						
Bacteria	-0.110	0.173	-0.068	0.233	0.384	0.282	0.019	-0.100	-0.099	0.276	1					
Algae	-0.357	0.281	0.252	0.229	0.370	0.187	0.256	0.377	-0.030	0.140	0.539	1				
Chl Ctn. Cells	-0.481	0.321	0.119	0.101	0.131	-0.029	0.270	0.496	0.014	0.019	0.067	0.607	1			
Cyano Cells	-0.459	0.303	0.085	0.069	0.069	-0.076	0.247	0.477	0.023	-0.010	-0.032	0.475	0.988	1		
Chl (Torch)	-0.599	0.199	-0.084	0.174	0.731	0.490	0.057	0.357	-0.169	0.308	0.352	0.502	0.567	0.526	1	
Cyano (Torch)	-0.573	0.179	-0.257	0.024	0.445	0.580	0.377	0.100	-0.159	0.151	0.480	0.548	0.481	0.421	0.652	1

0 = No Relationship
> 0.2 = Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

3.7 Weather Conditions

The Republic of Ireland experienced one of its hottest summers on record in 2018 that coincided with the sampling period in this study (Met Eireann, 2018). Drought conditions and national hosepipe ban were put in place for most of the country up to the end of August 2018 (O'Neill *et al*., 2019). 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 were observed. There stations were located in Knock, Markree and Mount Dillion. Decreases in the average monthly rainfall (Fig. 9**(A)**) and increases in temperature (Fig. 9**(B)**) were observed across the weather stations.

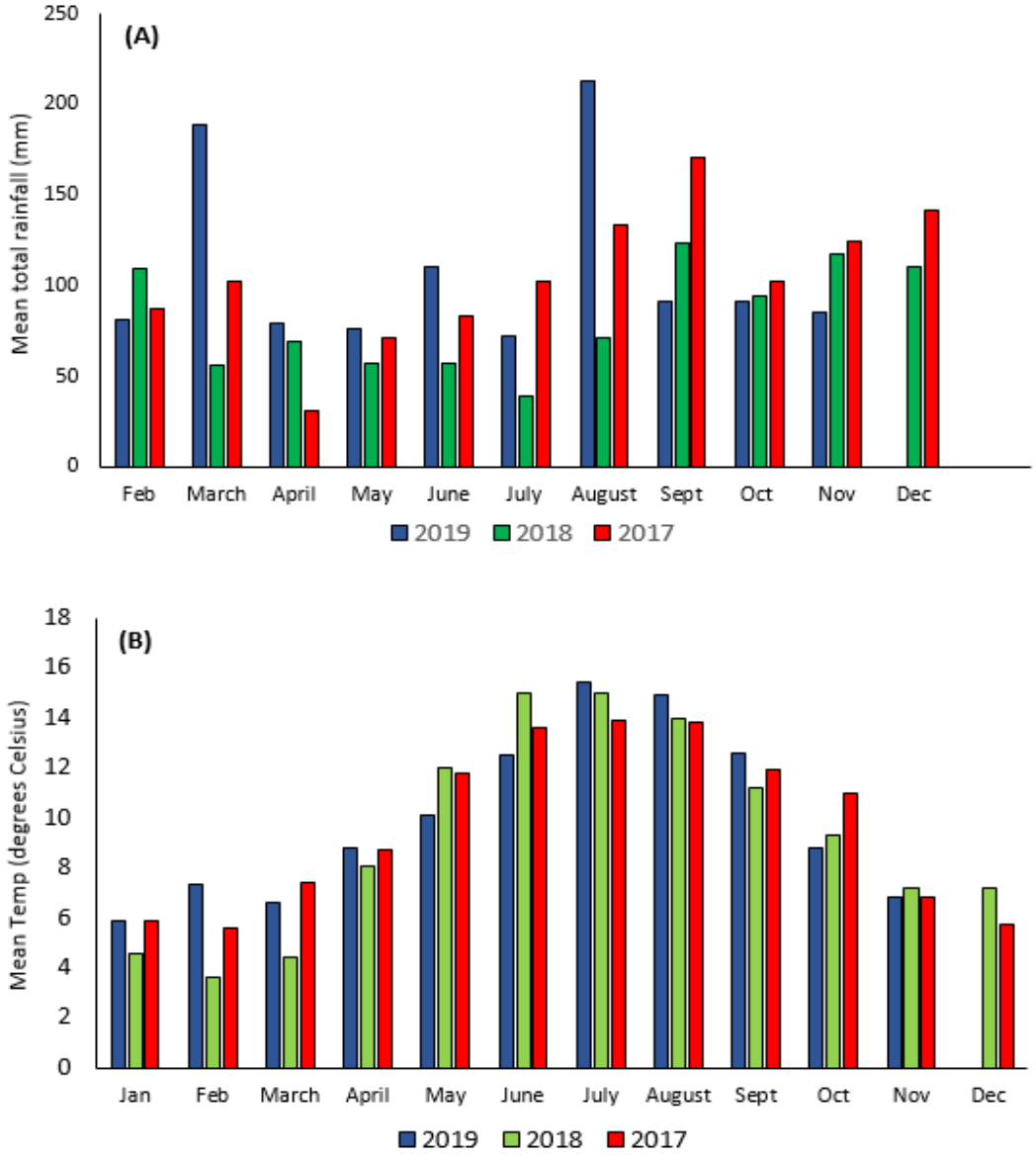


Fig. 9. (A) Mean total rainfall (mm) for three met offices nearby to the sampled perch farm, and **(B)** mean temperature in degrees Celsius recorded at same three met offices, over periods 2017 to 2019.

4. Discussion

4.1 Physicochemical Parameter Analysis

The highest nitrate concentrations of 3.27 mg/l and 3.25 mg/l observed for the FP and TP respectively are below the 50 mg/l maximum acceptable limit of SI No. 77 of 2019. When the levels were lowest at the end of May there was a water change in the pond, therefore the fish were removed. This process may have reduced the level of nitrates, with one of the main sources being fish waste, as well as decaying organic matter in the water (Jiménez-Montealegre *et al.*, 2002; Thajuddin and Subramanian, 2005), which would have also been partially removed in the process. Nitrites levels were below the limit of detection in March and highest in June with concentrations of 0.118 and 0.103 mg/l for the FP and TP respectively. The temperature was highest at this point ranging between 19 and 21°C and the bacterial counts were also highest in June overall. As this initial steps in this process involves the oxidation of ammonia to nitrite (Helfrich and Libey, 1990; Hargreaves, 1998), Fig.3 illustrates the decrease of ammonium levels at the start of June, followed by an increase in the nitrite levels, respectively, which may in part account for this increase in nitrite concentrations. The partial change of water in the pond at the end of May could have contributed to the rise in ammonium. A sudden decrease in bacterial and algal numbers evident from Fig.'s 5 and 8 may have impacted the nutrient recycling process. The ammonium ion tends to be harmless to fish unless extremely high concentrations are reached (Boyd and Lichtkoppler, 1979). The highest phosphate concentrations were detected in May for both ponds. Lund (1965) stated that phosphorus levels can decrease in the summer, and as the summer months progressed, the phosphate concentrations measured during this study did in fact decrease. This may in part be due to the high levels of bacteria in the summer which are major competitors of algae for the uptake and utilisation of inorganic phosphorus (Lund, 1965).

4.2 Algal Enumeration

The algal population remained steady across the sampling months but peaked when the temperatures were at the highest (19°C and 21°C) between May and July. The decrease in the algal numbers in the TP compared to the FP in August may have been due to the removal of weeds and duckweed from the outer area of the TP which may have led to a dilution of the algal population. It is a common trend that algal cells reach the highest concentration during the summer months due to increasing irradiance, longer daylight hours and higher temperatures. Xuemei *et al.* (2011) found that algae numbers were higher in summer than winter as is the general trend of algal growth in lakes or ponds. However, the decrease from summer to winter was only marginal, from 3.45 x 10^3 to 1.46 x 10^3 cells/l, whereas in this project cell

numbers declined to much lower levels in winter, decreasing to 9 cells/ml in the first week of November.

PCA analysis indicated a strong correlation between temperature the chlorophyll content measured using the AlgaeTorch, with a coefficient score of 0.791. This would be expected as temperature and daylight are two of the most important factors in terms of phytoplankton growth, so an increase in temperature will lead to an increase in phytoplankton. This correlation was not, however, present for the chlorophyll analysis carried out in the lab, even though this parameter correlated to the on-site chlorophyll measurements. This result may imply that although certain parameters have been proven to share relationships, this may not always be reflected in the data as other variables may interfere or an increased number of data points may be required.

There was a moderately strong positive correlation between algae and bacterial enumeration data in both the FP and TP, with coefficient scores of 0.555 and 0.539, respectively. This correlation is commonly observed due to the symbiotic relationship shared between these two biological entities. Algae require nitrogen as an essential element for building structural and functional proteins (Hu, 2004). It is available in the soil organic matter (SOM); however, nitrogen is not in a bioavailable form for algae to utilise. Nitrogen-fixing bacteria convert the nitrogen (nitrogen fixation) into a form that can then be utilised by algae (Neospark, 2014; Thajuddin & Subramanian, 2005). The rate of nitrogen fixation largely depends on the bacterial species present in the water and the concentration of ammonia (Hargreaves, 1998). This nitrogen fixation process highlights the important interdependent relationship that exists between algae and bacteria. Another aspect of this dynamic relation involves organic matter, on which bacteria thrive (Amon and Benner, 1996; Baines and Pace, 1991; Blancheton *et al.*, 2013). One of the principal sources of organic matter in the rearing water is primary production by microalgae, followed by excreta and feed pellets (Baines and Pace, 1991; Moriarty, 1997). Aerobic bacteria present in the water body break down this organic matter into CO_2 and ammonia (Phang, 1991). Algae then utilise the CO_2 for photosynthesis and release oxygen during the process, which in turn oxygenates the water for the fish (Neospark, 2014). Algae also uptake the ammonia as well as heavy metals, reducing the availability of toxic substances for fish to consume (Neori *et al.*, 2004).

Phosphorus was also a determining factor for plankton richness in the study carried out by Xuemei *et al*. (2011) on an artificial lake, whereas transparency negatively correlated with plankton communities. This negative correlation was related to the negative correlation achieved between transparency and the presence of algae, indicating that algae have a major impact on the turbidity of water (Xuemei *et al*., 2011). This finding agrees with the moderately strong positive correlation achieved between turbidity and chlorophyll-containing cells in both the FP and TP in this study.

4.3 Cyanobacterial Population Enumeration

The Cyanobacteria population accounted for the majority of the chlorophyll-containing cells detected/recorded throughout the sampling period in both ponds. This observation is highlighted further with the use of the moving average trend line Fig.'s 5 and 6. While certain species of Cyanobacteria, for example, *Microcystis* sp., can release toxins into the water and exhert detrimental effects on other organisms, Cyanobacteria are also beneficial for processes such as nitrification. Throughout this process, the nutrients are taken up by the cells and are therefore removed from the water, increasing the water quality in terms of nutrient pollution. In a study carried out by Liu *et al*. (2018) for the treatment of aquaculture using Chlorophyta, prior to inoculation with the green microalgae, Cyanobacteria were responsible for the partial removal of the pollutants from the aquaculture water, highlighting the importance of Cyanobacteria in the rearing water.

Following the PCA analysis, the highest correlation for the FP and the TP was the positive one observed between chlorophyll-containing cells and the cyanobacterial cells with coefficient scores of 0.990 and 0.988 for the FP and TP, respectively. This clearly corresponds to the data displayed in Fig.'s 5 and 6, reiterating the observation that the Cyanobacteria phylum represented most of the chlorophyll source present in pond 1 at the perch farm compared to algae. There was a strong positive correlation between the same two parameters when measured on site using an algal torch with a score of 0.652, which indicates that both on site and in lab measurements of phytoplankton produced similar trends. There was a moderately strong positive correlation between algal counts and chlorophyll-containing cells for the FP with a score of 0.441. Whereas a strong positive correlation was established for the same parameters in the TP, with a score of 0.607. This would suggest that the trends for algal numbers in the TP were more in line with the overall trends for chlorophyll-containing cells compared to the algal numbers in the FP. This may be due to the presence of fish in the FP, which would have impacted the algal numbers to a higher extent, by uptake into the diet for example.

In the case of nitrates, the data for nitrate concentration in both the FP and the TP was negatively correlated with chlorophyll and cyanobacterial parameters measured. This would indicate that the presence of chlorophyll-containing cells/pigment, the majority of which corresponded to Cyanobacteria, had a negative impact on nitrate levels. Phytoplankton are known for the uptake and removal of certain nutrients from the water and the coefficient scores reflect this fact. This finding is comparable to results determined in a study carried out by Choi *et al*. (2010), where the growth of Cyanobacteria and algae inhibited the maximum nitrification rate by a factor of 4 in an autotrophic bioreactor. Hu *et al*. (2000) also established similar results in an assessment of the removal of nitrate from

groundwater by Cyanobacteria, with *Synechococcus* sp. displaying the highest rate of nitrate removal.

4.4 Algal Community Profiling

Chlorella and *Monoraphidium* were the most common algae present in both the FP and TP in March and both species remained quite dominant in April. *Chlorella* has been reported as one of the most effective algal species at nutrient uptake, particularly nitrogen and phosphorus (Wang *et al*., 2010).In this study, the nitrate, nitrite and ammonium concentrations all decreased in early/mid-June when the *Chlorella* population remained prevalent. The effectiveness of *Chlorella* sp., in particular, *Chlorella vulgaris*, at nutrient removal is also evident from its common use in the treatment of wastewater (Min *et al.*, 2011; Abdel-Raouf *et al.*, 2012; Choi and Lee, 2012; Godos *et al.*, 2012; Delrue *et al.*, 2016). *Chlorella kessleri,* synonymous with *Parachlorellakessleri,* has also shown great potential for pollutant removal from aquaculture wastewater. Liu *et al*. (2018) inoculated aquaculture wastewater with five Chlorophyta species with *P. kessleri* exhibiting the greatest rate of nutrient uptake in terms of COD, nitrogen and total phosphorus. *Monoraphidium sp.* have also been reported for successful nutrient uptake. In a biodiesel production study carried out by Holbrook *et al*. (2014) *Monoraphidium* reduced concentrations of nitrates and phosphates to <5 mg/l and <1 mg/l, respectively. Therefore, *Monoraphidium sp.* are potentially useful organisms for phytoremediation of aquaculture water if the cell densities are increased. Sanchis-Perucho *et al*. (2018) discovered that the nutrient removal efficiency of a consortium of *Monoraphidium* and *Scenedesmus* sp. was more effective than the removal of nitrogen and phosphorus compared to *Chlorella*.

There was an increase in algal diversity for the month of April, with Chlorophyta dominating in both the FP and TP. The most numerous Chlorophyta observed, other than *Chlorella* and *Monoraphidium*, included *Pandorina* sp., *Chlamydomonas* sp., *Dictyosphaerium* sp., *Kirchneriella* sp. and *Scenedesmus* sp., with *S. obtusus*, *S. quadricauda*, *S. obliquus*, *S. opaliensis* and *S. acuminatus* all identified. The presence of different clonal populations for some algae species, such as a four- and eight-colony formation of *Scenedesmus* sp., compared to single-celled organisms may be attributed to the selective pressures in aquatic environments. For example, a study carried out by Zhu *et al*. (2015) demonstrated that upon exposure to *Daphnia* filtrate, acting as a predator, the *Scenedesmus* sp. increased the rate of the formation of four- and eight-celled populations. The presence of both the four- and eight-celled *Scenedesmus* sp. at the collaborating farm in this study may be indicative of the selective pressure that was present in the rearing water for the duration of the study, due to the abundant diversity that was evident from all the samples analysed. There may also have been selective pressures due to the dramatic changes in

meteorological and environmental conditions, ranging from snow in March to drought in the summer months. Crytophytes and Euglenophyta were also observed in April in the form of *Chroomonas* sp. and *Cryptomonas* sp, and *Trachelomonas* sp., respectively. *Pediastrum* sp. was observed in the sample for May, which was not present prior to then. This species remained present until October, after which it was not observed.

Diatoms, mainly *Cyclotella* and *Stephanodiscus* sp., were the most frequent species observed in the month of May for the TP and June for the FP and the TP. According to Stoermer and Julius (2003) diatoms tend to be specific to certain habitats which allows for their use as indicators of water quality, with *Stephanodiscus* considered to be one of the most common and ubiquitous freshwater diatoms. In July, *Merismopedia* sp. was present which had not been present prior to then. *Synura* sp. were quite dominant in July for both the FP and TP. There was an increase in the presence of *Scenedesmus* sp. in August compared to any other month.

The species diversity for both the FP and the TP was very similar every month, however with one major exception. In the month of September, the TP was completely dominated by *Cryptomonas* sp. Contrastingly, very large diatoms, which resembled *Aulacodiscus*, *Hyalodiscus* and *Cyclotella* sp. dominated the FP. The presence of these diatoms seemed to cause a decrease in other species present, possibly due to feeding or out-competing with other species for nutrients. In fact, the algal counts for mid-September were lower for FP compared to the TP.

According to Vuorio *et al*. (2007), when analysing multispecies communities of phytoplankton, enumeration procedures can be complicated and more information regarding water quality can be determined by phytoplankton community analysis compared to basic nutrient or chlorophyll *a* measurements. Therefore, it was important to perform a two-step algal analysis procedure in the form of flow cytometry and microscopy. Other factors can also be problematic for the algal identification process. Stoermer and Julius (2003) state that the average size of diatomic cells decreases after each vegetative life cycle, which can lead to variability in cell morphology of the same species. Environmental conditions such as salt levels can also alter diatom morphology (Stoermer and Julius, 2003). As well as that Small *et al*. (2016) stated that in terms of the capacity of photoautotrophic systems, such as algae, to remove nutrient waste from the water depends largely on energy uptake from sunlight, which is very unpredictable in a climate such as the one in Ireland. With variations in climate change and increasing temperatures worldwide, certain microalgae species may not be able to grow, and as they are a source of oxygen in the ponds, the use of a natural means of production oxygen for biological processes may no longer be an option.

Resident bacteria and other microbes, which can limit or influence microalga growth due to the availability of nutrients, contaminated microalgae populations in the pill pond. Compared with previously described closed photo-bioreactors (Han *et al*., 2019), a pill pond system has a much lower investment and operational cost but higher volume, making it more suitable for treatment of aquaculture wastewater. A recent life cycle analysis (LCA) conducted by Stez *et al*. (2015) intimated that a pill pond is a sustainable way to use microalgae-bacterial flocs for aquaculture wastewater remediation and recycle biomass for aquaculture feed. Han *et al*. (2019) indicated that two critical factors should be considered for advancing this type of aquaculture process. Firstly, location and pond depth need to be properly evaluated so as to improve light transmittance and photosynthesis rate. Secondly, the relationship between microalgae and bacteria should be thoroughly investigated to be fully elucidated. Van Den Hende *et al*. (2014) reported that microalgae-bacterial flocs contributed to the removal of 28% COD, 53% BOD_5, 31% TN, and 64% TP in aquaculture wastewater (12 m^3 raceway pond), suggesting that the threat of bacteria to microalgae is putatively low when a beneficial cooperation is established.

5. Conclusion

The results of this study conducted at an aquaculture perch farm in the Republic of Ireland provide a baseline for the rearing water microalgae and physicochemical ecosystem interactions. In pond aquaculture that often relies upon natural wastewater treatment process, there is much less control over environmental conditions compared to closed tanks, which is why the ecosystem dynamic needs to be understood and possibly manipulated for successful and sustainable fish production. Identification of the most influential biological species in more depth would provide the opportunity of transplantation of specific microbial assemblages when required for certain processes, i.e. the addition of a specific bacteria for nitrification or the fertilisation of a specific algal species for nutrient removal or oxygen supplementation during the daytime. However, in order for this to be possible and beneficial, the function of each species needs to be determined. In addition, more sustainable and effective disease control measures need to be implemented for successful management and eradication of unwanted pathogens and possibly for control of the algal population, once identified. Without the baseline information, the required knowledge to inform prevention measures rather than undergoing treatment processes would be unfeasible. This constitutes the first study that reported good agreement between use of real-time laboratory-based techniques and in field monitoring technologies for enumerating microalgae and bacterial communities where it is envisaged that use of these combinational approaches will aid the future development of aquaculture processes. Having an in-depth knowledge of this characterisation would also be the basis for future

diagnostic applications such as the design and development of diagnostic molecular kits.

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