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Title: Cultivable microalgae diversity from a freshwater aquaculture filtering system and its potential for polishing aquaculture derived water streams

Running head: Microalgae from aquaculture for bioremediation

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Abstract

Aims:

Microalgae are ubiquitous in aquatic environments, including aquaculture farms, but few studies have delved into their phytoplankton diversity and bioremediation potential. In this study, the cultivable phytoplankton of a rainbow trout freshwater aquaculture farm was isolated, phylogenetically analysed and used to assemble a consortium to polish an aquaculture derived effluent, with low concentrations of ammonium, nitrite, and nitrate.

Methods and Results:

Through standard plating in different selective media, a total of 15 microalgae strains were isolated from sludge from a rotary drum filtering system which removes suspended solids from the water exiting the facility. Based on 18S rRNA gene sequences, isolates were assigned to nine different genera of the *Chlorophyta* phylum: *Asterarcys*, *Chlorella, Chloroccocum*, *Chlorosarcinopsis*, *Coelastrella*, *Desmodesmus*, *Micractinium*, *Parachlorella, and Scenedesmus*. Species from most of these genera are known to inhabit freshwater systems in Galicia and continental Spain, but the *Coelastrella*, *Asterarcys* or *Parachlorella* genera are not usually present in freshwater streams. In an on-site integrative approach, the capacity of a consortium of native microalgae isolates to grow on aquaculture derived effluents, and its nutrient removal capacity were assessed using a raceway pond. After 7 days, removal efficiencies of approximately 99, 92 and 49% for ammonium, nitrite and nitrate were achieved concomitantly with a microalgae biomass increase of ca. 17%.

Conclusions:

Sludge from the aquaculture filtering system presents a high diversity of microalgae species from the *Chlorophyta* phylum, which application in a consortial approach revealed to be efficient to polish aquaculture derived effluents with low nutrient content.

Significance and Impact of Study:

The use of native microalgae consortia from aquaculture systems can contribute to the development of efficient treatment systems for low-nutrient wastewater, avoiding nutrients release to the environment and promoting water recirculation. This may further strengthen the use of phycoremediation at the industrial scale, as an environment-friendly strategy.

*Keywords***:** Phytoplankton diversity, Green microalgae, Aquaculture water streams, Nitrogen removal, Microalgal consortium

Introduction

Microalgae are a highly diverse group of photosynthetic microorganisms ubiquitous to every aquatic ecosystem, which can also be found in terrestrial environments (Hoffmann, 1989) and even on human made structures (Rifón-Lastra and Noguerol-Seoane, 2001). Due to their role as first trophic level in the ecosystems they occupy, they are directly impacted by environmental changes (anthropogenic or natural), though not all microalgae species are affected in the same way, as they are not equally sensitive (Wu et al., 2017). The diversity of phytoplankton in water systems is highly dependent on nutrients availability, temperature, light, grazing pressure, with parameters like water quality, N:P and C:N ratios, pH and silica concentration playing a significant role in the species composition and the lineages present, as well as in their productivity and physiology (El-Sheekh et al., 2000; Granéli et al., 2008).

In the northwest region of Spain, Galicia, the algal catalogue of rivers and freshwater systems places the number of identified *Chlorophyta* species at over 400 (making it the most prevalent phylum), followed by *Cyanophyta* and *Bacillariophyceae*, respectively (Margalef, 1955, 1956; Seoane, 1993; Rodriguez, 2005). These numbers have since been bolstered by 70 more species by further phytoplankton diversity studies (Rodríguez and Rodríguez, 2004, 2007a, 2007b). Freshwater aquaculture facilities obtain the water necessary for fish production from nearby water streams. It stands to reason that the microalgal diversity present in aquaculture systems should be strongly influenced by the phytoplankton species in those nearby water streams, which then could suffer further changes in abundance and structure resulting from the increased nutrient concentrations in this new environment generated by intensive aquaculture.

Over the last decades, fish production through aquaculture has been growing, surpassing the yield of wild fisheries in some parts of the world (Han et al., 2019). This industry utilizes enormous water volumes, generating great volumes of wastewater carrying increased amounts of suspended solids, and dissolved nutrients, such as nitrogen, and phosphorus, which are often released into aquatic ecosystems, causing environmental issues, such as eutrophication of the receiving water streams. Indeed, eutrophic water bodies have documented effects on indigenous microalgal species, including an increase in cell density, resulting in algal blooms and a decrease in phytoplankton diversity as less tolerant species are outcompeted by those which are more adaptable (Shanthala et al., 2009).

Environmental issues caused by aquaculture discharges are amplified in land-based facilities, since a concentration effect of pollutants might occur for being set in delimited areas. Ammonium (NH_4^+), which is mainly excreted by fish as a result of protein metabolism, is the main nitrogen form in aquaculture effluents (Wright, 1995). Therefore, it is crucial to decompose this nitrogen compound, especially in recirculating aquaculture systems (RAS) (Turcios & Papenbrock, 2014). Rotating biological contactors, trickling filters, bed filters, fluidized bed reactors, bio-flocs, and wetlands, are the most common biotreatment processes used in aquaculture (Mook et al., 2012). Aerobic bacterial filters are often used in intensive aquaculture systems to remove ammonium by its conversion into nitrate through nitrification by nitrifying bacteria. These systems are expensive and usually require an additional anaerobic treatment to promote denitrification of the nitrate produced, which is also an expensive and fragile system (Milhazes-Cunha & Otero, 2017). In a recent study at laboratory scale, Santorio et al. (2021) tested more compact and energy-efficient alternatives, an aerobic granular sludge reactor operated in sequencing batch mode and a continuous flow granular reactor (CFGR), to remove nitrogen compounds from intensive aquaculture water streams, producing high quality effluents and treating large flows, respectively.

Microalgae based systems are versatile alternatives or complements to these technologies as these microorganisms can take up both ammonium and nitrate present in wastewater (Milhazes-Cunha & Otero, 2017). Moreover, the use of such systems adds the benefit of the production of biomass that can be used for other ends (de Carvalho et al., 2020). Microalgae growth requirements are nearly only light, and nutrients present in every water stream, albeit at distinct concentrations. In fact, research on the treatment of aquaculture effluents has demonstrated the potential of algae for this purpose, with different species and treatment systems, such as raceway ponds or revolving algal biofilm cultivation systems (Han et al., 2019). For the successful application of microalgae for wastewater treatment, the selected microalgae should be adequate for the growth conditions and the environment where they will be cultivated so that only suitable species are used. Indigenous strains isolated from a specific environment would fit this description as they are already adapted to the abiotic conditions of such a site, which should fulfil the microalgae's nutrient requirements (Renuka et al., 2015).

Microalgae present in the aquaculture filtering system are subjected to more challenging physico-chemical conditions and potentially have the longest retention time in the aquaculture circuit. In this study, it is hypothesized that this farm sector provides conditions for hosting a wide diversity of microalgae, and that some of those might be well adapted to the variable conditions present in these water streams. Thus, foregathering information on the microalgae composition of this matrix, through isolation and identification, can be an advantage when aiming for their use in bioremediation processes. Also, the use of microalgae to treat wastewater in a consortial approach can be a promising alternative to improve process efficiency. When using a consortium of well-adapted strains, its synergistic interactions could have a broader potential in treating the wastewater due to the more diverse metabolic network, even under unfavourable environmental conditions, than monocultures. Some of these metabolic functions are difficult or perhaps impossible for single species cultures. The enrichment culture technique is commonly used to obtain microbial consortia with desired metabolic properties. However, the complexity of the microbial communities within the obtained consortia is high. The use of less complex but equally effective microbial consortia is often preferable especially when its upscaling at an industrial scale is envisaged. Moreover, the fact that not all of the existent strains within the enrichment can be cultivated and grown at the lab could limit its application for industrial purposes. Therefore, the assembling of an effective consortium harbouring a reduced number of culturable microbial species is often advantageous (Puentes-Téllez & Falcao-Salles, 2018).

This study sought to isolate and identify the culturable phytoplankton diversity present in the sludge collected from the water filtration system of a rainbow trout (*Oncorhynchus mykiss*) fish farm in Galicia, northwest of Spain. Afterwards, the set of isolated native microalgae was used in a consortial approach to polish real water streams with low nutrient content. It was hypothesized that native microalgae could be used to thrive in the aquaculture facilities effluents from where they were retrieved, as these isolates are already adapted to this environment, thus having a leverage in removing nutrients.

Materials and methods

Study area and sampling

The survey was performed in the rainbow trout aquaculture farm Grupo Tres Mares S.A. (43.0035˚ N, 9.2491˚ W), Santo Estevo de Lires, Galicia, in the northwest of Spain. This farm has a rotary drum filter which receives water from

the fish tanks. These systems are widely used to retain and remove suspended solids from process water, both in flow-through and in recirculating systems, allowing for the continuous filtration of high volumes of water without interruption of flow (Wang et al., 2021). This way, solids in the water with sufficient size are retained by the mesh openings. Samples of the sludge present in the retention drum from the water filtering system of the aquaculture facility were collected. The collection took place in September during the dry season, when the water stream that supplies the farm was partly recirculated due to the low water level of the nearby river (Ría de Lires).

Isolation of microalgae strains

Three different growth media were prepared for microalgae isolation: BG-11 (Zhu et al., 2013), optimum *Haematococcus* medium (OHM) (Monteiro et al., 2010) and a modified Provasoli *Haematococcus* medium (PHM) as described by Monteiro et al. (2011). Serial dilution of the sludge samples (0, 10x, 10^2x, 10^3x, 10^4x, 10^5x) was performed and further pour plated, in triplicate, on the three media. Plates were incubated at 25 ˚C, with light provided at an intensity of approximately 40 µmol photons m^{-2} s^{-1}, with a 16h/8h L/D cycle. Individual colonies visually identified as microalgae were picked with sterile loops and streaked onto the same type of solid medium, under sterile conditions, until isolated colonies were obtained. After unialgal colonies were obtained, they were replated onto BG-11 and stored at 25 ˚C, with light provided at ca. 40 µmol photons m^{-2} s^{-1}, with a 16h/8h L/D cycle.

Molecular identification of microalgal isolates

DNA extraction, BOX-PCR genetic fingerprinting and molecular identification by 18S ribosomal DNA amplification and sequencing

DNA extraction was carried out according to Wan et al. (2011). Microalgae isolates biomass was collected using sterile loops and resuspended in 6% (w/v) Chelex-100 buffer. The suspension was incubated for 10 min at 100 ˚C and then cooled on ice for 10 min. The suspension was vortexed and centrifuged for 1 min at 10,000 *g* and the supernatant collected and stored at -20 ˚C.

BOX-PCR is a genomic DNA fingerprinting method based on the repetitive element sequence-based polymerase chain reaction (Rep-PCR) of widespread evolutionarily conserved repetitive DNA elements, such as BOX, existent in prokaryotes and eukaryotes' genomes. For each strain, the PCR amplification applied to these BOX elements produces a unique band pattern on agarose gel (Tacão et al., 2005).

Therefore, BOX-PCR fingerprinting was performed to select, from within the obtained isolates, those that might potentially belong to different algal species or strains, using the primer BOX-A1R (5' CTA CGG CAA GGC GAC GCT GAC G 3'). The reaction mixture (20 µL) consisted of ultrapure water (12.6 µL), BOX-PCR A1R primer at 10 µmol L^{-1} (1.6 µL), Supreme NZY Taq 2x Green Master mix (5 µL) and DNA template (0.8 µL). Amplification was performed in an iCycler Thermal Cycle (Bio-Rad, California, USA) using the following programme: initial denaturation step (95 ˚C for 7 min) followed by 30 cycles consisting of denaturation (94 ˚C for 1 min), annealing (53 ˚C for 1 min) and extension (65 ˚C for 8 min), and a final extension step (65 ˚C for 16 min). PCR products alongside with 1-kb molecular weight ladder (GeneRuler DNA ladder mix, Thermo Scientific) were loaded onto 1.5% agarose gel for electrophoresis run. The banding patterns were analysed with Bionumerics software v6.1 (Applied Maths, Belgium). Isolates displaying different profiles were selected for identification.

Identification of the selected microalgae isolates was done through amplification of the 18S ribosomal DNA gene region using EUF (5' TCAGAGGTGAAATTCTTGGATTTA 3') and EUR (5' AGGGCACGACGTAATCAACG 3') primers. PCR mixture consisted of: ultrapure water (10.8 µL), EUF and EUR primers (10 µmol L^{-1}) (1.2 µL of each), NZYTaq 2x Green Master Mix (14.4 µL) (NZYTech, Portugal), DMSO (1.2 µL) and DNA template (1.2 µL). Amplification was done in an iCycler Thermal Cycler (Bio-Rad Laboratories, California, USA) using the following conditions: initial denaturation step 95 ˚C for 5 min; 30 cycles of denaturation at 95 ˚C for 1 min, annealing at 57 ˚C for 1 min, extension at 72 ˚C for 1 min and 30 s, and a final extension step at 72 ˚C for 10 min. To confirm the amplification´s success, electrophoresis was performed in 1.5% agarose gel (30 min at 90 V) and further visualized under an UV light (Bio-Rad Laboratories, California, USA). PCR products were purified using GRS PCR & Gel Band Purification Kit (GRiSP, Portugal), following manufacturer's instructions, and sequenced by StabVida (Portugal). The genomic sequences were manually trimmed using BioEdit software to remove ambiguous sequences in their distal (5' and 3') ends. The resulting sequences were subjected to nucleotide BLAST search against the National Centre for Biotechnology Information (NCBI) database to identify the closest strain to each microalgae isolate. The obtained 18S rDNA gene sequences were deposited in the NCBI database under the accession numbers MT732259 to MT732273.

Phylogenetic analysis of isolates

The top blast 18S rDNA gene sequences against each sequence of the isolated microalgae strains were retrieved from NCBI database and used for the phylogenetic analysis using MEGAX software. The rDNA data set was aligned using ClustalW with default settings and the aligned sequences were trimmed by deleting poorly aligned regions at both alignment ends, resulting in a matrix of 489 positions in each sequence of the final dataset. The evolutionary model that best fit the dataset was determined by the Bayesian information criterion. The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) and evolutionary distances were determined according to the Kimura 2-parameter method (Kimura, 1980). Tree robustness was estimated by bootstrap analysis based on 1000 replications (Felsenstein, 1985).

Capacity of microalgae consortia to polish water from aquaculture

In an on-site integrative approach, the effluent exiting a pilot CFGR, which partly treated the water recirculated in the aquaculture facility, was redirected into the raceway pond for polishing. The experiment was carried out batch-wise in a 40 cm x 20 cm x 20 cm single-loop raceway pond reactor with a working volume of 5 L. Motion in the raceway was achieved using a paddlewheel at 30 rpm and illumination provided by two LED strips working in 16h/8h L/D cycle. Before adding the aforementioned volume to the raceway pond, the effluent exiting the CFGR was first centrifuged at 5,700 *g* for 10 min to remove excess suspended solids.

A microalgae consortium composed by the retrieved culturable isolates was used as inoculum. Colonies of each isolate were picked from the agar plates and used to prepare monocultures inoculum in BG-11 liquid medium. Afterwards, each monoculture suspension was maintained at 25 ºC, with a 12h/12h L/D photoperiod, with light intensity (approximately 40 µmol photons m^{-2} s^{-1}), with continuous agitation, for one week. The mixed microalgae consortium inoculum was then obtained by mixing, in equal proportions, each monoculture inoculum to attain a starting dry weight cell density of ca. 0.03 g L^{-1}, and stored at 25 ºC, with a 16h/8h L/D photoperiod, with light

intensity (approximately 40 µmol photons m^{-2} s^{-1}), with continuous agitation. Cell concentration was determined as biomass dry weight using Whatman 0.47 µm filters. Before any chemical analysis, all samples were centrifuged for 5 min at 5,700 *g* and filtered using 0.45 µm pore size filters. The ammonium (NH_4^+-N) concentration was determined in accordance with Bower and Holm-Hansen (1980), while the concentrations of nitrite (NO_2^--N) and nitrate (NO_3^--N) were determined in accordance with the Standard Methods (APHA, 1999), all performed in triplicate. The concentrations of total organic carbon (TOC) and phosphate (PO_4^{3-}-P) were determined by catalytic combustion (Analyser model TOC-L CSN, Shimadzu, Japan) and ion chromatography (861 Advanced Compact IC system, Metrohm, Switzerland), respectively. The samples' pH was measured using a Hach probe.

Removal rate (R_i, mg L^{-1} d^{-1}) and specific removal rate (R_x, mg mg^{-1} TSS·d^{-1}) of NH_4^+-N, NO_2^--N and NO_3^--N were calculated as described by Zhou et al. (2014), using the following equations:

$R_i = (C_0 - C_t)/(\Delta t)$ (Eq. 1)

$R_x = R_i / DW_0$ (Eq. 2), where C_0 and C_t are the concentration (mg L^{-1}) of each nitrogen form in the wastewater at the start and end of the experiment, respectively; Δt (day) is the time elapsed between the beginning and the end of the experiment, and DW_0 is the average initial cell density of inoculum (mg L^{-1}).

The removal efficiency (R_{Ef}, %) was calculated using equation 3:

$R_{Ef} = [(C_0 - C_t)/C_0] \times 100$ (Eq. 3), where R_{Ef} is the removal efficiency (%), C_0 is the initial concentration of the substrate (mg L^{-1}) and C_t is the concentration at the chosen time of the experiment (mg L^{-1}).

Results

Phytoplankton diversity in freshwater aquaculture

Overall, 15 different phytoplankton strains were isolated from the sludge retention rotary drum filter receiving wastewater produced in a freshwater aquaculture farm using three different agar-solidified media specific for microalgae and standard plating methods (Table 1). Eleven different algal strains were isolated from PHM, seven from OHM and three from BG-11, with four of the identified strains being isolated from the three growth media.

To identify the culturable microalgal diversity in the aquaculture sludge, molecular characterization was carried out through the analysis of 18S rDNA gene sequences from DNA extracted from the isolates. The amplification of 18S rDNA genes using universal eukaryotic primers displayed efficient amplification of a unique single band resulting in sequences that after trimming of their distal (5' and 3') ends varied in size from 546 bp to 873 bp. The BLAST analysis of the partial 18S rDNA gene sequences of the isolates indicated that the culturable strains belonged to nine different genera of the *Chlorophyta* phylum, distributed between the *Chlorophyceae* and *Trebouxiophyceae* classes. As shown in table 1, all isolates have high homology, ranging from ca. 98 to 100%, to closely related organisms found in the GenBank database. Four of the isolates (GTM-2 to GTM-5) were identified as four distinct strains of the *Chlorella* genus, closely related to different *Chlorella sorokiana* strains. The remaining strains consisted of genera from the *Chlorophyta* phylum: *Asterarcys* sp. (GTM-1), *Chloroccocum* sp. (GTM-6), *Chlorosarcinopsis* sp. (GTM-7), *Coelastrella* sp. (GTM-8), *Desmodesmus* sp. (GTM-9, GTM-10), *Micractinium* sp. (GTM-11, GTM-12), *Parachlorella* sp. (GTM-13, GTM-14) and *Scenedesmus* sp. (GTM-15).

A phylogenetic tree was constructed to confirm the isolates' identification through their evolutionary relationship (Figure 1). In the phylogram inferred using neighbour-joining method, the isolated strains are distributed between two

main clusters along with other closely related strains, separating strains according to the hierarchical taxonomic class that they belong to: *Chlorophyceae* or *Trebouxiophyceae*. Strains GTM-1 and GTM-8 clustered with other members of the *Asterarcys* and *Coelastrella* genera within the same clade. Strain GTM-15 clustered in a clade which strains from the genera *Scenedesmus*. *Tetradesmus* and *Acutodesmus* were also included. In addition, strains GTM-9 and GTM-10 were found to be closest to strains of the *Desmodesmus* genus, grouping separately in a clade. Altogether, the above referred five strains shared a common origin as all strains belonged to the alga order *Sphaeropleales* within the *Chlorophyceae* class. Strains GTM-6 and GTM-7 formed a separate lineage within the *Chlorophyceae* class, closely related to microalgal species of the *Chlorococcum* and *Chlorosarcinopsis* genera, respectively. The other branch of the phylogenetic tree was occupied by all the isolates that belong to the *Chlorellales* order (*Trebouxiophyceae* class). Among the isolates, strains GTM-13 and GTM-14 were included in a cluster dominated by sequences of species within the genus *Parachlorella* while strains GTM-11 and GTM-12 were closer to *Micractinium* strains. Interestingly, while strains GTM-2, GTM-3 and GTM-4 clustered with other strains from the genus *Chlorella*, GTM-5, also identified as another strain belonging to the *Chlorella* genera using BLAST, in the phylogram was closer to *Micractinium* strains.

Capacity of a microalgae consortium to polish a nutrient poor aquaculture water stream

A microalgal consortium composed of the isolated microalgae was applied in the polishing of partly treated aquaculture wastewater in a closed raceway pond. The water used as feeding for this polishing experiment consisted of a 5 L parcel of a partly treated effluent from a pilot CFGR and was transferred to the raceway pond on the same day of its collection. This water's physico-chemical properties were pH of 5.02, 1.62 mg NH_4^+-N L^{-1}, 0.07 mg NO_2^--N L^{-1}, 1.51 mg NO_3^--N L^{-1}, 1.20 mg PO_4^{3-}-P L^{-1} and 5.72 mg TOC L^{-1}. Data on the removal of ammonium, nitrite, and nitrate as well as the evolution of microalgae biomass content throughout the experiment are shown in Fig. 2. The analysis of the water in the raceway pond at the begging of the experiment revealed an ammonium concentration higher than expected (3.5 mg NH_4^+-N L^{-1}), when compared with the above-mentioned value for the same parameter in the influent, before being added to the raceway system. This was a consequence of residual amounts of growth medium from the inoculum, despite the biomass cleaning step. The concentration of NH_4^+-N decreased steadily until day-4, when it was nearly completely removed (ca. 99%) (Table 2). During the first two days, the concentrations of NO_2^--N and NO_3^--N remained stable, but increased slightly on day-3, after which the residual amount of nitrite in the system was nearly all consumed (ca. 92%). Nitrate consumption began on day-3, reaching a removal efficiency of ca. 49% by the end of the assay. In addition, 46% of the PO_4^--P present at the beginning of the experiment was removed (data not shown). Data on the removal rate and on the specific removal rate of each nitrogen form is shown on Table 2. Removal rates decreased in the order NH_4^+-N > NO_3^--N > NO_2^--N which coincided with the different inorganic nitrogen forms' availabilities in terms of concentration in the feeding medium. Cell density remained relatively stable throughout the 7-day trial, with no visually distinct growth phases. By the end of the trial, ca. 17% of increase in biomass content was observed.

A second experiment under laboratory conditions was carried out using an initial algal concentration of 0.14 g L^{-1}, and an ammonium initial concentration of 1.6 mg NH_4^+-N L^{-1}. After 7 days, approx. 97% of NH_4^+ had been removed. While the removal of this compound was successful, approx. 42% of the remaining biomass had been lost in the same

period of time (data not shown). This biomass loss was attributed to a short acclimatization period, as the microalgae in the consortium were transferred from a nutrient rich liquid medium (BG-11) to a low nutrient aquaculture mimicking medium. Higher biomass productivity is expected after longer acclimatization periods.

Discussion

In this study, 15 cultivable microalgae strains of the *Chlorophyta* phylum were isolated from the sludge collected from the retention drum of a water filtering system in a freshwater aquaculture facility in Galicia, Spain. A consortium of such strains was able to remove the trace nutrient present in the effluent from a pilot reactor treating water exiting fish tanks from an aquaculture facility.

Based on a BLAST-based species assignment, the isolated microalgae diversity belonged to nine different genera distributed between two phylogenetic classes: *Chlorophyceae* and *Trebouxiophyceae*. In general, the phylogenetic reconstruction based on the 18S rRNA gene dataset supported the taxonomic identification at genus level. The phylogenetic tree increased the confidence of the findings regarding the identity of the isolated strains as they clustered closely with other strains of the same genera. The single exception was strain GTM-5, that through BLAST search was identified as belonging to the *Chlorella* genus but in the phylogram clustered most closely with strains of the *Micractinium* genus, contrary to that observed for the other strains also annotated as *Chlorella* species (strains GTM-2, GTM-3 and GTM-4). This incongruence was probably due to differences in the sequence lengths used for BLAST analysis and for the phylogenetic reconstruction. A molecular sequence of about 646 bp was used for BLAST analysis indicating that this microalgal isolate was closely related to a *Chlorella sorokiniana* strain (100% of sequence similarity), while for the sequence-based phylogenetic analysis a sequence of about 489 bp was used, due to the trimming of the ends of the poorly aligned sequences. *Chlorella* and *Micractinium* are members of the class *Chlorophycaea*, sharing a common origin and thus the trimming of the ends of the sequences lead to this divergence. In addition, although sequences of strains GTM-1 and GTM-8 were annotated as *Asterarcys* and *Coelastrella* species, respectively, they grouped into the same genetic cluster of the phylogram with a high index of nucleotide similarity (100%). This was also due to the shortening of the molecular sequences. To overcome these limitations, sequencing of other molecular markers such as the ITS or the 23S gene region could be useful for the discrimination of closely related species and thus to ascertain the taxonomical positions of the isolates to a better extent. However, recently, it was reported that both ITS and 23S markers also have shown to exhibit limitations in species assignment (Ferro et al., 2018). Nevertheless, it is also important to notice that even though the trimming of the sequences after alignment lead to some mismatches, the phylogram still revealed genetic variation within strains assigned to the same genus. Based on 18S rRNA gene sequences, the closest relative to strains GTM-2, GTM-3 and GTM-4 was *Chlorella sorokiniana* with 100, 98 and 100% of similarity, respectively. However, in the phylogram, these strains were separated in different branches within the same clade; strains GTM-2 and GTM-4 clustered together and are clearly distant from the sister strain GTM-3.

In addition, strains GTM-2 and GTM-4, along with strains GTM-1 and GTM-8 also displayed high degrees of similarity between each pair (99.6 and 100%, respectively), with the differences in sequence lengths possibly being the main contributor to BLAST identification as different organisms in the latter pair. Further research into other

identifying DNA sequences, such as the ITS and 23S region sequences could help clarify whether these strains actually belong to the same organisms, as well as their species identity.

Literature on the algal diversity in the NW of Spain revealed that several species of the *Scenedesmus*, *Chlorella*, *Chlorosarcinopsis* and *Micractinium* genera have been identified in the region (Margalef, 1956; Rifón-Lastra and Noguerol-Seoane, 2001; Vasconcelos and Cerqueira, 2001; Rodríguez and Rodríguez, 2007a, 2007b;), while the remainder genera had not yet been reported in this part of Spain. By broadening the search to the whole continental Spain, species of *Chlorococcum* and *Desmosdesmus* genera are also found to inhabit freshwater systems in the country (Cobelas Alvarez and Gallardo, 1986; Fanés Treviño et al., 2009). However, no report was found on the presence of species belonging to the *Coelastrella*, *Asterarcys* or *Parachlorella* genera in freshwater systems in continental Spain. Nevertheless, the latter genera had previously been described close to the northern border of Portugal (Bock et al., 2011).

All the identified microalgal genera include well known freshwater species, though some, like *Coelastrella* sp., also include species documented to inhabit soils, wastewaters, and peat pools. Some of these strains are not usually associated with freshwater streams but derived from other close environments. Having been retrieved from sludge collected from the wastewater filtration system of the aquaculture facility, perhaps they could have had the opportunity to grow in this environment as a result of the change of conditions (from river to aquaculture setting), thus facilitating the establishment of less common microalgae in freshwater.

In this study, different growth agar media were used to broaden the diversity of the collected algal species as there is not any unique medium suitable for the isolation of all algal species. Therefore, the use of different media with different compositions and concentrations of key components such as carbon, vitamins, salts, and nutrients, could improve the isolation of a great microalgae variety. Perhaps the presence of vitamins in the growth media could have contributed to the growth of a greater variety of algal strains in PHM and OHM in comparison with BG-11, though the possibility that several isolated strains grew in the three media, but their colonies were not able to be replated has to be considered.

Diatoms are one of the most prevalent groups of microalgae in wastewater (Casé et al., 2008; Kulabtong et al., 2019). In this study, none of the growth media used were specific for these organisms, which require key elements like silica for cell division (Jøsrgensen, 1952). Therefore, no diatom isolates were obtained. Notable, however, was the absence of cyanobacteria in the present survey, as these Cyanophyta have been among the most prevalent organisms reported in other aquaculture systems (Kulabtong et al., 2019; Qiao et al., 2019). Their absence from this study may be justified by the choice of isolation media, as perhaps these were not adequate for the Cyanophyta present in the aquaculture farm, though BG-11 is a known growth media for cyanobacteria. Another possible explanation lies in the DNA extraction step, as the procedure was not successful for several colonies. Without the necessary DNA samples for their identification, possible cyanobacteria strains could have gone unnoticed.

Other studies on the diversity of microalgae in aquaculture systems display a greater range of genera and species. While researching the phytoplankton diversity in an industrial *Litopenaeus vannamei* aquaculture system, and using high-throughput and morphological identification approaches, Qiao *et al.* (2019) found a total of 41 genera distributed through nine phyla, with green microalgae, cyanobacteria and diatoms within the dominant groups. Green microalgae and cyanobacteria were especially abundant, namely the *Picochlorum* and *Synechococcus* genera, accounting for most

of the total phytoplankton. Likewise, when investigating shrimp and prawn ponds, Kulabtong et al. (2019) found 36 different species through morphological identification, with green microalgae dominating the phytoplankton diversity (21 species), although cyanobacteria displayed higher total biomass in the system. Because these research works feature different environments (fresh and saltwater, temperate and tropical rivers), different sample types (sludge and water samples) and different identification approaches (cultivation vs morphological identification and high-throughput sequencing), differences in the diversity of the identified microalgae are expected. In addition, the aforementioned aquaculture systems also focus on the production of different fish or shrimp species and on different scales which directly ties to the characteristics of the systems' water, ultimately conditioning the phytoplankton diversity in the sampling sites. It is worth taking into account that because the present study only looked into the culturable microalgae that could be grown in solid media, some strains might have been disregarded, and could not be identified in this way. As a result, the total diversity in the sludge of the aquaculture system could have been underestimated.

Following the assessment of phytoplankton diversity in the aquaculture facility, a consortium of the native isolated culturable strains was assembled and its potential to polish the effluent leaving a pilot CFGR, treating wastewater generated from freshwater fish aquaculture during water recirculation season, was evaluated. The removal of free ammonia from the water in the aquaculture system is of great concern as concentrations over 0.08 mg L^{-1} (or 3.0 mg NH_4^+-N L^{-1} of ammonium) can be toxic to rainbow trout. Even a 1.6 mg NH_4^+-N L^{-1} of ammonium is only tolerable in the short-term exposure (Liao and Mayo, 1972). However, given the water's acidity, this value could theoretically be higher, as the chemical balance favours NH_4^+ over NH_3. Although carbon removal from aquaculture effluents is important, its removal is not as important an issue as ammonium due to the toxicity of the latter for fish. Thus, for a preliminary proof of concept to assess the feasibility of the microalgae consortium in a raceway pond to treat extremely low loaded wastewater aiming for water recirculation, ammonium removal was the focus, which was successfully accomplished.

Previous studies on the topic of aquaculture wastewater treatment have found success using native microalgal cultures for this purpose. Indigenous species hold the benefit that they can be effectively more adapted to that environment, increasing their chance to survive and proliferate, and thus achieve higher nutrient removal rates than non-native strains. In fact, in the present study, the native microalgae consortium was effective in assimilating inorganic nitrogen and phosphorus for growth, and nutrient uptake occurred from the beginning of the trial without the need of an acclimatization period. Furthermore, research on this field has shown that often multi-species cultures have positive effects on the productivity of algal cultures, utilizing nutrients more efficiently, can also contribute to a faster removal of target pollutants (Stockenreiter et al., 2016; Tossavainen et al., 2018). Enrichment processes are more commonly used to obtain well adapted consortia, but isolation of native species is also often adopted for the selection of potential candidate microalgae strains for wastewater remediation. Through enrichment, the obtained microbial consortia are complex and usually composed of a large number of culturable and unculturable species which could hamper its upscaling. Microbial consortia formed by the assemblage of cultivable native strains is an alternative to obtain mixed populations, that allow for effective and more controlled treatment processes, valuable when application at larger scales is envisaged.

Generally, in the presence of multiple nitrogen sources, the order in which each nitrogen source is consumed is directly tied to the energy efficiency of their consumption. Microalgae will firstly take up NH_4^+, and so long as it remains available, the other nitrogen sources will hardly be removed. This is because NH_4^+ is the most energetically efficient nitrogen source available and therefore its assimilation is less energy consuming than NO_2^- or NO_3^- (Perez-Garcia et al., 2011). Once the NH_4^+ concentration becomes low enough, and so less available, the consumption of NO_2^- and NO_3^- begins leading to near complete removal of NO_2^- and NO_3^-. The increase in NO_3^- and NO_2^- concentrations on day-3 was possibly linked to the consecutive decrease in biomass, due to the release of intracellular material into the medium. The increase in NO_2^- concentration might also have been a result of the reduction of NO_3^- into this compound by organisms in the reactor system either from the CFGR or native to the aquaculture facility itself, thus becoming more bioavailable for consumption. Although the microalgae species in the consortia efficiently removed NH_4^+, in the present study, the removal rate (0.487 mg NH_4^+-N L^{-1} d^{-1}) was relativity low. This slow removal could be a consequence of the limited algal growth due to the low levels of available nutrients and carbon sources.

The rather low increase in biomass concentration can likely be linked to the overall low concentrations of key nutrients such as the nitrogen, phosphorus, and carbon sources in the water stream, as well as the ratio between them, namely N:P. The molar N:P ratio of this aquaculture effluent was 9:1 but, as a result of nutrient increase from the inoculum, this value was closer to 4:1 in the raceway pond. This latter ratio implies a deficiency in nitrogen, as further studies have placed the optimum N:P ratio for freshwater algae growth and nutrient removal in the range of 6.8:1 to 10.0:1 (Li et al., 2019). The low pH of the water used to feed the raceway pond could also have negatively affected the growth and yield of the culture, further impairing the production of biomass (Lv et al., 2019). Furthermore, this low increase of the microalgal biomass content, could also be due to the initial adaptation of the microalgae species to both the physico-chemical and microbiological composition of the effluent. Often, after an initial acclimation period, biomass productivity tends to increase. This is particularly important, as the microalgal biomass generated from the treatment of the aquaculture effluents can then be exploited for the recovery of value-added products, e.g., the production of bioenergy, animal feed, pharmaceuticals, or fertilizers (Han et al., 2019). Further use of the produced microalgae biomass could be a way to help this industrial sector in its integration in the circular economy concept.

In this work, throughout the experiment, biomass progress was followed only quantitatively. Changes in the microbial composition of the consortium are expected to occur, especially in the long run, but as this was a short-term assay aiming to prove the concept for the use of an autochthonous cultivable microalgae consortium in bioremediation processes, changes in microbial composition were not analysed.

Although biomass growth was low, removal efficiency showed potential, with ammonium removal efficiency showing comparable results to other studies on the removal of nitrogen from aquaculture wastewater (Table 3).

Tossavainen et al. (2019) used mixed algal cultures to treat fish aquaculture wastewater and obtained an NH_4^+-N removal ranging from to 98.9 to 99.5%, similar to the present study, where a removal efficiency of 99.6% was observed, although a higher NH_4^+-N concentration was present in the media. Nasir et al.(2015), using an unialgal *Chlorella* sp. inoculum, successfully treated wastewater derived from African catfish aquaculture, obtaining an ammonium removal of 98.5% after 10 days. Ansari et al. (2017) utilized unialgal cultures of *S. obliquus*, *C. sorokiniana*, and *Ankistrodesmus falcatus* for the treatment of Nile tilapia aquaculture wastewater. After 14 days of

batch cultivation, removal efficiencies of 86.85, 88.71 and 98.21% for NH_4^+-N and of 77.70, 75.76 and 80.85% for NO_3^--N were obtained for the unialgal cultures of *S. obliquus*, *C. sorokiniana*, and *A. falcatus,* respectively. The NO_3^--N removal efficiency obtained by the microalgal consortium used in this study is undoubtedly lower but the available NO_3^--N concentration in the water stream was also lower than that in the study performed by Ansari et al. (2017). Additionally, it is possible that if this culture had remained active for an additional 7 days, similar removal efficiencies could have been achieved. Gao et al. (2016), on the other hand, utilized a membrane photobioreactor and, with this set-up, obtained an average total nitrogen removal efficiency of 86.1%. Future research on the feasibility to use poorly nutrient charged aquaculture to cultivate microalgae could benefit from utilizing a continuous system as this would address the obstacle presented by low concentration of N and P and potentially treat larger volumes of effluent. The use of raceway ponds is promising for the treatment of aquaculture streams for being an environmentally sustainable option – they use natural sunlight, microalgae in such systems trap atmospheric CO_2, and the space occupied can sometimes be availed of already existing tanks. In this study, the 5 L raceway pond was illuminated with LED strips working in a 16h/8h L/D cycle which seemed to be enough to prompt biomass growth. Nevertheless, when projecting an upscaling for such a system, benefiting from natural sunlight would be economically and logistically advisable.

Bioprospecting of indigenous microalgae from a freshwater aquaculture farm revealed the presence of a diverse microalgae population with 15 cultivable microalgal strains belonging to nine different genera from two different classes within the *Chlorophyta* phylum. The use of a native mixed microalgal culture was successful in the removal of ammonium from the partially treated aquaculture wastewater in a raceway pond. This is especially meaningful as the removal of this pollutant is of great concern when water recirculation for the fish tanks is aimed. Further long-term studies could help to ascertain the viability of the application of the designed microalgae consortia in raceway pond systems to treat aquaculture water streams aiming for recirculation. Evaluation of the species within the assembled consortium over time could also be helpful to trace which species are more prevalent and more functional to the treatment. Additionally, it would be interesting to explore this consortial approach in treating water streams with different N rate variations, since in an aquaculture system, both in flow-throw and in RAS, those fluctuations are expected to occur.

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Conflict of Interest

The authors declare no conflicts of interest.

Credit author statement

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Figures captions

Fig. 1 - Phylogenetic tree of the isolated strains from the aquaculture facility's sludge and closely related taxa. Boxes indicated the strains isolated in this work. The tree was generated using the neighbour-joining method tree with 1000 bootstrap replicates. Bootstrap values (%) are showed near each node. The scale bar represents 0.01 substitutions per site.

Fig. 2 – Time-course profiles of ammonium (NH_4^+-N, ●), nitrite (NO_2^--N, ■) and nitrate (NO_3^--N, ▲) removal during polishing of the low nutrient water stream by the microalgae consortium along with the variation of the microalgae dry weight biomass concentration (×).

Table 1 - Microalgal taxa and corresponding BLAST result, including closest genetic relative, accession number, query cover, identity percentage and isolation site. The growth media from which each strain was isolated is also included.

Microalgae isolate (accession number)	Sequence length (bp)	Closest relative BLAST result	Closest related strain origin	Query cover (%)	Identity (%)	Isolation Media
GTM-1 (MT732259)	645	*Asterarcys quadricellulare* isolate FACHB-2316 (MH176109.1)	n.r.	100	100	PHM
GTM-2 (MT732260)	648	*Chlorella sorokiniana* isolate NLMX (MN011866.1)	n.r.	100	100	PHM, OHM
GTM-3 (MT732261)	629	*Chlorella sorokiniana* strain NKH6 (LC505542.1)	n.r.	100	97.7	OHM
GTM-4 (MT732262)	656	*Chlorella sorokiniana* strain DPK-5 (KX966287.1)	Water body, India	100	100	PHM
GTM-5 (MT732263)	646	*Chlorella sorokiniana* NKH18 (LC505550.1)	n.r.	100	100	PHM
GTM-6 (MT732264)	638	*Chlorococcum* sp. z1 (MK954470.1)	n.r.	100	100	PHM, OHM
GTM-7 (MT732265)	648	*Chlorosarcinopsis* sp. YACCYB260 (MH651263.1)	n.r.	100	99.9	PHM
GTM-8 (MT732266)	617	*Coelastrella* sp. IS3 (MN719510.1)	Poultry wastewater, Australia	100	100	PHM
GTM-9 (MT732267)	873	*Desmodesmus* sp. GTD9C2 18S (JQ315186.1)	n.r.	100	99.5	OHM
GTM-10 (MT732268)	645	*Desmodesmus* sp. ZFY_TSS20171115-0871-0818 (MH624152.1)	n.r.	100	100	BG-11, PHM, OHM
GTM-11 (MT732269)	654	*Micractinium* sp. MM0001 (MF959935.1)	Seawater, South Korea	100	100	PHM
GTM-12 (MT732270)	546	*Micractinium belenophorum* strain CCAP 271/1 (FM205880.1)	n.r.	100	99.5	PHM
GTM-13 (MT732271)	640	*Parachlorella* sp. BX1.5 (LC473527.1)	Natural water, Japan	100	100	OHM
GTM-14 (MT732272)	641	*Parachlorella hussii* strain ACOI 939 (HM126551.1)	n.r.	100	100	BG-11
GTM-15 (MT732273)	637	*Scenedesmus* sp. MF2 (MN850521.1)	Dongpo Lake, China	100	99.8	BG-11, PHM, OHM

n.r.- not reported at the respective NCBI page.

Table 2 - Removal rate (R_i), specific removal rate (R_x) and removal efficiency (R_{Ef}) of the nitrogen forms by the microalgae consortium. The concentrations of the nitrogen forms on day-0 ($C_{0\ mean}$) and on day-7 ($C_{7\ mean}$) are presented as mean (± S.D.) (n=3).

Nitrogen form	$C_{0\ mean}$ (mg L^{-1})	$C_{7\ mean}$ (mg L^{-1})	R_i (mg L^{-1} d^{-1})	R_x (mg mg^{-1}_{TSS} d^{-1})	R_{Ef} (%)

NH_4^+-N	3.42 (± 0.22)	0.01 (± 0.01)	0.49	1.2×10^1	99.6
NO_2^--N	0.07 (± 0.002)	0.005 (± 0.001)	0.009	0.2	92.0
NO_3^--N	1.49 (± 0.02)	0.76 (± 0.02)	0.10	2.6	49.3

Table 3 – Nitrogen removal efficiency from aquaculture derived water streams through phytoremediation with microalgae

Microalgal strain(s)	Wastewater source	Concentration (mg L^{-1})		Removal efficiency (%)		Cultivation system	Reference
		NH_4^+-N	NO_3^--N	NH_4^+-N	NO_3^--N		
Mixed microalgal culture	Freshwater rainbow trout pre-treated in a CFGR	1.62	1.51	99.6	49.3	5 L raceway pond batch culture	This study
Mixed microalgal culture	Freshwater catfish and pike perch aquaculture	7.4 - 9.7	-	98.9 - 99.5	-	2 L bottle batch cultures	Tossavainen et al., 2019
Chlorella sp.	Freshwater African catfish aquaculture	0.71	-	98.5	-	20 L PBR batch culture	Nasir et al., 2015
S. obliquus *C. sorokiniana* *Ankistrodesmus falcatus*	Nile tilapia aquaculture	5.32	40.67	86.85 88.71 98.21	77.70 75.76 80.85	1 L conical flask batch culture	Ansari et al., 2017
Chlorella vulgaris	Saline white-leg shrimp aquaculture	-	2.00	-	86.1	5 L MPBR continuous culture	Gao et al., 2016

Fig. 1

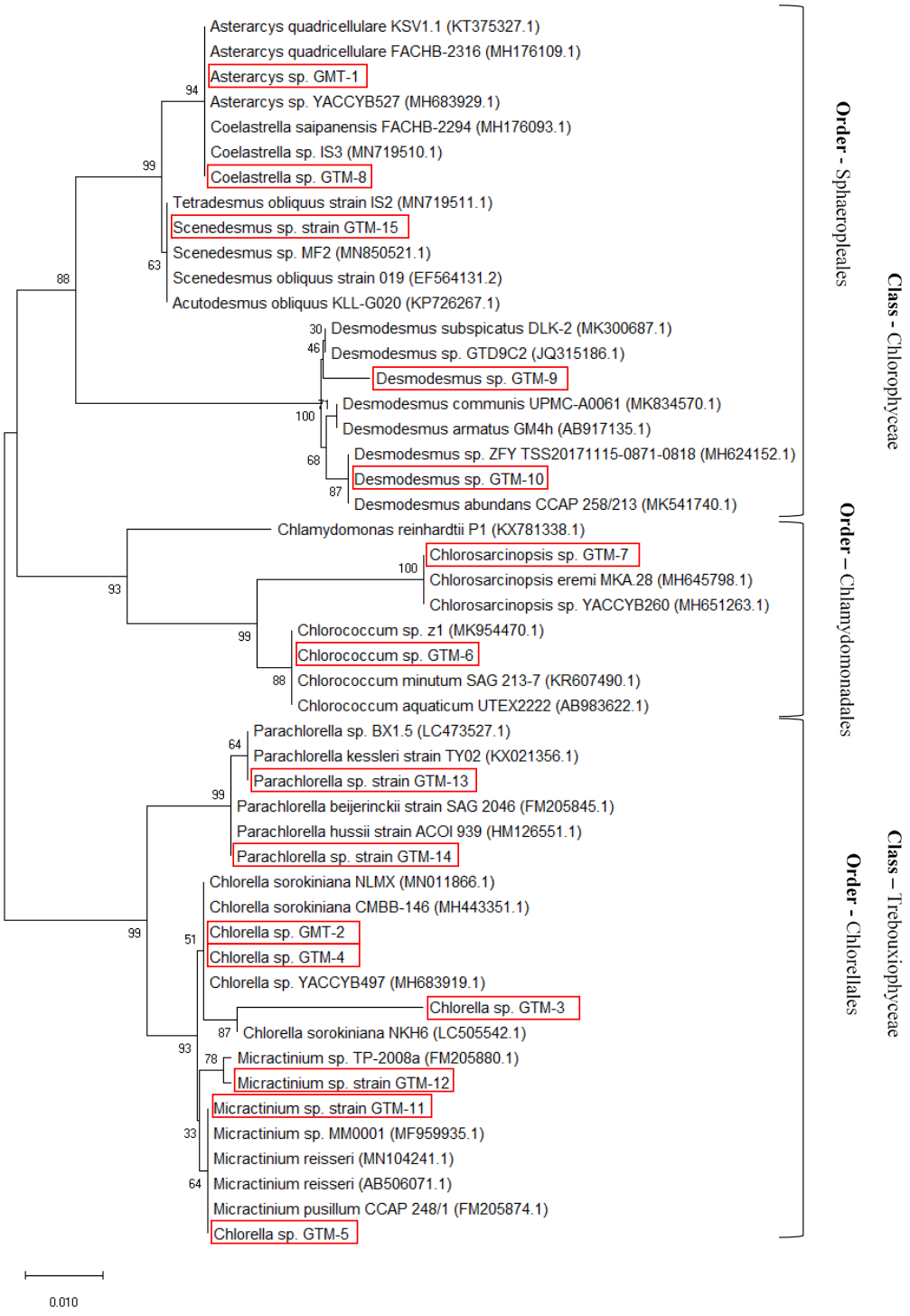

Fig. 2