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PII: S0044-8486(19)33172-2

DOI: https://doi.org/10.1016/j.aquaculture.2020.735377

Reference: AQUA 735377

To appear in: *aquaculture*

Received date: 21 November 2019

Revised date: 4 March 2020

Accepted date: 14 April 2020

Please cite this article as: S. Naughton, S. Kavanagh, M. Lynch, et al., Synchronizing use of sophisticated wet-laboratory and in-field handheld technologies for real-time monitoring of key microalgae, bacteria and physicochemical parameters influencing efficacy of water quality in a freshwater aquaculture recirculation system: A case study from the Republic of Ireland, *aquaculture* (2019), https://doi.org/10.1016/j.aquaculture.2020.735377

Synchronizing use of sophisticated wet-laboratory and in-field handheld technologies for real-time monitoring of key microalgae, bacteria and physicochemical parameters influencing efficacy of water quality in a freshwater aquaculture recirculation system: A case study from the Republic of Ireland

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Highlights

Flow-cytometry correlated with in-field AlgaTorch® 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

There has been growing interest in exploiting microalgae as a natural process for low cost wastewater treatment and for water quality control and remediation in aquaculture. This constitutes the first study to report on a strong relationship between use of sophisticated wet-laboratory flow-cytometry equipment and in-field AlgaTorch® technologies for determining microalgae and bacteria population dynamics in a freshwater pill-pond aquaculture farm over a 10-month monitoring period producing Eurasian Perch, *Perca fluviatilis* in the Republic of Ireland. Nitrate levels and temperature were the most significant factors influencing microalgae numbers in rearing and treatment ponds as determined by Principle Component Analysis. Variance in climate, namely drought conditions that occurred during monitoring period, did not affect microalgae or microbial numbers. *Chlorophyta, Bacillariophyta* and *Cryptophyta* were the most dominant algal divisions observed in this

recirculation aquaculture system, many of these are recognized as a natural source of beneficial prebiotics for fish. 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. Furthermore, holistic datasets can be utilised for smart agriculture by way of informing management tools for future remote monitoring and decision-making by producers.

Keywords

Microalgae, freshwater aquaculture, waste treatment, resource utilization, sustainability

1. Introduction

Aquaculture has become the fastest-growing food sector in the world (FAO, 2018; Ruiz-Salmón *et al.*, 2020). 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; O'Neill et al., 2019; O'Neill et al., 2020). 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 (Tahar *et al.,* 2018; Tahar *et al.*, 2019). 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 (O'Neill et al., 2020).

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 either harmful of beneficial blooms in the rearing water that can effect 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. There is pressing need to monitor and manage microalgae, bacteria and key parameters in rearing water on fish farms that both detects and mitigates for the emergence of pathogens in order to make remedial management actions in real time (O'Neill et al., 2020). However, biomass analysis tends to be an insufficient method for speciation of microalgae as lacks distinctive features (Xuemei *et al*., 2011). This highlights the importance of traditional time-consuming microscopic analysis in aquaculture.

There are many approaches to enumerating microalgae that have been traditionally limited to using conventional slide methods, such as the Sedgewick-Rafter slide, the Palmer-Maloney slide, the Petroff-Hausser slide and the standard haemocytometer method (Guillard, 1978; Han *et al*., 2019). The most common method for phytoplankton enumeration, especially for multispecies communities, is the Utermöhl method that requires an inverted microscopic with sophisticated optics in order to ensure reliable results (Vuorio *et al*., 2007). There is also considerable inter-variation between operators of this method, and variation between microscopes used that affects reliability and robustness (Vuorio *et al*., 2007). The use of real-time enumeration methods in marine aquaculture, such as flow-cytometry, enables rapid-counting and differentiation of more than 10,000 cells or targets per second that can add to accuracy of algal determinations in a non-destructive manner (Endo *et al*., 2010). Recently, researchers have reported on the use of handheld monitoring devices, such as AlgaTorch®, for taxonomic classification of algae through measuring fluorescence (Szymański et al., 2017). However, comparative use of conventional and sophisticated wet-laboratory equipment (such as flow-cytometry) with on-farm monitoring devices (such as AlgaTorch®), has yet to be reported for characterization and monitoring of freshwater aquaculture that relies on using natural biological processes for controlling water quality.

Thus, the aim of this timely study was to gain a comprehensive understanding of the role and relationship of microalga species with key bacterial and physicochemical parameters in freshwater aquaculture over a 10-month monitoring period using both wet-laboratory and on-farm measurements in order to define and enhance freshwater aquaculture.

2. Materials and Methods

2.1 Study Site and Sampling

Samples were collected from a freshwater fish farm located in Boyle, Co. Sligo, 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.

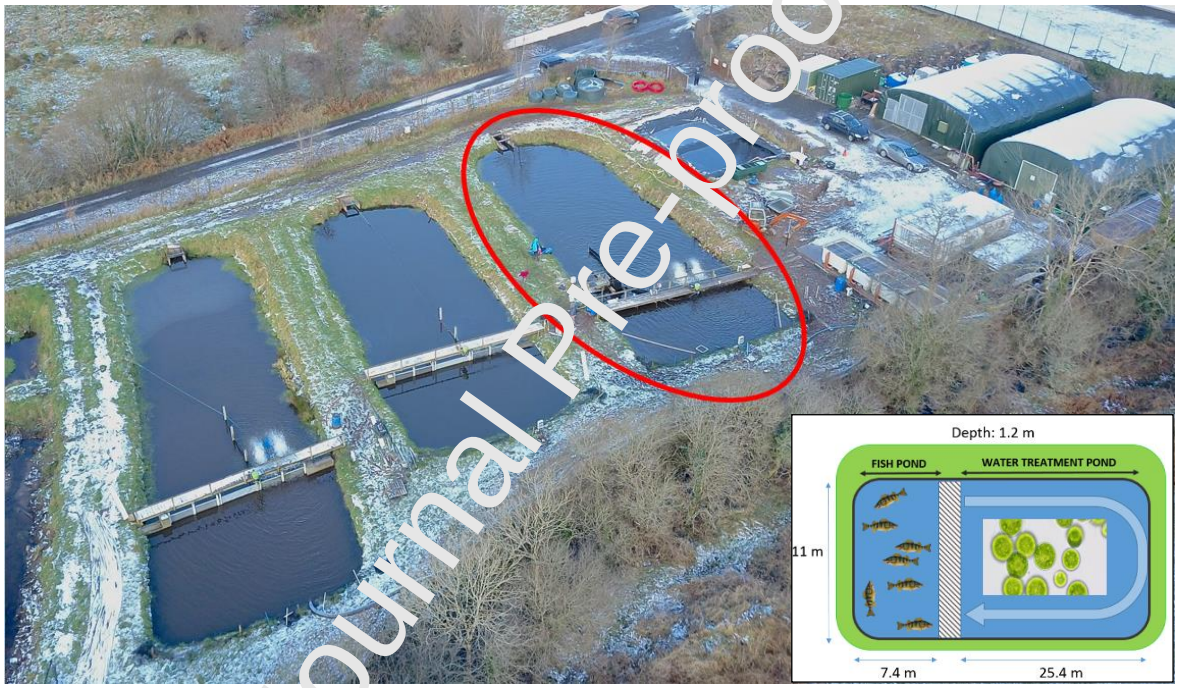

Fig. 1. Perch farm flow through ponds - aerial view, with insert schematic of Pond 1 layout.

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 and devices for physicochemical parameter analysis at the perch farm including acceptable limits.

Parameter	Measurement	R^2 Coefficient	Result Ranges (mg/l)		MAC (mg/l)	
			Fish Pond (FP)	Treatment Pond (TP)	SI No. 77 (2019)	Boyd (1998)
Nitrates	Photometric [Spectroquant® Nitrate Test – 1.09713]	0.998	< 0.01 – 3.27	< 0.01 – 3.25	50	0.2 – 10.0
Nitrites	Photometric [Spectroquant® Nitrite Test – 1.14776]	1	< 0.01 – 0.12	< 0.01 – 0.10	0.03	< 0.3
Ammonium	Photometric [Spectroquant® Ammonium Test – 1.14752]	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 [Spectroquant® Phosphate Test – 1.14848]	0.999	0.17 – 1.66	0.10 – 2.20	0.025	0.005 – 0.2
Carbonate Hardness	Titration [MColortest™ Carbonate Hardness Test – 1.08048	-	70.06 – 650.57	50.04 – 630.55	-	50 – 200

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 were 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.

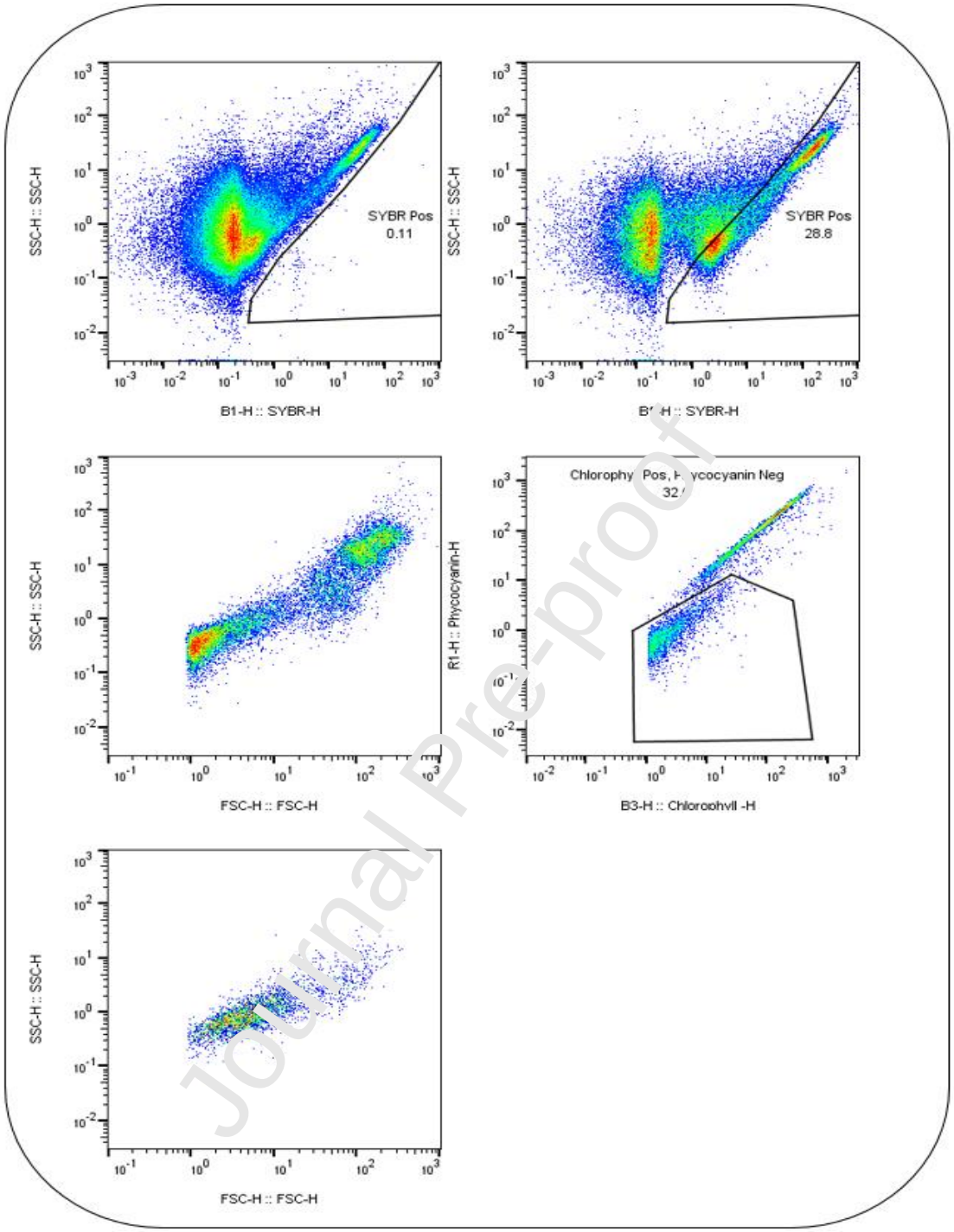

Fig. 2. Flow cytometry algal gating process involving **(A)** Gating for autofluorescence in the unstained sample, **(B)** Gating for relevant living organisms in the SYBR Green stained sample, **(C)** Chlorophyll positive population of the living cells viewed under FSC vs SSC, **(D)** Gating to eliminate cyanobacterial population and **(E)** Algal population of interest for enumeration viewed under FSC vs. SSC.

2.4 Microalga Profile Development and In-field AlgaeTorch Monitoring

Identification using a standard inverted microscope for morphological analysis and photographic identification keys was used in conjunction with flow cytometry (FCM) for

enumeration to maximise the information obtained.In addition to FCM analysis of microalgal populations, the chlorophyll and Cyanobacteria populations were also measured *in situ* using the AlgaTorch® (bbe Moldaenke). The AlgaTorch® 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).

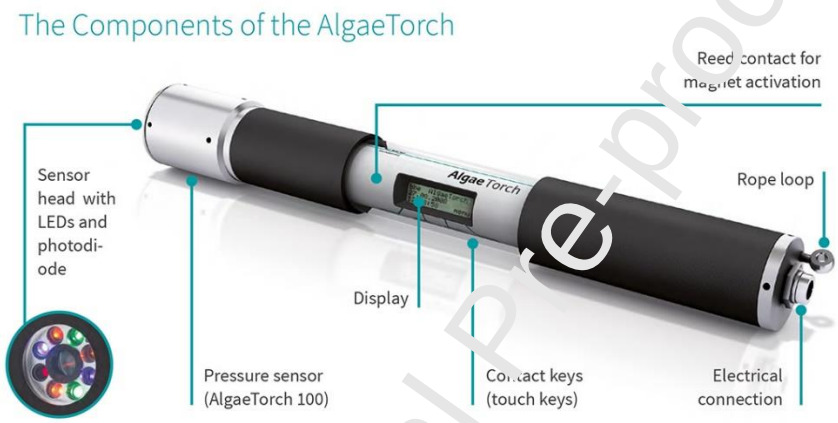

Fig. 3. AlgaeTorch diagram outlining the main components for operation (Source: bbe Moldaenke).

2.5 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 were 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.

2.6 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.

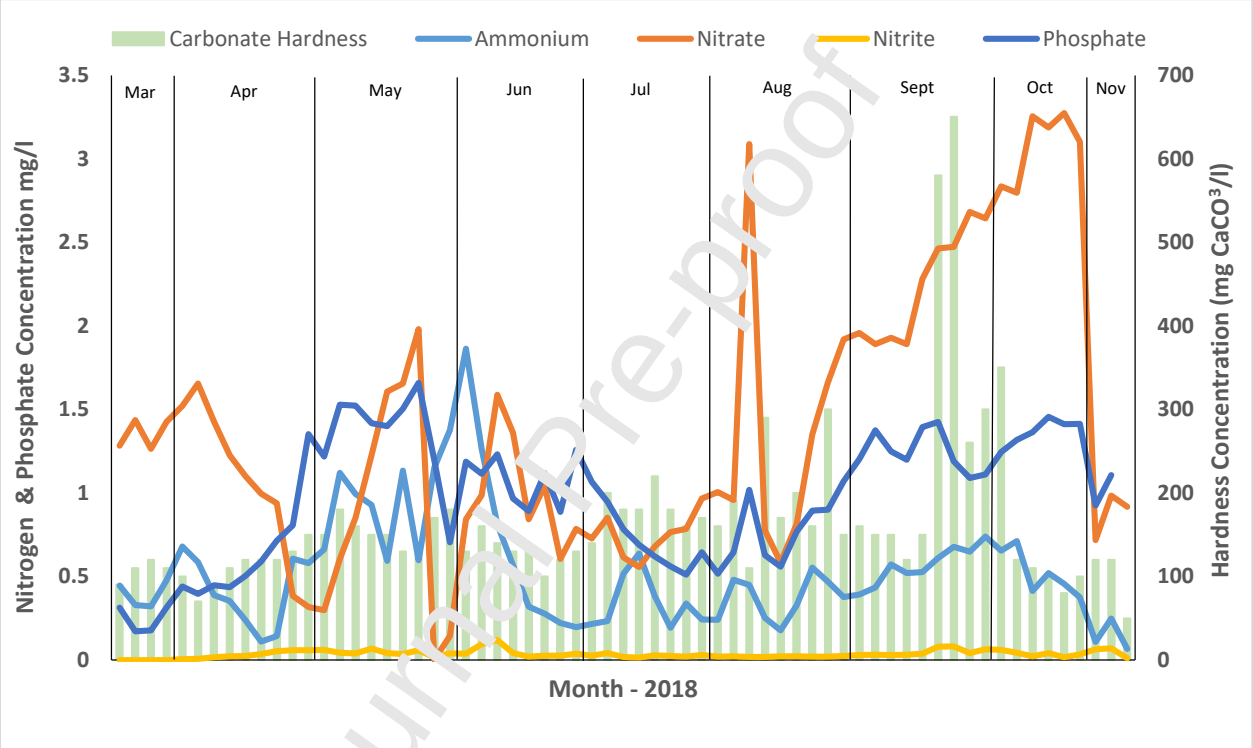

Fig. 4. Trends for nitrogen, phosphate and carbonate hardness for the FP at the perch farm, 2018.

There were no statistically significant differences between the data obtained for both sections of the pond regardless of the test used for analysis, as reported for nitrate (p= 0.9455), nitrite (p=0.9347), ammonium (p=0.7567), phosphate (p=0.9215) and carbonate hardness (p=0.0619).

3.2 Microalgal Enumeration

The algal numbers from March until November of 2018 in the perch farm are displayed in Figure 5 expressed as algal cells per ml, which was measured using FCM. As stated in the gating methodology for algal enumeration, the desired population included the chlorophyll population excluding cells positive for PC, therefore the graphs below do not include the majority of the Cyanobacteria numbers. The algal population in terms of 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.

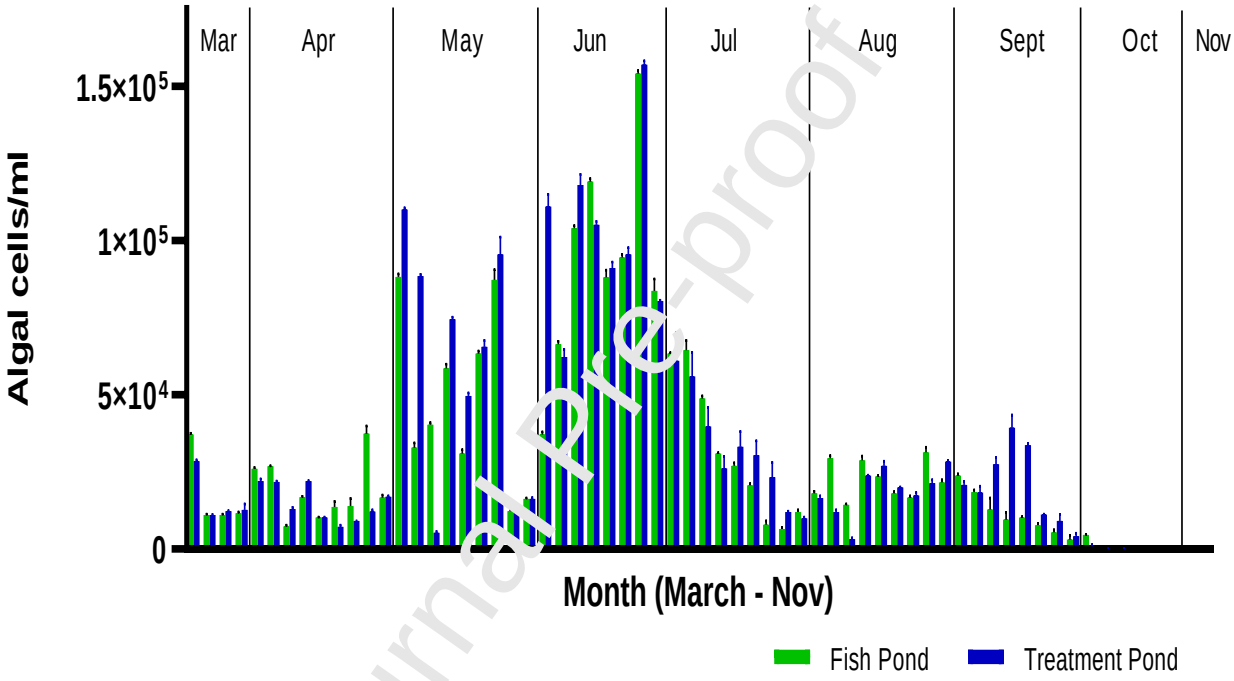

Fig. 5. Algae cells/ml in from March to November 2018 in Pond 1 at the perch farm.

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.

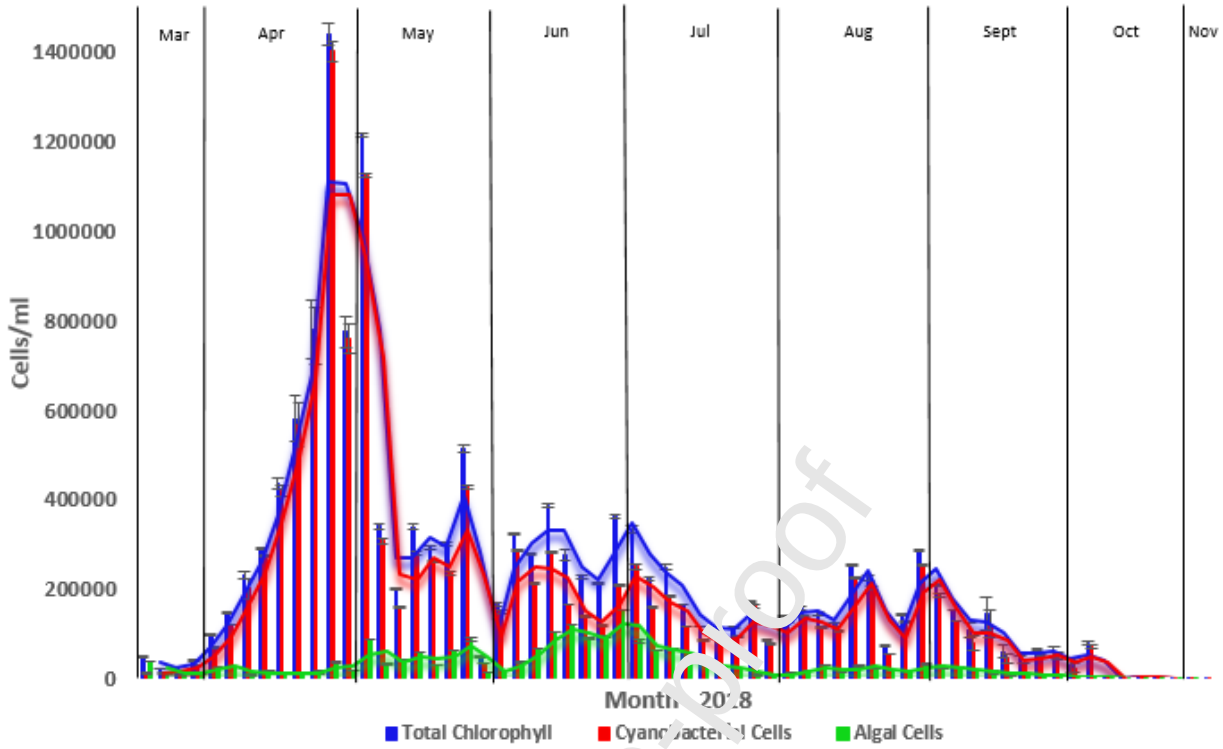

Fig. 6 Enumeration of total chlorophyll-containing cells consisting of cyanobacterial and algal cells in the FP at the perch farm, 2018.

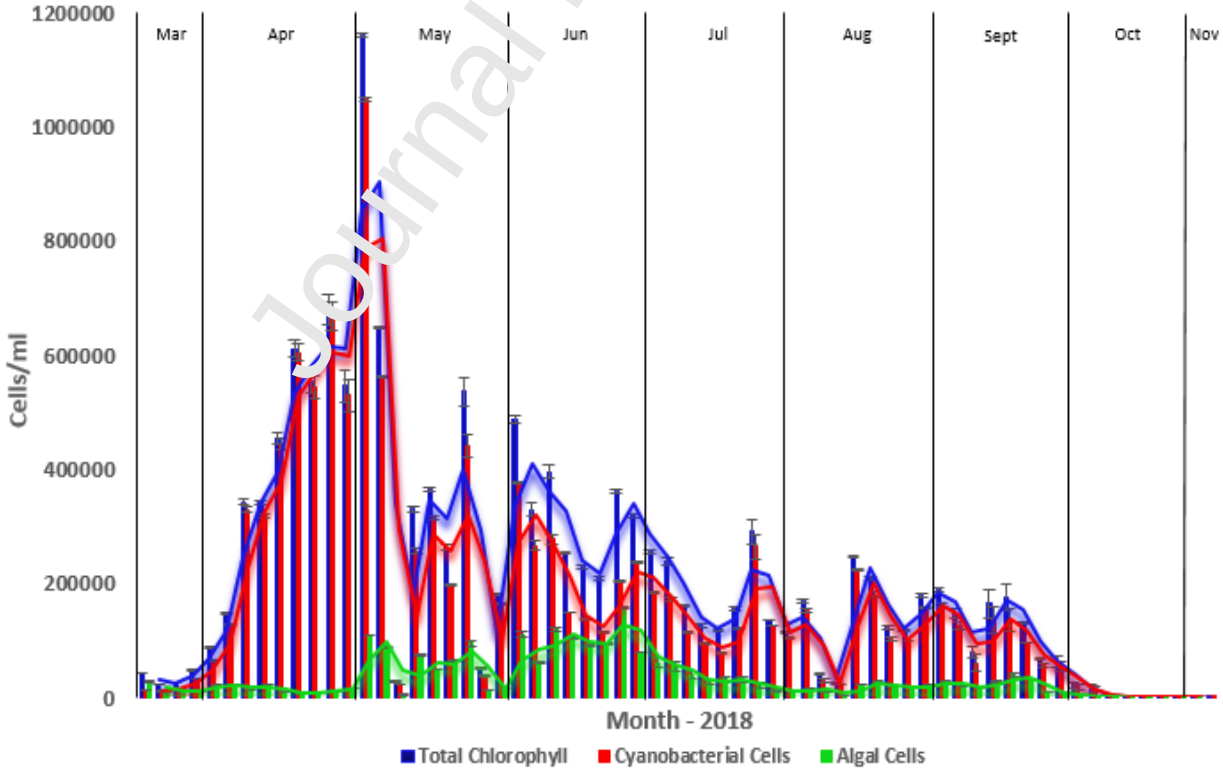

Fig. 7 Enumeration of total chlorophyll-containing cells consisting of cyanobacterial and algal cells in the TP at the perch farm, 2018.

3.4 Microalgal Community Profiling

Through use of the inverted light microscope, the microalgal profile and species diversity for each month was determined. The dominant species for each month are presented in Table 3 for the FP and Table 4 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 3 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	**Species**		
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 4 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	**Species**		
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 6.33x10^{6} cell/ml. The trend for this analysis from March to November 2018 is displayed in Fig. 8 expressed as $\log_{10}$ cells/ml.

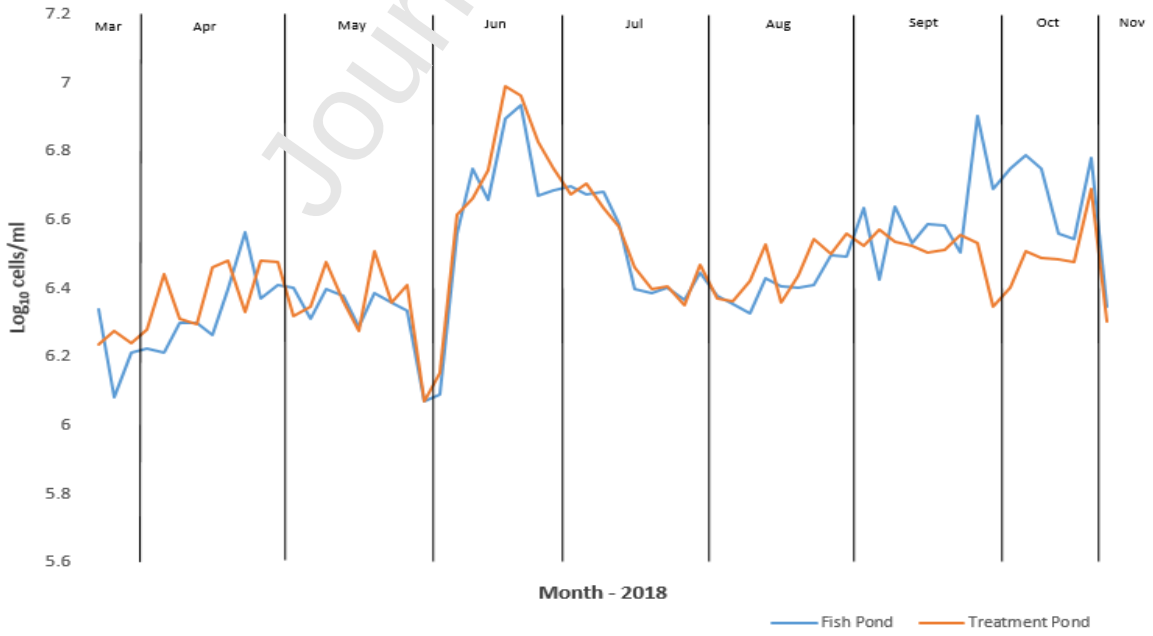

Fig. 8 Bacterial enumeration (log10 cells/ml) from March to November 2018 in Pond 1 at the perch farm.

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 Algaltorch® that is in good agreement with FCM determinations, with results in µg/l. Tables 5 and 6 display the correlation matrix scores obtained for the parameters analysed in this study.

Table 5 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	-0.1[illegible]5	1							
Feeding Rate	-0.093	-0.091	-0.076	0.068	0.420	0.367	-0.292	-0.060	0.[illegible]0	1						
Bacteria	-0.030	0.216	-0.080	0.257	0.402	0.304	-0.002	-0.121	0.100	0.[illegible]	1					
Algae	-0.369	0.230	0.043	0.190	0.329	0.264	0.336	0.319	-0.160	0.[illegible]	0.[illegible]55	1				
Chl-Ctn Cells	-0.463	0.366	0.065	0.284	0.069	-0.043	0.327	0.484	-0.151	-0.115	[illegible]	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 6 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	0.282	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	-0.0[illegible]	1						
Bacteria	-0.110	0.173	-0.068	0.233	0.384	0.282	0.019	-0.100	-0.099	0.27[illegible]	1					
Algae	-0.357	0.281	0.252	0.229	0.370	0.187	0.256	0.377	-0.030	0.14[illegible]	0.53[illegible]	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.

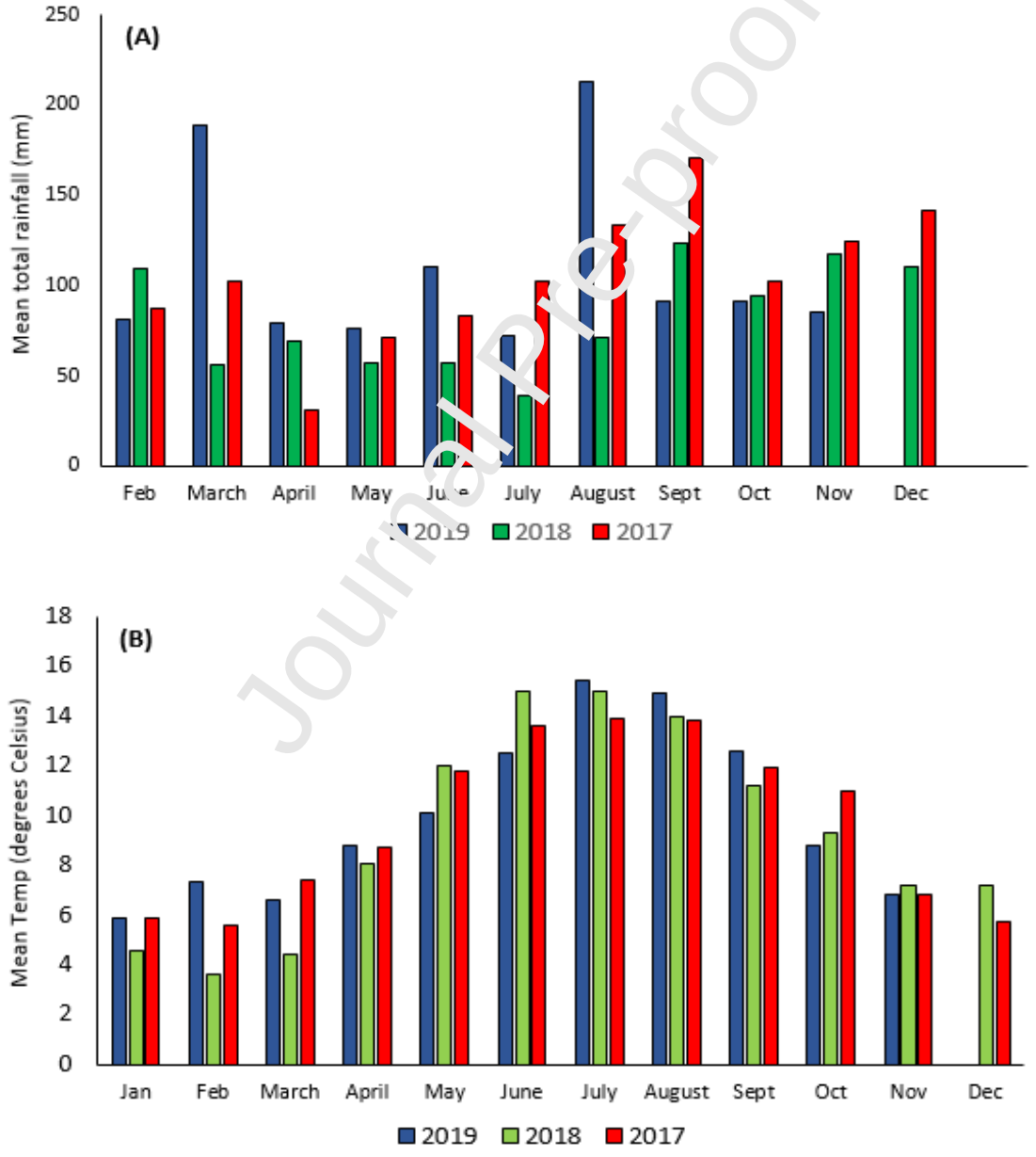

Fig. 9. (A) Mean total rainfall (mm) for three met offices nearby to sampled fish farm, and **(B)** mean temperature in degrees Celsius recorded at same three met offices, over periods 2017 to 2019.

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 (Hargreaves, 1998; Helfrich and Libey, 1990), 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 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 AlgaTorch®, 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.

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* sp., 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 (Chen et al., 2012; Choi and

Lee, 2012; Delgadillo-Mirquez et al., 2016; Delrue et al., 2016; Godos et al., 2012; Min et al., 2011; Nurdogan and Oswald, 1995). 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 be 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).

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. Findings from this present study provides knowledge regarding microbial interactions, and ecology of these systems, that prevent the utilization of microbial communities in the assessment, improvement and control of aquaculture farms (). 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 ; Bentzon-Tilia, Sonnenschien & Gram, 2016). 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.

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 cyanobacteria 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 (Abdel-Raouf *et al*., 2012; Choi and Lee, 2012; Delrue *et al*., 2016; Godos *et al*., 2012; Min *et al*., 2011). *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 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, 21% 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 rely 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. Limitations associated with findings relate to the fact that flow cytometry is highly specialised and not broadly available to support aquaculture industry, but could be provided as a specialist contract service. 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.

Acknowledgements

The authors wish to thank the collaborating aquaculture farm, Bord Iascaigh Mhara (BIM) and European Monetary Fund (EMF) for co-funding this research under the Knowledge Gateway Scheme (17KGS004).

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CRediT roles:

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Siobhan Kavanagh: funding acquisition, investigation, methodology, supervision, writing original manuscript and editing revision.

Mark Lynch: investigation, methodology, supervision, writing original paper and making edits to revision

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

Flow-cytometry correlated with in-field AlgaTorch® 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