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O uso de *Corbicula fluminea* na remediação de *blooms* cianobacterianos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Joana Luísa Lourenço Estevinho Pereira, investigadora em pós-Doutoramento, e co-orientação da Doutora Daniela Rebelo de Figueiredo, investigadora em pós-Doutoramento, e do Professor Doutor Fernando José Mendes Gonçalves, professor Associado com Agregação, todos do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar.

Aos meus portos de abrigo nos meus dias mais tempestuosos

o júri
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palavras-chave

Corbicula fluminea; cianobactérias; blooms cianobacterianos; bioremediação; taxa de filtração; pseudofezes; caracterização de cianobactérias; compensação de danos.

resumo

Os *blooms* cianobacterianos estão associados à eutrofização em massas de água. Isto pode ser um problema para os restantes organismos, já que algumas cianobactérias podem ser tóxicas, não só para organismos aquáticos, como para os que ingiram água contaminada ou que acumulem toxinas nos seus tecidos. É assim da maior relevância o desenvolvimento de estudos que explorem formas de controlar/remediar águas afetadas pelo problema. Tal constituiu a principal motivação do presente estudo, cujo objetivo geral foi a avaliação da capacidade de um bivalve de água doce, *Corbicula fluminea*, enquanto agente bioremediador nestes cenários. Embora *C. fluminea* seja uma espécie invasora problemática na Europa e América do Norte, a sua distribuição já é muito alargada, pelo que o aproveitamento do seu elevado poder de filtração poderá ser uma boa estratégia de gestão da peste via compensação de danos resultantes do seu impacto em ecossistemas invadidos. Numa primeira fase do trabalho foi caracterizado (com recurso a ferramentas de microscopia e de tipagem genética) um conjunto de culturas laboratoriais de cianobactérias representativo da composição mais comum dos *blooms* ocorrentes em massas de água nacionais, incluindo espécies potencialmente produtoras de cianotoxinas. Alguns dos *taxa* de cianobactérias consideradas foram *Anabaena cylindrica*; *Microcystis* ou Oscillatoriales. Na segunda fase do trabalho foi avaliada a capacidade de *C. fluminea* para filtrar e ingerir cada uma das cianobactérias, em ensaios de curta duração com cada cianobactéria, oferecida em densidade típica de *bloom*. Os resultados obtidos revelaram baixas taxas de filtração em muitos casos, sobretudo quando comparadas com as taxas de filtração atingidas quando foi oferecida uma microalga verde (*Raphidocelis subcapitata*). No entanto, a análise de balanço de massa de clorofila *a* no sistema (filtrada e ingerida, retida em pseudofezes e não filtrada) permitiu algumas anotações importantes. Foi notória a relevância da agregação e sedimentação de cianobactérias filtradas promovida por *C. fluminea*, através das pseudofezes. Este processo tem semelhanças com o que é conseguido com a aplicação de químicos floculantes, usados para remover matéria orgânica e organismos microscópicos indesejados da coluna de água (por sedimentação e posterior aspiração), assim abrindo caminho para a continuação da exploração da possibilidade de utilização de *C. fluminea* como agente de remediação de *blooms* cianobacterianos.

keywords

Corbicula fluminea; cyanobacteria; cianobacterial blooms; bioremediation; filtration rate; pseudofaeces; cyanobacteria characterization; offseting damage.

abstract

Cyanobacterial blooms are frequentetly linked to eutrophication in waterbodies and can be a problem for other organisms since some cyanobacteria are toxic, not only to aquatic organisms, but also to all those ingesting contaminated water or organisms that had previously accumulated toxins in their tissues. Studies exploring new strategies to control/remediate affected waters are thus of major relevance. This motivated the present study, where the assessment of the suitability of the freshwater bivalve *Corbicula fluminea* as a bioremediator agent within such scenarios was aimed. Although *C. fluminea* is a problematic invasive species, mostly in Europe and North America, its distribution is already wide, thus the exploitation of its powerful filtration capacities can become a valuable add-on in this pest's management via compensating damage resulting from its impacts in invaded ecosystems. In a first stage, microscopic characterisation and genetic typing were carried out over a set of laboratory cyanobacteria cultures. These represent the most common *taxa* composing blooms that have been monitored in national waterbodies, including strains potentially producing cianotoxins. Some of the *taxa* considered were *Anabaena cylindrica*, *Microcystis* and Oscillatoriales. In a second stage, the capacity of the clams to filter and ingest each cyanobacteria was assessed, using short-term assays where each cyanobacteria was offered to the clams respecting densities typically found in blooms. The results often showed low filtration rates, especially compared to those reached as a green microalgae (*Raphidocelis subcapitata*) was offered. However, chlorophyll *a* mass balance analysis in the test system (Chl *a* filtered, retained in pseudofaeces and non-filtered) allowed some important outcomes. The agreggation and settling of filtered cyanobacteria promoted by *C. fluminea* through the production of pseudofaeces was notorious. This process resembles the use of flocculants to remove organic matter and microrganisms from the water column into the bottom for further aspiration, thus opening avenues to continuing assessing the possibility of using *C. fluminea* as a remediation for cyanobacterial blooms.

The use of *Corbicula fluminea* in the remediation of cyanobacterial blooms

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Chapter I. Introduction

I.1. Cyanobacteria and cyanobacterial blooms

Cyanobacteria are primary producers essential to the carbon cycling in aquatic ecosystems (Brito et al. 2012). This means that cyanobacteria only require, for the good progression of their life cycle, water, CO_2, inorganic substances (such as phosphorus and nitrogen) and light. Thus, their energy metabolism is generally preceded by photosynthesis, where sunlight and CO_2 feeds the production of energy-rich molecules and oxygen. Despite that, some species can live in total darkness, while some others can have heterotrophic ability (Chorus and Bartram 1999). According to Fay (1965), the cyanobacteria that grow in the dark generally grow in substrates with sucrose, although some species prefer glucose (Allison et al. 1937; Allen 1952; Kiyohara et al. 1960; Kiyohara et al. 1962).

The cyanobacteria are framed in the group of gram-negative photosynthetic prokaryotes, which is with no defined nucleus and organelles (Paerl et al. 2001), being one of the most diverse group regarding morphology, physiology and metabolism (Wiegand and Pflugmacher 2005). The most common forms found in the wild are unicellular, solitary or in colonies; undifferentiated, nonheterocystous filaments, also solitary or in colonies; filamentous, with the presence of heterocysts, which are differentiated, nitrogen-fixing cells (Paerl et al. 2001). Heterocysts allow the fixation of atmospheric dinitrogen through a process assisted by a nitrogenase enzyme (Ressom et al. 1994; Paerl et al. 2001; Wiegand and Pflugmacher 2005). It is known that cyanobacteria present a low sterol content, which makes them a poor resource for zooplankton. Additionally, the outer cell layer of cyanobacteria contains an endotoxic lipopolysaccharides (LPS), which is generally associated with *Escherichia coli*, *Salmonella* sp., *Vibrio cholerae*, *Yersina pestis*, and *Pseudomonas aeruginosa*. This LPS favors cyanobacterial toxicity or modulates their toxic effects (Wiegand and Pflugmacher 2005).

Although constituting a natural component of phytoplankton such as, for example, green microalgae, cyanobacteria are more prominently involved in eutrophication processes of freshwater ecosystems because they can more easily cope with conditions of low N:P

levels, water stability (lentic character), reduced transparency, increase of water temperature, pH and conductivity (Codd 2000; De Figueiredo et al. 2004; Macário 2013). Eutrophication mostly occurs due to the aging of water bodies, resulting in an increase in nutrients, biological activity and organic matter load, with human activities having an important role in the acceleration of this process (Carmichael 2008). Human activities promote the input of nutrients in water bodies through e.g. agricultural runoff or discharge of inadequately treated sewage, then potentiating a rapid growth of cyanobacteria and consequently impressing a considerable impact on the water quality (Carmichael 2008; Macário 2013). Under these conditions, cyanobacteria can often outcompete microalgae due to their ecophysiological abilities (Merel et al. 2013).

Vanderploeg *et al.* (2001), while quoting other authors, stressed out that some colonial cyanobacteria such as *Microcystis*, *Anabaena* or *Aphanizomenon*, tend to be favored in eutrophic water bodies with excessive phosphorus loading. Despite eutrophication scenarios have important negative impacts in ecosystems and potentially in human health (see section I.2 for specific consequences of cyanobacteria-driven eutrophication scenarios), little is known about the formation of cyanobacterial blooms and the cyanotoxins variants that can be present in the affected waters (Srivastava et al. 2013). This can be immediately illustrated by the lack of a consistent definition of "bloom". One accepted ecological definition follows: a significant production of biomass in a short period of time, with negative consequences, such as the decrease of phytoplankton diversity (Merel et al. 2013). However, this definition can change significantly in other arenas. For example, when a water body with recreational purposes or even drinking water supply sources are concerned, blooms are defined by cell concentrations with the ability to cause problems or becoming nuisances for the users of those water bodies (Coalition 2009).

Blooms formation occurs through a few steps, starting with the seeding and initial growth of cyanobacteria, and then undergoing a rapid exponential growth phase; this growth phase is followed by a plateau phase where the cyanobacteria density is maximal accounting to the system's carrying capacity, and finally a die-off phase occurs. Depending

on the conditions, the plateau phase can last for a long time period or can be shorter (Coalition 2009).

I.2. Hazard potential of cyanobacterial blooms

It is known that cyanobacterial blooms have the ability to deteriorate the water quality, then affecting the ecology of the water body and surrounding area (Backer 2002). Besides generally affecting the trophic balance within freshwater ecosystems, some mass-developing cyanobacteria are capable of producing toxins. These toxins have been associated with the death of animals, birds and fish in many countries and linked to several forms of human illness (Lawton and Codd 1991). The first study associating cyanobacteria to animal poisonings regards Lake Alexandrina (Adelaide, Australia), where the death of sheep, cattle and horses was attributed to drinking water contaminated with a cyanobacterium, more specifically, *Nodularia spurnigerza* (Francis 1878; Valenttne 1878). Since then, cyanobacterial toxins started to be recognized as a cause for the death of wild animals, farm livestock, pets, fish and bird in many countries. They are even suspected of having caused human illness in Australia, North America and Europe (Carmichael 1986; Codd and Poon 1988; Carmichael 1989; Falconer 1989; Codd et al. 1989; Falconer 1991). In Portugal, the common occurrence of cyanobacteria blooms has been noticed in natural lakes, reservoirs and large, slow flowing rivers (Vasconcelos 1999). Potentially toxic cyanobacteria that frequently occur in these systems are *Microcystis spp.*, *Aphanizomenon flos-aquae*, *Aphanizomenon gracile*, *Planktothrix sp.*, *Anabaena flos-aquae*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*, but so far, clear links between these occurrences and harmful effects are scarce in Portugal (Vasconcelos 2001; Saker et al. 2003; Valério et al. 2005; de Figueiredo et al. 2006).

By definition, harmful algal blooms (HAB) occur when an algal bloom can affect the environment, such as plants and animals, or affect human health in an adverse way. There are areas in the world where important impacts in public health were correlated to cyanobacterial blooms, both via ingestion of contaminated water and dermal contact with

contaminated recreational waters (Backer 2002). Cyanobacteria toxins which commonly can harm human health are hepatotoxins (microcystin; nodularin; cylindrospermopsin), neurotoxins (anatoxin-a; anatoxin-a(s); saxitoxins; β-N-methylamino-L-alanine) and dermatotoxins (aplysiatoxins; lynbyatoxins) (Backer 2002; WHO 2003; De Figueiredo et al. 2004; Wiegand and Pflugmacher 2005; Macário 2013; Merel et al. 2013). The most common ways of poisoning by cianotoxins are through ingestion of toxin-producing cyanobacteria. However, toxins can also be assimilated after their release into the water column following the death of cyanobacteria cells (Fulton and Paerl 1987; Vanderploeg et al. 2001). Carmichael (1992) considered *Microcystis aeroginosa* as one of the most dangerous species in this sense due to the capability to develop in a wide range of nutrient concentrations and the capacity to be toxic for both aquatic and terrestrial organisms. The impacts of HAB associated with *Microcystis* and other cyanobacteria include the disruption of ecosystem function, adverse health (human and wildlife) effects, and aesthetics impairment with consequences on the touristic exploitation of water bodies and their landscape (Vanderploeg et al. 2001).

In fact, HAB can present strong impacts on local economies, often triggered by the restriction or interdiction of recreational activities in affected water bodies, therefore being directly prejudicial to its touristic exploitation (Merel et al. 2013). Steffenson (2008) compiled a series of cases of blooms and their impacts on the Australian economy but there are not many of such studies worldwide although their relevance (Merel et al. 2013). The better known case studied by Steffenson (2008) regarded neurotoxic *Anabaena* blooming during 1991 in the Darling River, Central Australia, and causing losses to the dedicated tourism sector of around $1.5 million. The author also highlights that during 1991, another bloom occurred in Hawkesbury Nepean River, New South Wales, Australia, causing losses to the tourism sector of about $6.7 million lower compared to the previous year. Moreover, once a bloom appears in surface water, monitoring the evolution of the phenomenon implies the repetition of expensive analysis, sometimes over several months, to guaranty the safe use or the ecological quality of the water in the long-term. For example, toxicity tests may cost over $1,000 per sample and monitoring several sampling sites may be

necessary. Therefore, in Australia, the cost estimation of bloom monitoring reaches over $8 million per year (Steffensen 2008).

Despite these references, the impact of HAB in the economy remains poorly studied and understood, and more studies are needed to better cope with the harmful effects of cyanobacteria, including those regarding human health. From a strictly economical point of view, extreme health consequences of human intoxication can represent high expense, both when acute (leading e.g. to liver failure) and long-term (leading to e.g. cancer or neurodegenerative diseases) exposure occurs (Merel et al. 2013). Luckily, most known cases of human health effects of HAB result from moderate human intoxication, generally associated with gastro-enteritis. However, this symptom is associated with numerous more severe pathologies if the patients are treated without considering the possible relation to cyanotoxin poisoning (Merel et al. 2013).

I. 3. Current management options targeted at cyanobacterial blooms

An important signal indicating a high rate of cyanobacterial growth in a given water body is the alteration of the watercolor and the formation of easily visible and thick scums (Merel et al. 2013). Scums usually trigger a reply by the competent authorities that almost invariably involves the use algaecides to control/eradicate the bloom. The most commonly used algaecide for the purposes copper sulfate (Chorus and Bartram 1999). This use of copper explores the vulnerability of cyanobacteria to Cu^2; the ion affects the electron transport in the photosystems and the activity of fundamental enzymes (Chorus and Bartram 1999; Le Jeune et al. 2006). Although their efficiency to eliminate blooms, algaecides promote cell lysis and, therefore, the releasing of intracellular toxins that may have been produced (Jones and Orr 1994; Peterson et al. 1995). As *Microcystis aeruginosa* was exposed to copper sulfate (650 µ/L for 24 hours), cell membrane alterations were observed and these were related to the increase extracellular microcystin-LR (Kenefick et al. 1993). In addition, Cu^{2+} tends to precipitate and accumulate in sediments, promoting toxic effects in the non-target benthic biota and inherently allowing the re-establishment

of the blooms. Recent studies suggest the use of hydrogen peroxide as an alternative to copper sulfate (Matthijs et al. 2012) and other algaecides are available in the market that may be as effective as these two in controlling the blooms. However, the use of algaecides must be avoided as much as possible, since they constitute a short term remediation and, because they tend to have a synthetic nature, they may represent an ecological and public health risk *per se* (Griffiths and Saker 2003). Moreover, these solutions are generally costly, which can often become a factor preventing the successful management of the problem. For example, Steffensen (2008) referred that the Australian SA Water spends over $1 million a year in algaecides and disposal of copper contaminated water.

Available strategies to control or remediate the ecosystems affected by cyanobacteria are hence costly and producing undesirable or even hazardous side-effects. Low-cost solutions that can be efficient in approaching the problem and concomitantly present reduced additional hazardous potential are largely unavailable. Following on the growing trust put on biological control agents (e.g. parasites, predators or antagonists) as ecofriendly alternatives to chemical control methods targeted at aquatic invasive species (Oduor et al. 1999), the possibility of using biological agents to control or remediate cyanobacteria blooms should be given some attention. In fact, some studies have been made on the suitability of organisms or substances released by organisms as biological control agents against cyanobacterial blooms. Promising options considering the interplay between efficiency, cost and environmental safety include bacteria, protozoan predators, macrophyte competitors, fish as biomanipulation agents, plant extracts with cianotoxic properties and vegetal straw (Kim et al. 2007; Cobo 2015). These studies provide an ideal context for the present study, where the filtering potential of the Asian clam, *Corbicula fluminea*, upon cyanobacteria was assessed.

I.4. The Asian clam *Corbicula fluminea*

The Asian clam, *Corbicula fluminea*, is a freshwater bivalve that belongs to the phylum Mollusca, Class Bivalvia (Pelecypoda), Subclass Heterodonta, Order Venerioda and

Superfamily Corbiculoidea (Corbiculacea) (Britton and Fuller 1980). It is a medium-sized clam with maximal adults shell length ranging between 19 and 56 mm (Aldridge and McMahon 1978; Ituarte 1985; Hornbach 1992; Mouthon 2001; Sousa et al. 2008b) with the umbonal region being frequently found eroded. In most populations, the longevity of this clam ranges between 1.5 to 3 years (Mouthon 2001), although some authors recognize longer longevity for the clam, ranging between 1 and 5 years (Sousa et al. 2008a).

As represented in Figure 1, the life cycle of the Asian clam comprises internal incubation of the offspring in the demibranchs, which are released as pediveligers that grow into juveniles maturing typically after reaching a shell length of 10 mm (Ituarte 1985; Kraemer and Galloway 1986; Mouthon 2001). This clam is commonly described as a hermaphroditic species, with the ability of autofertilization and cross fertilization, both occurring in the paleal cavity, while the embryonic development occurs in the demibranchs (Kraemer and Galloway 1986). Caffrey et al. (2011) reported two reproductive seasons in natural populations being one between May and July while the other between September and December. This reproductive pattern is apparently dependent on the population and the environmental framework. For example, Rosa et al. (2014) studied the seasonal dynamics of the population used as a source for the clams tested in the present study, and found several sequential reproductive events, being the most notorious in June, August-September and November (Rosa et al. 2014b). High offspring numbers are typical of the Asian clam. Each individual can generally release up to 700 juveniles $clam^{-1}$ day^{-1} (Aldridge and McMahon 1978). Although high mortality of pediveligers is usually recorded (King et al. 1986; Kraemer and Galloway 1986; Sousa et al. 2008b), dense populations can establish under favorable conditions configuring a biological invasion.

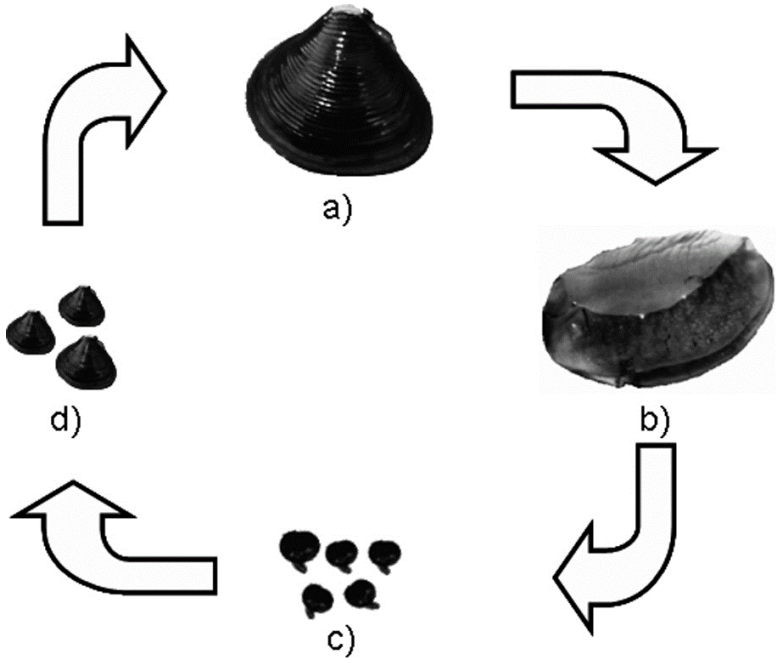

Figure 1: Representation of the life cycle of *C. fluminea*. a) adult organism; b) inner demibranch with larvae; c) small juveniles (with a completely developed foot and D-shape configuration); d) small adults (adapted from Sousa et al. 2008a).

In fact, *Corbicula fluminea* is considered one of the 100 worst invasive species in the European freshwaters (DAISIE, 2008). This clam earned its place among the most successful invasive species, since, in less than 100 years, the species invaded all continents with the exception of Antarctica (Crespo et al. 2015). Its native range comprises Asia, Africa and Australia (Mouthon 1981; Araujo et al. 1993; Ituarte 1994) and the invasion of European freshwaters was first recorded in Portugal (river Tagus estuary) and France (River Dordogne estuary; Mouthon 1981). Shortly after the species was recorded in the Netherlands (Blanken 1990), Germany (Kinzelbach 1991), Spain (Araujo et al. 1993) and Belgium (Swinnen et al. 1998) were invaded, as summarized by Mouthon (2001). Invasive species share several typical characteristics. They usually show wide amplitude of geographical distribution and are able to colonize a high diversity of habitats. More specifically, invasive species commonly present high genetic variability and phenotypical plasticity, which allow them a great resilience to environmental fluctuations and changes; rapid growth, early sexual maturity, great fecundity and short amount of time between generations, often

translating into r-strategist populations; females with the ability to colonize a new ecosystem alone; an association with human activities (e.g. trade as aquarium items, potential gastronomic applications, accidental transport in trading boats in the case of *C. fluminea*), and high dispersal potential (Lodge 1993; Alcaraz et al. 2005; Céréghino et al. 2005). Common negative impacts by invasive species, including *C. fluminea*, are the increased likelihood of the introduction of new diseases and parasites, competition with the native biota and the operation of significant changes in abiotic conditions; they are hence generally considered significant promoters of biodiversity loss (Ruiz et al. 1997; Dukes and Mooney 1999; Kura & Revenga 2003; Sousa et al. 2008b; Butchart et al. 2010; Crespo et al. 2015). This biodiversity loss can occur with the reduction of genetic diversity and genetic pool of native populations, extinction of endemic species and changes in the functionality and constitution of habitat (Vilà et al. 2010). An important characteristic of *C. fluminea* that is worth further noticing is its ability to alter the ecological characteristics of the invaded ecosystems (Sousa et al. 2008b; Sousa et al. 2009), particularly by creating and modifying habitats via disruption of the benthic-pelagic carbon dynamics (see section I.5 for details) and intensive shell deposition in the substrate (Ilarri et al. 2012), this supporting the touting of this species as an ecosystem engineer (Werner and Rothhaupt 2007).

I.5. *C. fluminea* filtering capability

The success of *C. fluminea* as an invasive species is frequently linked to its feeding capacities and inherent plasticity, as well as to the ability to alternate between filter-feeding from the water column and pedal feeding by filtering re-suspended material from sediments (Way et al. 1990). *Corbicula fluminea* is able to consume a significant amount of organic material in the streambed via pedal feeding, when suspended material is scarcer (Hakenkamp and Palmer 1999). However, the major feeding option of *C. fluminea* is through filtering from the water column and they are very competent in such activity. The species is actually a powerful filter feeder as shown in table 1. However, filtration rates have been recorded in different units, under different experimental contexts and using different filtered items, with may difficult for the interpretation of the rates obtained. Still, Cohen et al. (1984)

achieved records as high as 33 mL h^{-1} g^{-1}, under favorable conditions, such as no contamination, favorable size and shape of filtered items.

Table 1: Literature records on the filtration rate of *C. fluminea* on different food items. T stands for temperature; FW stands for fresh weight; DW stands for dry weight

Filtration activity	Filtered item	Notes	Reference
˚20 - 150 mL h^{-1}	Water column of Delta-Mendota canal	T = 20 - 24˚C	Prokopovich 1969
+200 - 800 mL $clam^{-1}$ h^{-1}	*Melosira sp.*	T = 18 - 27˚C	Mattice 1979
+ 347mL h^{-1} $clam^{-1}$; 177 mL h^{-1} g FW^{-1}; 1.561 mL h^{-1} g DW $^{-1}$	*Scenedesmus sp.*	T = 21 - 24˚C	Buttner and Heidinger 1981
*24.1 mL h^{-1} g DW $clam^{-1}$	phytoplankton of Potomac river	T = 26.5˚C; Different ages of clams	Cohen et al. 1984
+109 - 1370 ml hr^{-1}	*Chlorella vulgaris*	T = 20°C Shell length= 22.4 mm (SE= 0.24)	Lauritsen 1986
+30 - 140; 10 - 220; 25 - 610 mL $clam^{-1}$	*Chlorella vulgaris*; Microspheres (ø=1; 2; 5; 16 µm)	T = 15°C	Way et al. 1990
+7173.7 - 10971.1 mL $clam^{-1}$ h^{-1}	PolyVinylToluene beads. ø = 2.020 nm	T= 20˚C Shell length= 12.1 mm (±0.3)	Leff et al. 1990
*4.4 ± 0.6 mL g DW^{-1} min^{-1}	*Escherichia coli* strain JM83		Silverman et al. 1995
+100% removal of a oocyst suspension (1 x 10^6 L^{-1}) after 24h	*Cryptosporidium parvum* oocysts	Shell length= 1.5 - 2.5 cm	Graczyk et al. 1998

˚Pumping rate; +Filtration rate; *Clearance rate

I.6. The use of the Asian clam as a bioremediator

Mussels and clams are known by their great filtering ability (Parmalee and Bogan 1998). Due to this filtration ability, bivalves have been used as a bioindicators for a long time to identify pollutants in estuaries and coastal areas. However, little is known in this context for freshwater bivalves (Sousa et al. 2008a). Conversely, the ability of some bivalve pests, such as Asian clam and zebra mussel, to accumulate contaminants, as well as their high tolerance and resilience, is better known (Karatayev et al. 2007; Elliott et al. 2008; Rosa et al. 2014a; Binelli et al. 2014; Silva et al. 2016). The exploitation of those abilities can be understood as a branch within pest management programs, promoting the offsetting of the nuisance's negative impact to a certain extent.

Rosa et al. (2014a) and Silva et al. (2016) already demonstrated the powerful filtering abilities, and the ability to accumulate metals of *C. fluminea*, suggesting its use in bioremediation settings or as an agent assisting water purification in invaded but polluted ecosystems. The use of *C. fluminea* as a bioremediator in eutrophic waters was suggested by Cohen et al. (1984), after demonstrating a direct relationship between clam density and the improvement of water quality in the Potomac river (USA). The use of bivalves as a control tool for cyanobacterial blooms was also suggested (Pires et al. 2007; Gulati et al. 2008; Triest et al. 2015; Waajen et al. 2016), but experimental studies supporting this possibility are very scarce. Vanderploeg et al. (2001) examined the filtering activity of *Dreissena polymorpha* on *Microcystis aeruginosa*. These authors observed that although the clams kept the filtering activity at high and continuous rate, they did not ingest the cyanobacteria and produced large amount of pseudofaeces, which ultimately lead to the promotion and maintenance of the bloom. Waajen et al. (2016) demonstrated that quagga mussels (*Dreissena rostriformis bugensis*) can clear hypertrophic ponds by reducing the phytoplankton biomass, cyanobacteria included, inducing clear water states. More recently, Hardenbicker et al. (2015) showed a substantial suppression of coccoid cyanobacteria by *Dreissena polymorpha* and *Corbicula fluminea*.

If the use of *C. fluminea* as a bioremediator becomes experimentally supported and the application of the ecotechnology intended, its invasive character should always be an

important variable while designing the application methods. The introduction of invasive species is considered a major threat to biodiversity worldwide (Simard et al. 2012), responsible for economic losses around 5% of the gross income around the world (Pimentel et al. 2005; Pimentel et al. 2007; Simard et al. 2012).

I.7. Objectives and document layout

The overarching aim of this study was to assess the possibility of using the Asian clam, *C. fluminea*, to remediate cyanobacterial blooms. Such an assessment was assumedly at a laboratory scale because the state-of-the-art is still very short to provide the proper support to more realistic approaches. Also on this basis, monospecific cultures of cyanobacteria were used, representing the typical dominance of a given *taxa* in natural blooms; this option was purposely thought as a preliminary step for further studies using testing with the natural composition of cyanobacteria blooms. Several specific objectives were sequentially defined within the study as follows.

1) To characterize the morphology of common bloom-forming cyanobacteria in national water bodies that have been cultured for long in the laboratory.
2) To deliver the taxonomic affiliation of the cultured cyanobacteria based in both morphology and genetic typing.
3) To characterize the cyanobacteria cultures as to the inherent relationships between important biomass yield surrogate parameters such as chlorophyll *a*, optical density and cell density.
4) To inspect the filtering capacity of the bivalve *C. fluminea* on each cyanobacteria culture as offered in densities which are typical in natural blooms.
5) To assess the distribution of the cyanobacteria in the filtration test system in order to discern the mass percent that was actually ingested compared to the mass percent filtered, as well as to confirm the mass percent bypassing the digestive tract into pseudofaeces.

These objectives were tackled throughout this dissertation following a sequential order. The document begins with the introduction chapter (Chapter I), where the species used in the experimental work are introduced, as well as the ecological and economic context of both cyanobacterial blooms and invasive bivalves are discussed. Chapter II focus on the taxonomic affiliation and characterization of nine different cyanobacteria strains cultured in the laboratory, thus addressing objectives 1-3. Chapter III follows by describing the assessment of *C. fluminea* filtration and ingestion of cyanobacteria to assess the suitability of the clam to act as a remediation agent in affected systems (objectives 4 and 5). Chapter IV presents and overview of the conclusions of the study, drawing final remarks.

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Chapter II. Multilevel characterization of cyanobacterial cultures isolated from freshwater Portuguese water bodies

II.1. Introduction

Cyanobacteria are prokaryotic organisms with no defined nucleus and organelles (Paerl et al. 2001). They are primary producers and are essential to the carbon cycling in aquatic ecosystems (Brito et al. 2012). Cyanobacteria are separated in three major groups: 1) unicellular, but some with the ability to aggregate in colony; 2) undifferentiated, nonheterocystous filaments, also with the ability to aggregate; 3) filamentous forms containing differentiated cells, known as heterocysts (Paerl et al. 2001). Cyanobacteria that possess these heterocysts have the ability to fix atmospheric dinitrogen with the assistance of a nitrogenase enzyme, playing an important role in the nitrogen cycling dynamics (Paerl et al. 2001; Wiegand and Pflugmacher 2005; Moisander et al. 2012). These phytoplanktonic organisms are also often involved as key actors in the eutrophication of freshwater ecosystems. Depending on the environmental conditions, cyanobacteria can be considered as superior competitors compared to microalgae due to their ecophysiological abilities that include a wide tolerance range to light intensity, CO_2 concentration, nutrients availability, hydrologic characteristics and structure and function of the aquatic system (Merel et al. 2013). They can easily form blooms worldwide during summer months (De Figueiredo et al. 2004b; Codd et al. 2005). Cyanobacterial blooms are generally dominated by massive concentrations of colonies or filaments (Gazulha et al. 2012) and these assemblages can, in particular, impair other planktonic communities such those composed by microalgae (de Figueiredo et al. 2004a; Żak et al. 2012) and zooplankton (Fulton and Paerl 1987a; Vanderploeg et al. 2001; de Figueiredo et al. 2004a; Suikkanen et al. 2005).

Furthermore, strains of cyanobacteria belonging to the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis* and *Oscillatoria* (among others) are major promoters of harmful algal blooms (HABs) in freshwater systems, frequently being able of producing toxins, i.e. cyanotoxins (Carmichael 1992; De Figueiredo et al. 2004b). Among these

potentially toxic cyanobacteria, there are *taxa* such as *Microcystis* spp., *Cylindrospermopsis raciborskii*, *Planktothrix* (syn. *Oscillatoria*) *rubescens*, *Synechococcus* spp., *Planktothrix* (syn. *Oscillatoria*) *agardhii*, *Gloeotrichia* spp., *Anabaena* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Nostoc* spp., a few *Oscillatoria* spp., *Schizothrix* spp. and *Synechocystis* spp. (WHO 2003). Cyanotoxins are biologically active natural products that can be toxic to a large range of organisms (Wiegand and Pflugmacher 2005). The poisoning by cyanotoxins can occur through the ingestion of contaminated water and/or feeding on organisms that ingested and accumulated cyanotoxins (Carmichael et al. 2001). Toxins produced by cyanobacteria can be classified according to their effects on mammals: neurotoxins, hepatotoxins and dermatotoxins. The most common and concerning ones are microcystins, which are hepatotoxins, due to their acute and chronic effects (Vasconcelos and Pereira 2001; De Figueiredo et al. 2004b).

During blooms, dominant cyanobacteria usually do not occur as an individual species, but instead there is a co-occurrence of two or more species (Paerl et al. 2001). Although there is an interest in knowing blooms' composition towards a better ecological and human health management during their occurrence, the identification of a cyanobacterial species is not always easy under a microscope. Among some genera, there are several morphological features that can lead to ambiguity and compromise a proper identification, which makes the use of molecular approaches an essential step for characterization of bloom-forming cyanobacteria (de Figueiredo et al. 2010). Since every cyanobacteria contain the 16S rRNA gene, a structural gene in prokaryotes (Fulton and Paerl 1987b), this gene has been widely used for phylogenetic affiliation purposes (Palinska et al. 1996; Nübel et al. 1997; Otsuka et al. 1999). However, species discrimination based on this molecular approach has not been always consensual for some cyanobacteria (Stackebrandt and Goebel 1994; Palinska et al. 1996; Casamatta et al. 2005). Therefore, polyphasic approaches involving molecular, morphological and ecological features have been adopted for species classification (Komárek 2016). Additionally, cyanobacteria maintained under laboratory conditions may change their morphology and identification may become an enhanced challenge. They may lose some characteristics that are present in the nature or may not express them under optimal conditions (Garcia-Pichel et al. 1996; Palinska et al.

1996). Moreover, among strains of a same species, mutations may also occur during this period of culturing (de Figueiredo et al. 2010).

These difficulties in the taxonomic affiliation of cyanobacteria motivated the present chapter and ruled the methods herein used, as a support to the study exposed in chapter III. The aim in the present chapter was to characterize a set of cyanobacteria isolated from samples collected at several Portuguese freshwater bodies and maintained in the laboratory since longtime. Their morphology under the microscope served as a first basis for taxonomical identification, and molecular phenotypic affiliation followed for confirmation. Moreover, cyanobacteria chlorophyll yield was studied and the suitability of relationships between this biomass indicator and absorbance (440 nm) or cell density was investigated as a basis for the assays in chapter III.

II.2. Material and Methods

II.2.1. Cyanobacteria maintenance and culturing

A group of nine cyanobacteria isolated from Portuguese freshwaters were used in the present study, and they were assigned the following code numbers: #10; #11; #13; #15; #21; #22; 24; #26; #27. Long-term maintenance of the non-axenic cyanobacterial cultures in the laboratory was made in 100-mL Erlenmeyer vessels filled with 50 mL Woods Hole MBL culture medium (Nichols 1973). These stock cultures were kept at 20 ± 2ºC, under a $16h^{L}$:$8h^{D}$ photoperiod with light intensity of 130 μEm^{-2} s^{-1} provided by cool white fluorescent tubes (Fig. 1). Renewal was done fortnightly by harvesting about 10 mL from the old inoculum and inoculating in fresh, sterilized (autoclaved; 60-90 min, 120 ºC, 1 atm) MBL.

Figure 1: Freshly renewed stock cultures of cyanobacteria in Erlenmeyer vessels.

For downstream applications (see chapter III), cultures were grown in aerated 4 L glass vessels under the above temperature and light conditions, until reaching the stationary growth stage (Fig. 2). This required a 10-14-day growth period, depending on the species and strain.

Figure 2: Culture of cyanobacterium #21 in an aerated 4L vessel.

II.2.2. Morphologic characterization of cyanobacterial isolates

A sample of each long-term cyanobacteria culture was taken and mounted between a microscope slide and a cover slip. These extemporaneous preparations were observed under a microscope (Leica DM6) and photographic records were taken using a coupled camera (MC 190 HD). Taxonomic identification was performed using the references Hindák (2000), Rajaniemi *et al*. (2005), Komárek (2006), Komárek and Komárková (2006) and Willame *et al*. (2006).

II.2.3. Genetic characterization of cyanobacterial isolates

Total DNA was extracted from each cyanobacteria culture after the centrifugation of 2 mL of culture and re-suspension in 200 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Cell lysis was achieved following addition of 1 mg mL^{-1} of lysozyme and incubation at 37°C for 1 h. DNA extraction and purification was continued using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania), which culminated in DNA recovery in TE buffer for storage at -22°C.

DNA amplification of the 16S rRNA gene fragment through Polymerase Chain Reaction (PCR) was performed using a combination of the bacterial universal primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; Lane 1991) with the cyanobacteria-specific reverse primer CYA781R (5'-GAC TAC WGG GGT ATC TAA TCC CW-3'; Nübel et al. 1997). Synthesis of both primers was outsourced (STABVida, Oeiras, Portugal). The PCR was carried out on a Bio-Rad C1000^{TM} Thermal Cycler (Hercules, CA, USA) over each 35-µL reaction mixture containing 3 mM $mgCl_2$, 0,2 mM of each nucleotide, 1x PCR buffer containing $(NH_4)_2SO_4$, 10 µM of each primer, 1 U of Taq DNA polymerase and 2 µL of template DNA. The program for the PCR was set to an initial denaturation at 94°C for 5 min, and a final extension step for 10 min at 72°C. In between, 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C were included. A negative control reaction with no DNA template was carried out at the same time. PCR success was checked by electrophoresis at 80 V for 45 min on 1.5% agarose gel incorporated with Green Safe Premium (Nzytech, Portugal). A molecular weight marker

of 100 to 10000 bps (GeneRuler DNA Lader Mix, Thermo Scientific) (Fig. 14) was run in each gel to confirm the size of the target amplified DNA fragments (about 750 bps), under a UV transilluminator (G:BOX, Syngene). The sequencing of successfully amplified DNA sequences was outsourced (STABVida, Oeiras, Portugal). A basic search using the basic local alignment search tool for nucleotides (BLASTn) was performed to explore similarity of the cyanobacteria sequences against sequences deposited in the GenBank database.

I.2.4. Relationships among Chlorophyll a, absorbance 440 nm and cell density

A green microalga (*Raphidocelis subcapitata*) control treatment was set in the experiments described in Chapter III. Therefore, calibration curves between absorbance (Abs) read at 440 nm and cell density, as well and between Abs and Chl *a* concentration were needed. While the latter was obtained as described below for cyanobacteria, the former was available from previous studies in the laboratory. The establishment of this microalgae Abs x cell density calibration curve involved triplicate readings of the parameters pair in successive dilutions from a bulk culture, progressively adjusted so that deviations from linearity could be comprehensively covered by experimental data (N = 42; Abs 440 nm between 0.010 and 0.334) (Equation 1). Microscopic microalgal cell counting was made under a microscope (Olympus CKX41) using a Neubauer counting chamber.

Equation 1:

$$Cell\ density\ (cells\ mL^{-1}) = 6931 + 2.32\ e^{7}\ \times Abs_{440\ nm} - 9.97\ e^{6}\ \times {Abs_{440\ nm}}^{2}$$

For cyanobacteria, a linear relationship between cell density and absorbance at 440 nm was assumed *a priori*, as used in previous studies (de Figueiredo et al. 2004a) and generalised using Equation 2. Therefore, the calibration curve for each cyanobacterium was established on the basis of readings of the parameters pair in a sample taken from

each fully grown culture with no further readings on dilutions. Cyanobacteria cell counting was made under a microscope (Olympus CKX41 400x) in a Sedgwick-Rafter counting chamber.

Equation 2:

$$\text{Cell density}_{\,i}\ (\text{cells mL}^{-1}) = (\text{Cell Density}_{\,\text{culture}} \times \text{Abs}_{440\ \text{nm}\ i})/\text{Abs}_{\,440\text{nm culture}}$$

The construction of the Abs x Chl *a* calibration curve was based on successive dilution (from 1:2 up to 1:10, depending on the species) of each culture and measuring on each parameter pair. Three replicates were used to calculate an average value for each endpoint, and these average values were used to build up a calibration curve covering absorbance within 0.0013 - 1.081, depending on the species. Chlorophyll concentrations were determined using the trichromatic method (APHA 1995). Briefly, the microalgal/cyanobacterial suspension was vacuum-filtered through a Whatmann GF/C filter (1.2 µm pore), which was then frozen at -20ºC until further processing. When processing the samples, the chlorophyll retained in the filter was extracted with a solution of 90% acetone and maintained at 4ºC overnight, in foil-wrapped tubes to prevent chlorophyll degradation driven by light. Next, the extract was centrifuged for 10 min at 3500 rpm and the supernatant's absorbance was measured at 630, 647, 664, 730 nm. The resulting values of absorbance were used for the determination of Chl *a* using the trichromatic equations as detailed by APHA (1995).

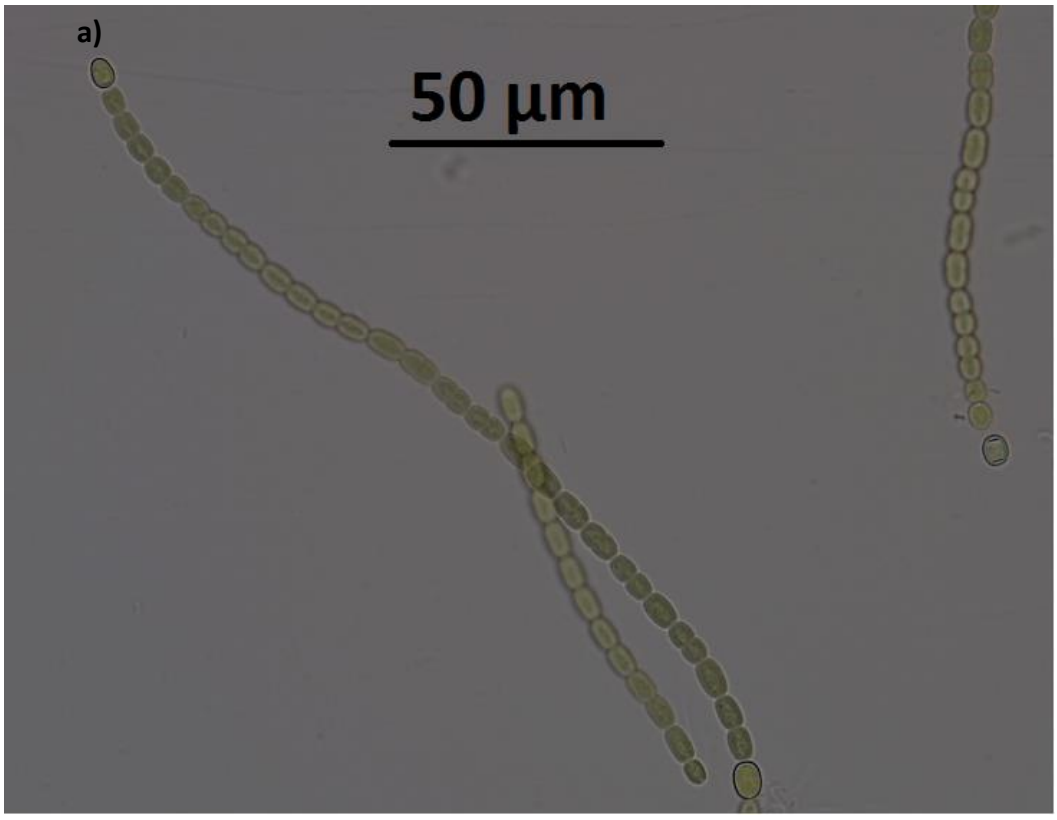
II.3. Results and Discussion

II.3.1. Morphologic characterization of cyanobacterial isolates

The relevance of conducting morphological characterization studies on the cyanobacteria was two-fold. First, by characterizing the current morphology of the cyanobacteria, a better

understanding on how the dimensions and forms available in the cultures can affect the filtration rates of *Corbicula fluminea* (see chapter III) could be reached. Second, morphological inspection can assist a proper taxonomic affiliation of cyanobacteria. In this way, all cyanobacteria cultures were examined under the microscope, and figures 3 - 11 show their morphological details. This allowed a preliminary taxonomic identification at the level of the order or genus, depending on the culture. The legends of the figures detail the most relevant features used for the proposed identification. Based on this microscopic inspection, to which genetic affiliation was also added (see section II.3.2.), the following identification is proposed for cultures.

Culture #10 fitted within the description of the order Nostocales (Fig. 3). This order presents some characteristics such as long, irregularly coiled trichomes surrounded by a diffuse mucilaginous envelope; also, the akinete development starts from the middle of the filament between two heterocytes (Rajaniemi 2005), although akinetes were not visible in culture.



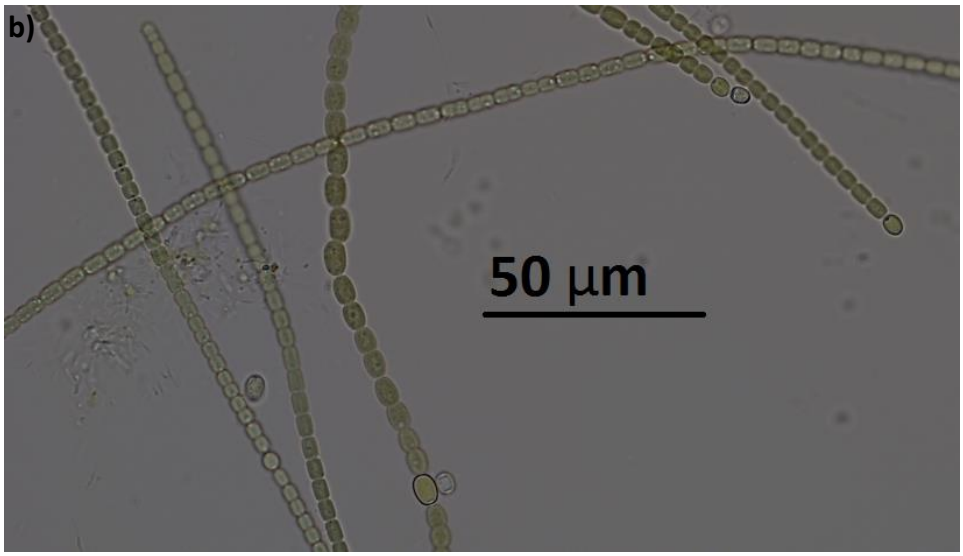
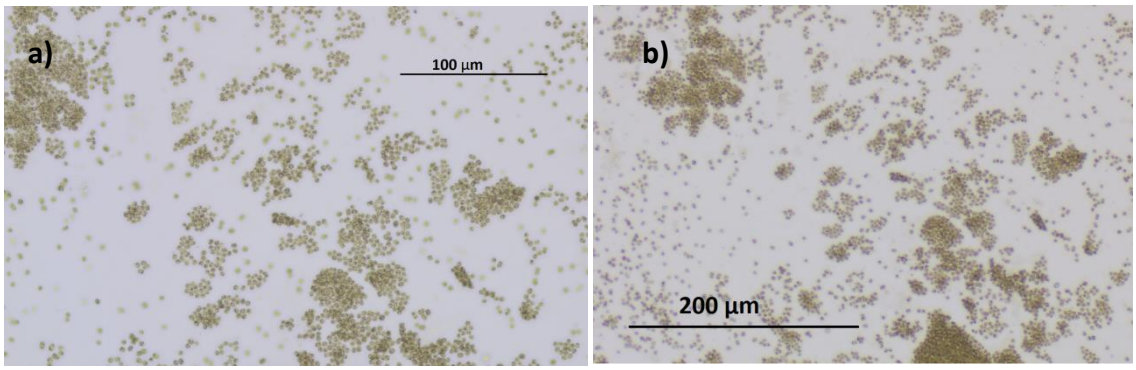


Figure 3: Microscopy image of cyanobacterium #10 *Nostocales*. In **a)**, it is possible to identify an individual organism and its natural form, morphology and heterocysts, while in **b)**, the low agglomeration tendency of this strain is documented, also with heterocysts.

Culture #11 corresponded to characters associated with the genus *Microcystis* (Fig. 4). This genus is characterized by irregularly-shaped and irregularly-sized colonies during blooms (Joung et al. 2006). Also, as assessed by Joung et al. (2006), *Microcystis* sp. cells range in size from 4 to 5 µm, and the colonies range in size from 52-200 µm, just as found in this study, although the prevalent desegregation of the colonies captured in the pictures.



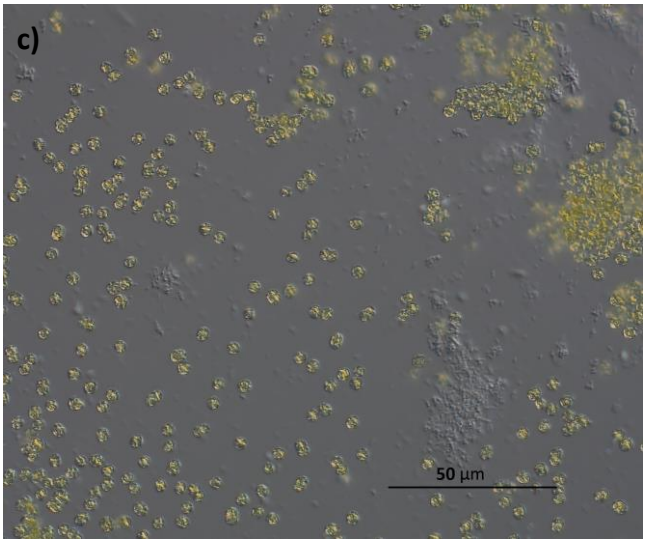


Figure 4: Microscopic image of cyanobacterium #11, preliminarily classified as *Microcystis*. In both **a)** and **b)**, the agglomeration that occurs naturally was documented, whereas in **c)** it is possible to identify individual organisms and some colonies.

Culture #13 showed characteristics corresponding to cyanobacteria from the genus *Cylindrospermopsis* (Fig. 5). This genus includes filamentous organisms with buoyancy control and ability to form akinetes, allowing easy dispersal and environmental resistance (Figueredo et al. 2007); one important characteristic is the presence of a terminal long-shaped heterocyst.

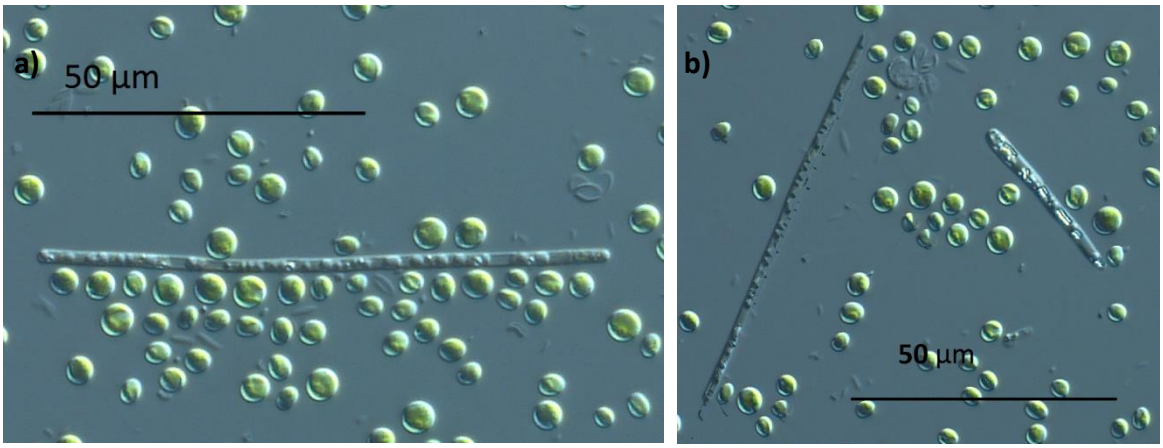


Figure 5: Microscopy image of cyanobacterium #13 *Cylindrospermopsis*. In **a)** and **b)**, it is possible to identify single organisms and their natural morphology, although the high contamination of the culture by green microalgae.

Culture #15 apparently corresponds to cyanobacteria belonging to the order Synechococcales (formely Chroococcales) (Fig. 6), which collects unicellular, nonheterocystous organisms (Brito et al. 2012).

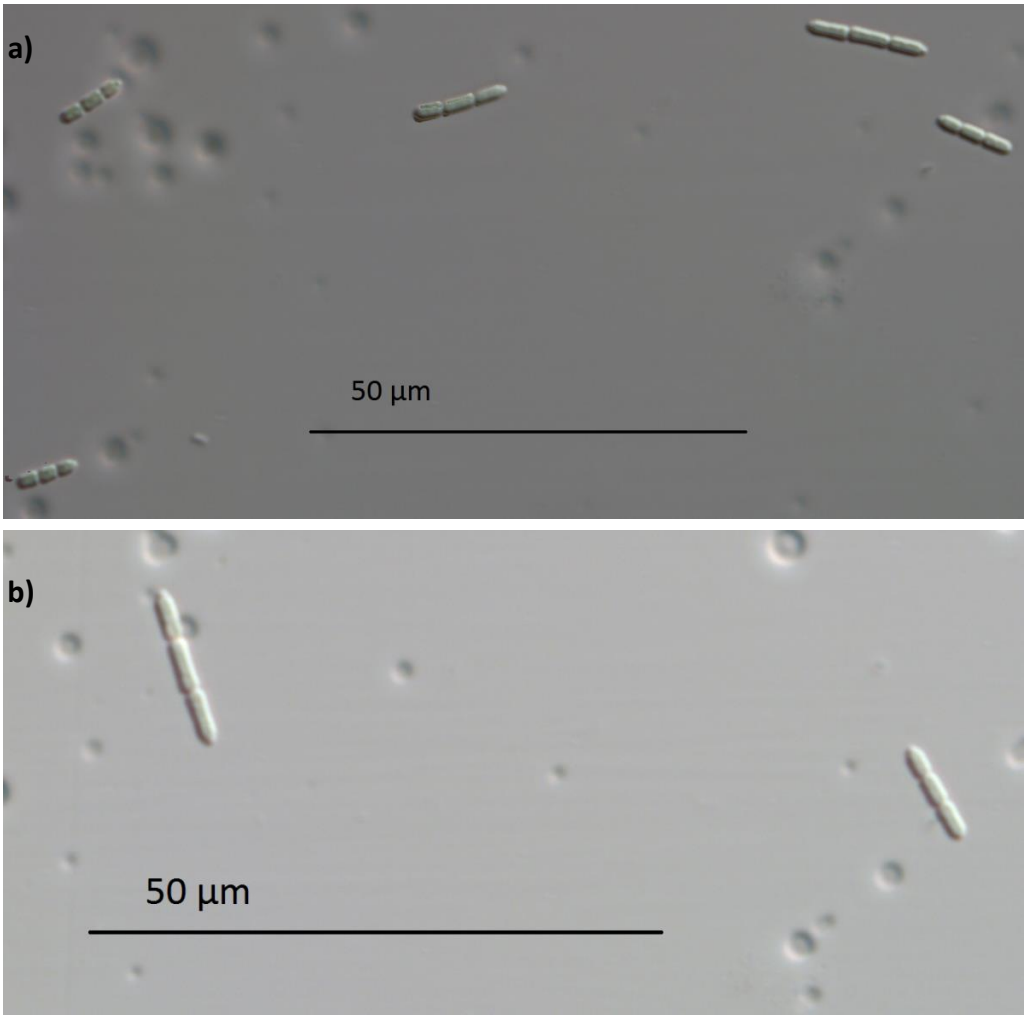


Figure 6: Microscopy image of a cyanobacterium #15 Synechococcales. In both **a)** and **b)**, it is possible to identify single organisms and their natural morphology.

The cultures #21, #22, #24 and #26 showed morphological characters related to the genus *Anabaena* (Fig. 7, 8, 9, 10 respectively). This genus is characterized by filamentous forms with nitrogen-fixing ability (Allen and Arnon 1955); planktonic species have gas vesicles; trichomes can vary from coiled to straight; position of akinetes, shape of terminal cells and width of vegetative cells are useful taxonomic characters for identification within the

Anabaena genus (Rajaniemi 2005). Culture #21, #22 and #24 apparently correspond to cyanobacteria belonging to the species *Anabaena cylindrica* (Fig. 7, 8 and 9, respectively), since the three cultures fit the typical morphology of the species, including a rounded shape of the terminal cell (denoted in the figures 7 – 9).

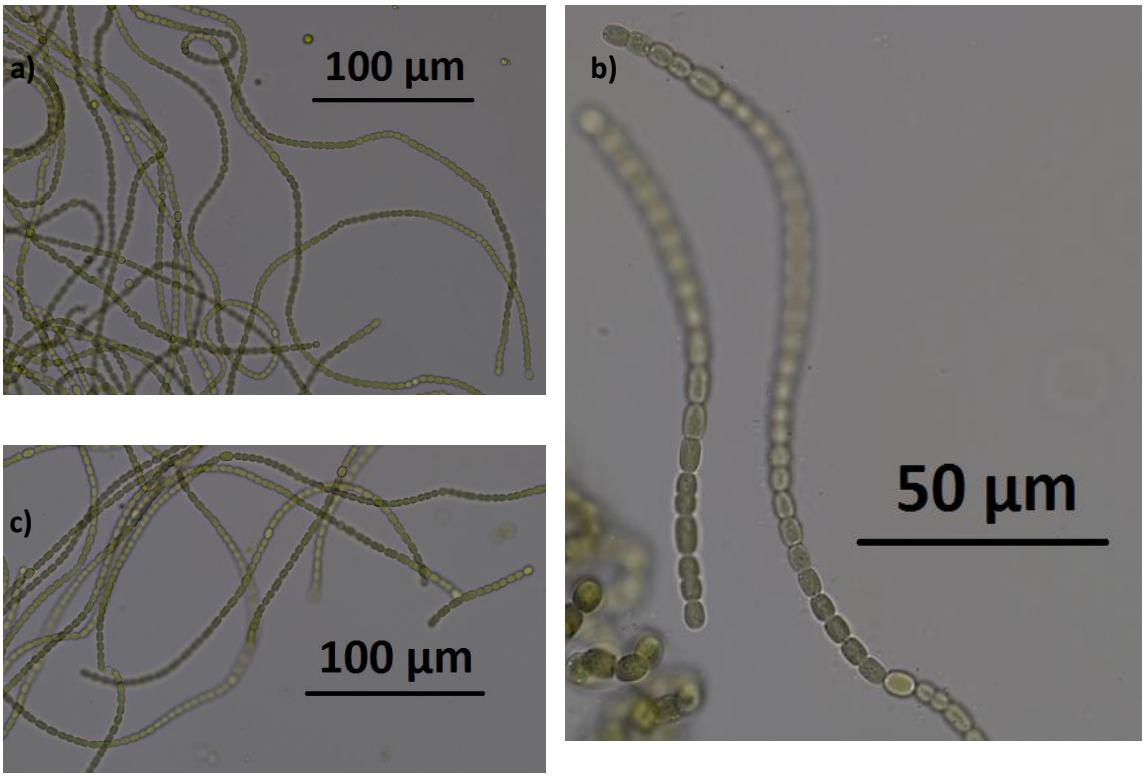


Figure 7: Microscopy image of cyanobacteria # 21 *Anabaena cylindrica*. In, **a)** and **c)**, the agglomeration tendency of this strain is documented, while in **b)**, it is possible to identify an individual organism and its natural form, morphology and heterocysts.

Although the identification of culture #22 also as *Anabaena cylindrica*, this culture clearly shows the tendency of the strain for producing longer filaments than the strain #21.

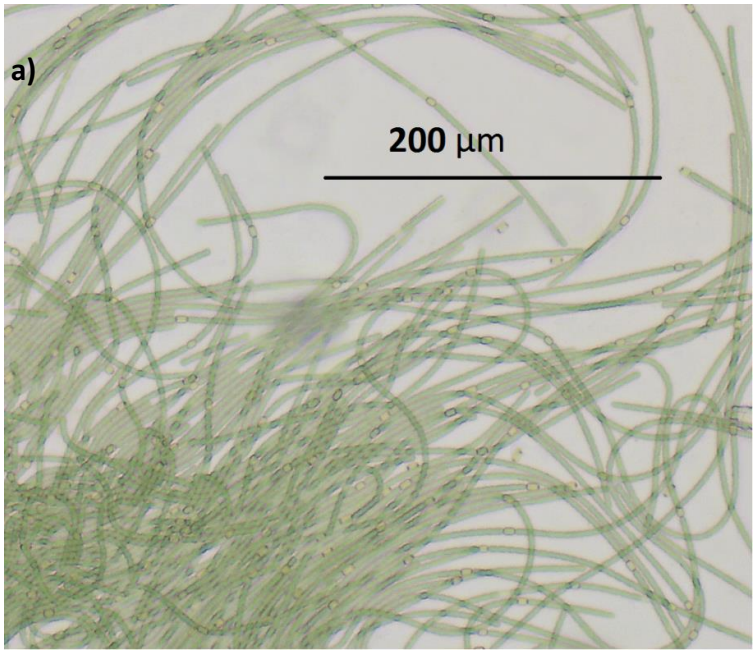


Figure 8: Microscopy image of cyanobacterium #22 *Anabaena cylindrica*. The agglomeration that occurs naturally was captured and single organisms (filaments) and their natural morphology were found. Heterocysts could also be observed.

Culture #24, as explained above, belongs to the species *Anabaena cylindrica*. However, it is a different strain.

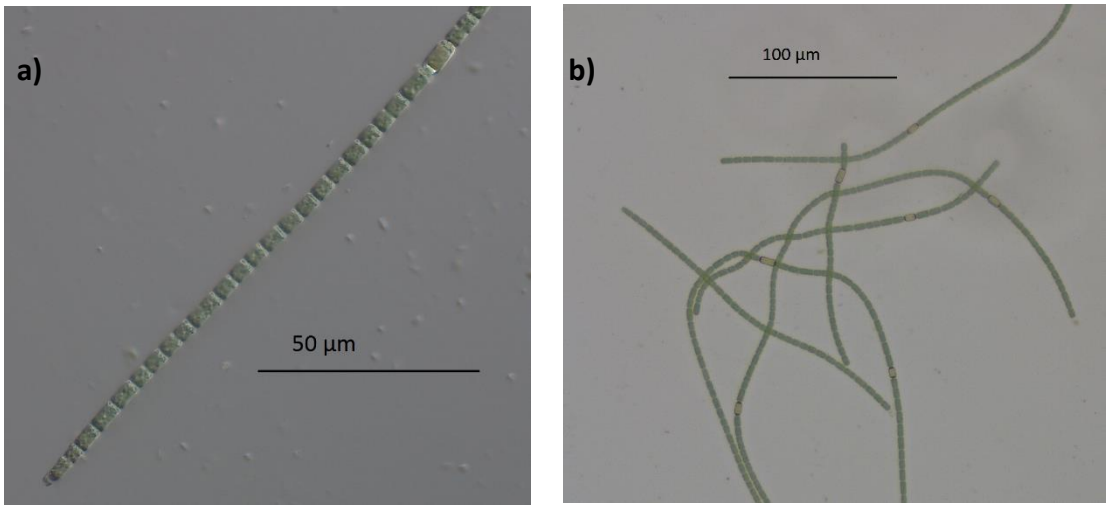
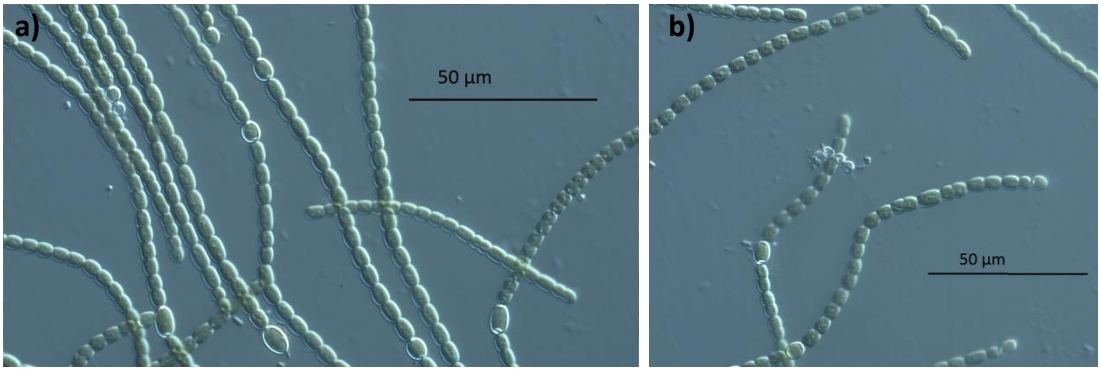


Figure 9: Microscopy image of cyanobacteria #24 *Anabaena cylindrica*. In both **a)** and **b)**, it is possible to identify single organisms (filaments) and their natural morphology; heterocysts could be identified in all filaments.

Culture #26 also showed characters corresponding to genus *Anabaena* (Fig. 10), although with different size and shape of vegetative cells when compared to *Anabaena cylindrica* identified above (figure 7 – 9).



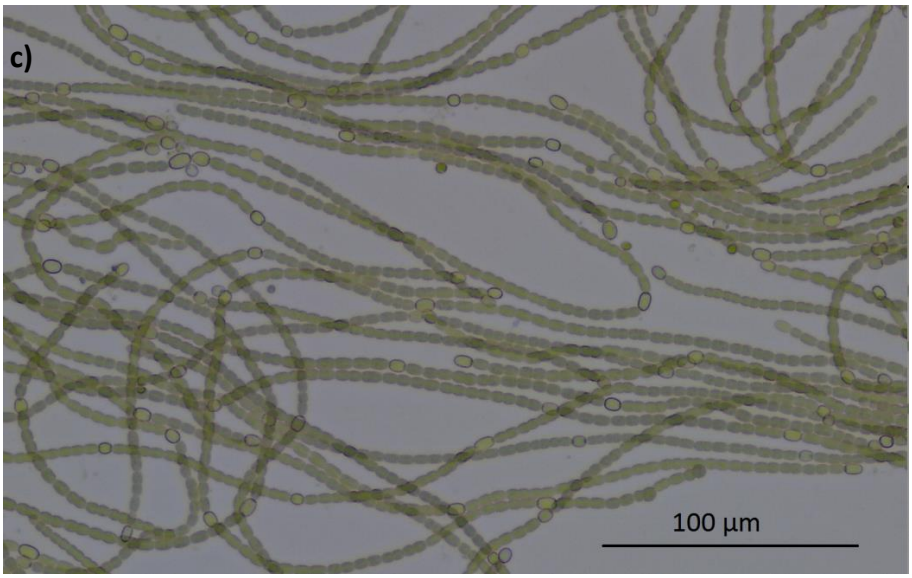


Figure 10: Microscopy image of cyanobacteria #26 *Anabaena sp*. In **a)** and **b)**, it is possible to identify single organisms (filaments) and its natural morphology, with heterocysts well represented, while in **c)**, the agglomeration tendency shown by the strain is clear.

Culture #27 corresponded to cyanobacteria belonging to the order Oscillatoriales (Fig. 11). This order can be distinguished by individuals with short filaments, trichal types without sheaths and without heterocysts and akinetes (Komárek 2006).

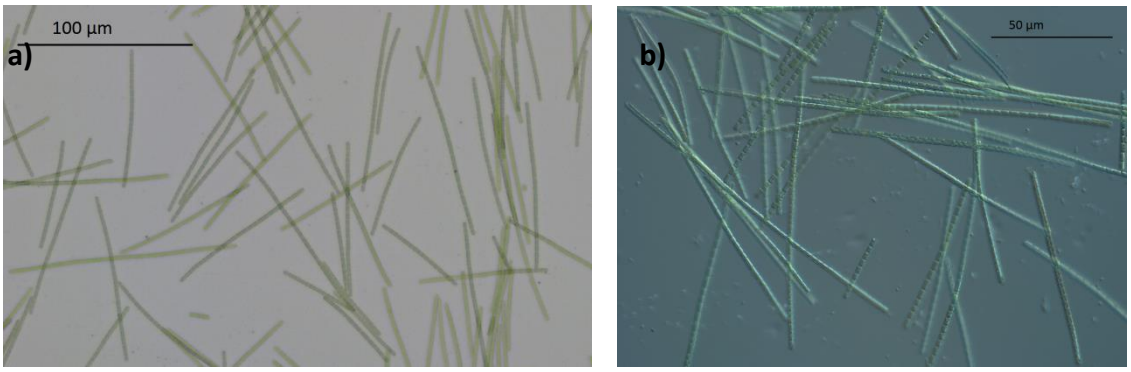


Figure 11: Microscopic image of cyanobacteria #27, preliminarily classified as Oscillatoriales. In **a)** and **b)** the agglomeration that occurs naturally was captured, while in **b)** it is also possible to identify several single organisms (filaments) and their natural morphology.

II.3.2. Genetic characterization of cyanobacterial isolates

The amplification of the 16S gene from the nine cultures was successful. Figure 12 shows the first gel image obtained, where amplified DNA from cultures #15, #27 and #10 was confirmed by the presence of a band of about 750 bps, which is the mark corresponding to the 16S rDNA target fragment (Nübel et al. 1997; Garcia-Pichel et al. 2001). Figure 13 shows the bands for the second DNA amplification batch, resulting in the unsuccessful DNA amplification for cultures #21, #26, #22, #24, #13, #10 (see absent 750 bps bands in Fig. 12). On the other hand, unspecific amplification is possibly denoted by the presence of smear and weak bands in Figs. 12 and 13. The non-amplification of DNA for some samples may indicate technical failure in upstream DNA extraction procedures. Poor quality of DNA, such as that with widely varying G+C content, or reduced quantity of DNA can constrain downstream sequencing (Wintzingerode et al. 1997; Garcia-Pichel et al. 2001) Primer degeneration or the existence of novel target sequences that are non-complementary to primers used are also sources of bias in DNA sequence-based taxonomical affiliation (Wintzingerode et al. 1997; Nübel et al. 1999; Garcia-Pichel et al. 2001). The formation of chimera molecules or even the amplification of template molecules with a different efficiency, although the same priming site are also common shortcomings affecting taxonomical affiliation of cyanobacteria using this molecular technique (Nübel et al. 1997). Since the cyanobacteria cultures used as a DNA source were non-axenic, and because the amplification targets highly conserved regions of the 16S rRNA gene common e.g. to other bacteria, misidentification may occur (Nübel et al. 1997).

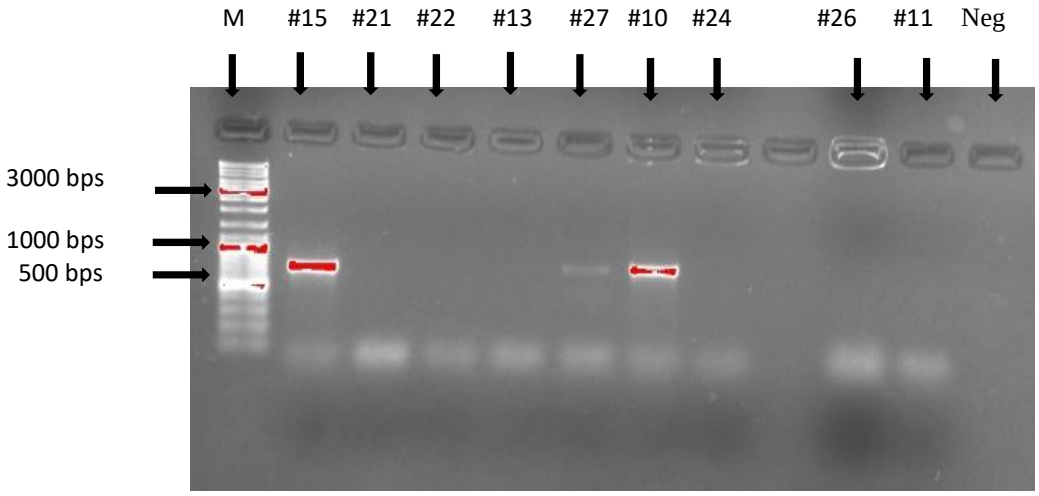


Figure 12: Gel image capturing the success of the first amplification batch. The first column contains the Ladder (Fig. 14), marking DNA fragment's size between 100 and 10000 bps. The following columns show the amplification outcome for samples #15, #21, #22, #13, #27, #10, #24, #26, #11 and, finally for the negative control (Neg). The red marks highlight good selective amplification in samples #15 and #10, respectively; a satisfactory amplification was achieved with sample #27, as interpreted from the band visible immediately before that marked red from sample #10.

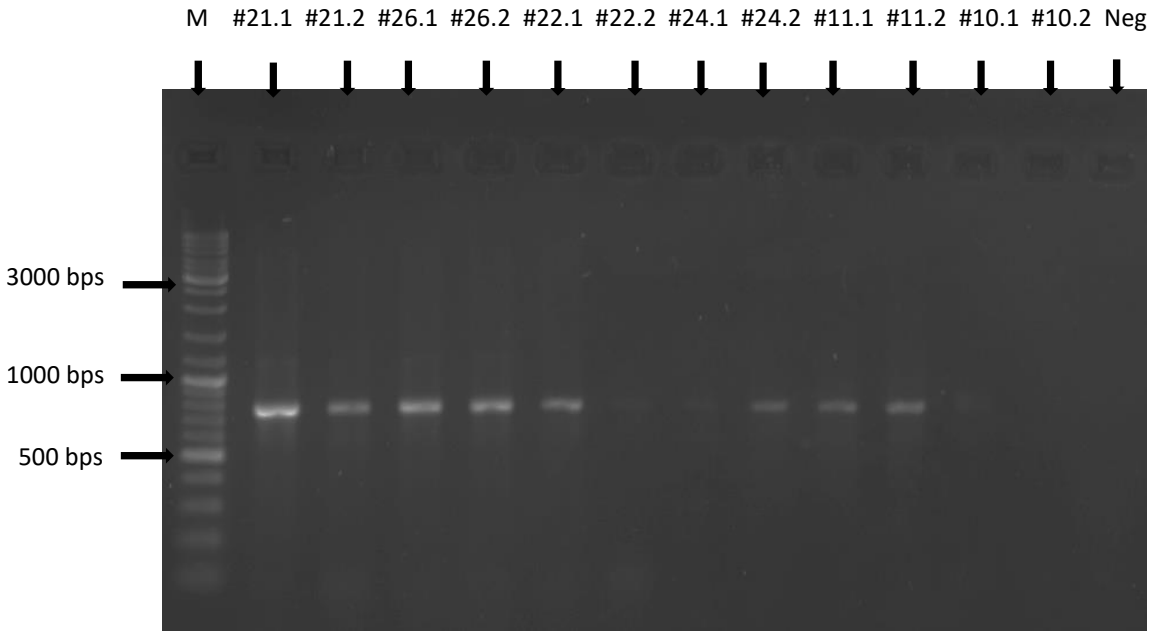


Figure 13: Gel image capturing the success of the second batch. The first column represents the Ladder Mix (Fig. 14), marking DNA fragment's size between 100 and 10000 bps. The following columns shows the

amplification outcomes regarding cyanobacteria #21 (sample 1) and (sample 2); #26 (sample 1) and (sample 2); #22 (sample 1) and (sample 2); #24 (sample 1) and (sample 2); #11 (sample 1) and (sample 2); #10 (sample 1) and (sample 2) and negative control (Neg). With the exception of #22 (sample 2), #24 (sample 1) and #10 (sample 2), all loaded samples resulted in a visible band.

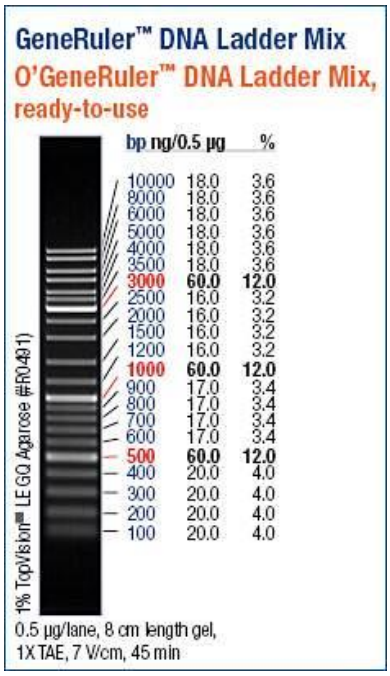


Figure 14: Clarification on the GeneRuler DNA Ladder Mix used on the electrophoresis (see above), with the correspondence between the visible bands and the size of DNA fragments (base pairs, bps) migrating in the electrophoresis.

Although the microscopic inspection can be imprecise leading to morphologic misidentifications (Hindák 2000; Li et al. 2000; Komárek and Komárková 2006) and non–consensual species discrimination for some cyanobacteria (Stackebrandt and Goebel 1994) (see II.3.1), genetic characterization may also be misleading (table 1), especially if non-axenic cultures are used and cultures are maintained for a long time (Rippka et al. 1979) as in the present study. In fact, some of our non-axenic cultures were contaminated with bacteria, which are visible in some of the images in section II.3.1. The presence of these bacteria, whose DNA was extracted along with that from the cyanobacteria, can interfere

with the DNA analysis, due to the use of one bacterial universal primer, the 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; Lane 1991) for 16S amplification. This type of contamination is evident for the cyanobacteria #10, where the sequencing outcome showed *Prosthecobacter debontii* strain FC3 (NR_026023), a bacterium (taxonomically: Bacteria; Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales), as the closest relative (98% similarity). Also, for cyanobacterium #11, the mixture of DNA from different sources was so intense that we could not discern the target sequence for comparison against reference sequences deposited in GenBank databases (note the absence of the samples from Table 1).

The sample from the cyanobacterium #27 contained reduced template DNA quantity, preventing successful sequencing and further comparison against the GenBank. However, the electrophoresis gel denotes a well visible band for this cyanobacterium (Fig. 12), which is contrary to the reported sequencing failure.

The taxonomic affiliation as assessed microscopically or molecularly was similar for cyanobacteria #21, #22 and #24. Still, molecular affiliation of sample #21, which was classified as *Anabaena* based on microscopic inspection, revealed identical percent similarity with *Anabaena cylindrica* and *Nostoc punctiforme* (Table 1). This denotes that the molecular identification within the order Nostocales is still unresolved and lead to the option of using the order as the taxonomic resolution level for this cyanobacterium in further studies. A similar outcome was found for sample #15, where equivalent percent similarity with two different species was retrieved, both belonging to the order Synechococcales (Table 1). Microscopic identification disagreed with molecular classification also for samples #13 and #26 at levels below the family. While microscopy indicated that culture #13 and #26 correspond to *Cylindrospermopsis* and *Anabaena*, respectively (Figs. 5 and 10), the genetic results indicate that #26 corresponds to and *Anabaena cylindrica* strain and #26 to *Cylindrospermum siamensis* (Table 1).

It is worth noting that some misleading affiliations can in part derive from limitations of databases and/or taxonomical resolution constrains, which is especially valid for cyanobacteria (Taton et al. 2006).

Table 1: Culture description and accession number, closest relative (after BLASTn search) and corresponding percentage similarity for the most similar 16S rDNA bacterial partial sequences (approximately 690 bp) in NCBI database. Molecular outcomes which are consistent with microscopic analysis outcomes are marked bold.

Culture	Closest relatives (NCBI accession NR)	Percentage similarity (%)	Phylogenetic affiliation
#13	*Anabaena cylindrica* strain PCC 7122 (NR_102457)	78	Bacteria; Cyanobacteria; Nostocales; Nostocaceae; *Anabaena*
#15	*Chamaesiphon minutus* strain PCC 6605 (NR_102459)	89	Bacteria; Cyanobacteria; Synechococcales
	Synechococcus elongatus strain PCC 6301 (NR_074309)	87	
#21	*Nostoc punctiforme* strain PCC 73102 (NR_074317)	79	Bacteria; Cyanobacteria; Nostocales
	***Anabaena cylindrica* strain PCC 7122 (NR_102457)**	**78**	
#22	***Anabaena cylindrica* strain PCC 7122 (NR_102457)**	**99**	**Bacteria; Cyanobacteria; Nostocales; Nostocaceae; *Anabaena***
#24	***Anabaena cylindrica* strain PCC 7122 (NR_102457)**	**99**	**Bacteria; Cyanobacteria; Nostocales; Nostocaceae; *Anabaena***
#26	*Cylindrospermum siamensis* strain SAG B11.82 (NR_117352)	84	Bacteria; Cyanobacteria; Nostocales; Nostocaceae

II.3.3. Relationships among Chlorophyll a, absorbance 440 nm and cell density

While relationships between cell density and absorbance are reliable in microalgae, as evident from its use in standard testing procedures (e.g. OECD 2013), the same does not apply to cyanobacteria. Due to the wide morphological variation that can occur between and within species, and the cyanobacteria ability to agglomerate or form colonies, microscopic counting is difficult (Lawton et al. 1999). These difficulties were confirmed during the microscopic counting made on fully grown cultures. Still, the equations relating Abs and cell counting are given in Table 2 as a general reference.

Table 2. Directly proportional relationships between Abs_{440nm} and cell density (cells mL^{-1}) written for each cyanobacterium on the basis of mean records (n = 5) taken in samples collected from fully grown cultures.

Cyanobacteria	Abs440nm-cell density relationship
#10 Nostocales	Cell density = $(1.56 \times 10^6 \times Abs_{440\ nm})/ 0.396$
#11 *Microcystis*	Cell density = $(1.39 \times 10^7 \times Abs_{440\ nm})/ 0.449$
#13 *Cylindrospermopsis*	Technical deficiency in sample preservation
#15 Synechococcales	Cell density = $(4.24 \times 10^7 \times Abs_{440\ nm})/0.673$
#21 Nostocales	Technical deficiency in sample preservation
#22 *Anabaena cylindrica*	Cell density = $(2.79 \times 10^6 \times Abs_{440\ nm})/ 0.626$
#24 *Anabaena cylindrica*	Cell density = $(4.81 \times 10^6 \times Abs_{440\ nm})/ 0.582$
#26 *Anabaena*	Technical deficiency in sample preservation
#27 Oscillatoriales	Cell density = $(9.98 \times 10^6 \times Abs_{440\ nm})/1.02$

It is important to note the comparison between #24 *Anabaena cylindrica*, with a higher relative cell density corresponding to lower relative Abs, and #22 *Anabaena cylindrica*. This is another evidence on how misleading absorbance readings may be.

Although the use of absorbance as a surrogate to estimate biomass for some colonial cyanobacteria has been considered unfeasible mostly due to technical interference of morphological features (Millie et al. 2002), and morphological heterogeneity within cultures (see II.3.1), our study was generally successful in establishing feasible relationships between absorbance readings and chlorophyll *a* quantification. This is indicated by the high R^2 values determined for every regression as shown in Figure 15. The least feasible regressions were those shown in fig. 15 with R^2 values between 0.88 and 0.93. These correspond to cultures #21, #26 and #27, i.e. Nostocales, *Anabaena* sp. and Oscillatoriales, respectively. All are filamentous bacteria, a morphologic trace that can *per se* constrain differential optical dispersion during spectrophotometric analysis depending on the orientation of the cells in the read sample at the reading moment. Furthermore, #27 Oscillatoriales and #21 Nostocales tend to aggregate forming large nodules that can easily interfere with the absorbance reading as well (see Figs. 7 and 11).

The relationships between Chl *a* and absorbance (Fig. 15) were established following dilution from fully grown cultures. Chl *a* content of these cultures markedly differed between cyanobacteria (range: 221.2 – 4842.553 μg L^{-1}), suggesting distinct growth capacity, thus, in theory, differential ability to contribute for bloom biomass. The differential Chl *a* yield that different cyanobacteria and microalgae might have in fully grown cultures is of interest in other arenas such as that exploiting Chl *a* as a bioproduct with applications in pharmaceutical, food and cosmetic industries (e.g. Halim and Danquah 2013). The higher the yield of a given cyanobacteria, the higher the industrial interest in exploiting it for Chl *a* production provided that no important additional requirements (e.g. culturing conditions) or constraints (e.g. production of toxins) hold.

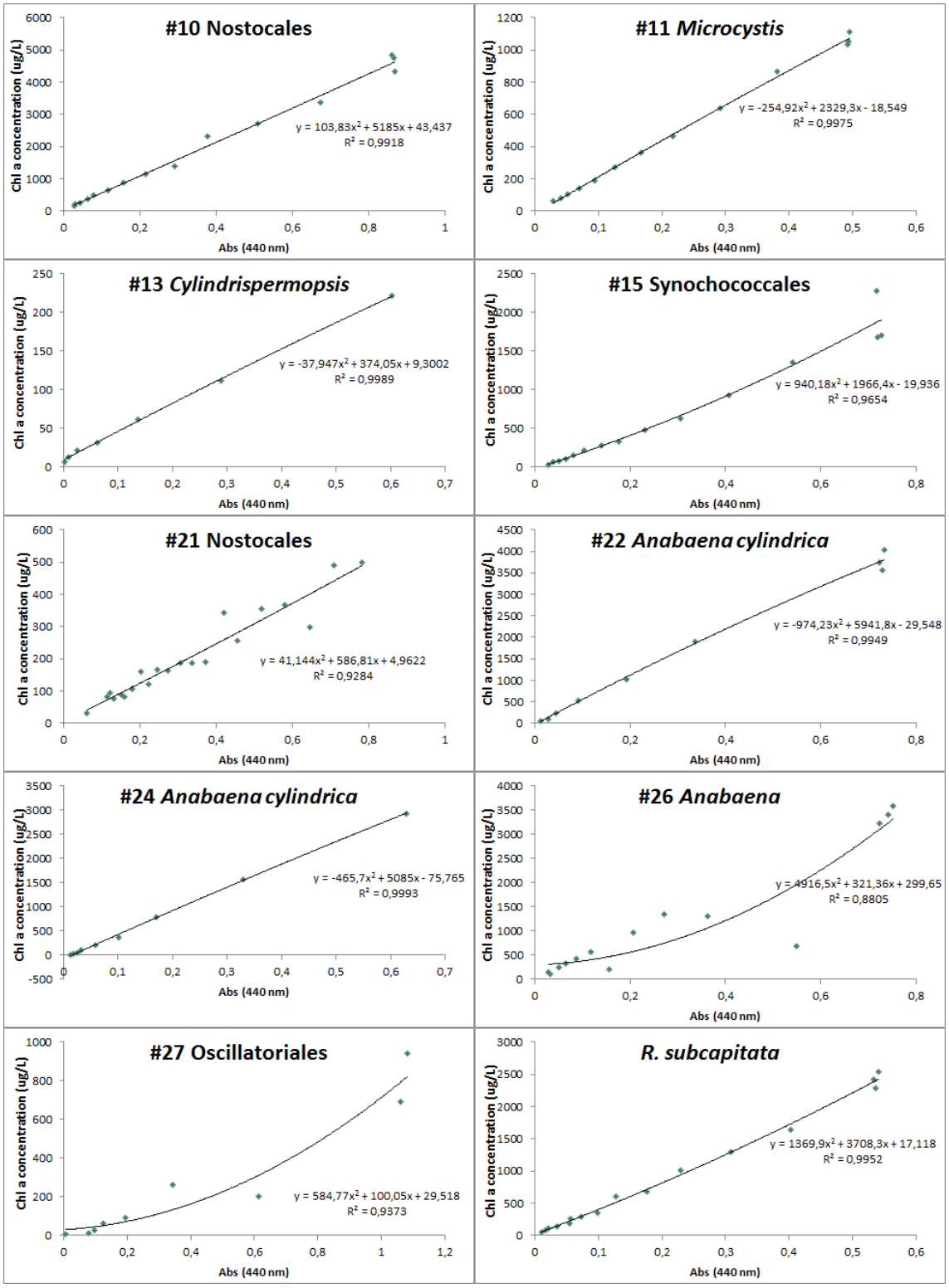


Figure 15: Relationships between Chl *a* content (µg/L) and Absorbance readings at 440 nm wavelength for each cyanobacteria used in the present study. Serial dilutions were applied to properly cover the curves with experimental data and allow a feasible fit: #10 using a 1:4 dilution; #11 using a 1:4 dilution; #13 using a 1:2

dilution; #15 using a 1:4 dilution; #21 using a 1:10 dilution; #22 using a 1:2 dilution; #24 using a 1:2 dilution; #26 using a 1:4 dilution; #26 using a 1:2 dilution; *R. subcapitata* using a 1:4 dilution.

II.4. Conclusions

The present chapter showed how difficult identification of cyanobacterial cultures can be, with problems arising when taxonomic affiliation is attempted based on microscopic morphology analysis but also when molecular approaches are applied. The current morphology of the cultured cyanobacteria differed from the morphologic characteristics described for type strains as identified using molecular techniques, suggesting that longtime maintenance under optimal conditions in the laboratory affects the cultured cyanobacteria somehow.

This morphological assessment is of major importance within the context of the present dissertation considering further planned studies such as those exposed in chapter III, where the filtering and feeding ability of *Corbicula fluminea* upon the cyanobacteria studied here were assessed. In fact, morphological features may play an important role in constraining filtering and ingestion rates by the bivalve. The chlorophyll *a* content of the cyanobacteria and calibration curves established between this variable and absorbance and cell density were performed allowing its use in routine assays, as those described in the next chapter.

II.5. References

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III. The potential of *Corbicula fluminea* as a cyanobacteria biofilter: filtration efficacy assessment

III.1. Introduction

Non-indigenous invaders are an important ecological problem worldwide. Some of them have high potential to affect food webs, biogeochemical cycles, net biodiversity balances, this all often leading to important impacts in specific sectors of the human economy (Kolar and Lodge 2001; Grosholz 2002). There are numerous examples supporting the touting of invasive species as a serious and concerning nuisance nowadays (Strayer et al. 1999), with the topic being growing under the spotlight as they were recognized as a major cause for biodiversity loss worldwide (Slingenberg et al. 2009).

One such example is that of the Asian clam, *Corbicula fluminea*, which is one of the 100 worst invasive species in European freshwaters (DAISIE 2008) coming from native distribution ranges in Asia, Africa and Australia (Mouthon 1981; Ituarte 1994; Sousa et al. 2007; Sousa et al. 2008a). The rapid growth of *C. fluminea*, alongside with early sexual maturity, short life span, high fecundity and its capability to associate with human activities, make the species one of the most troublesome invaders in freshwater aquatic ecosystems (Sousa et al. 2008a). By bearing these attributes, the Asian clam can potentially overlap and out-compete native species in the exploitation of resources (Atkinson et al. 2011), this being a primary ecological impact in invaded ecosystems. Also, the species can affect hydrology, the biogeochemical cycles and the biotic interactions by two main mechanisms: (i) assimilation-dissimilation, which is the uptake and release of energy and materials; (ii) physical ecosystem engineering, which reflects the physical modification of the ecosystem substrate impacting from individuals up to ecosystems (Sousa et al. 2008a; Sousa et al. 2009). Complying with the first mechanism, *C. fluminea* can alternate between preferred filter feeding but can also use pedal feeding by filtering re-suspended material from sediments when suspended material is scarcer (Way et al. 1990; Hakenkamp and Palmer 1999). This interplay between filtering from the water column, with consequent deposition

in the sediment by feces and pseudofaeces (which provide improved dietary provisioning for other benthic species; Basen et al. 2013), and pedal feeding where deposited particles can be mobilized and filtered (Hakenkamp and Palmer 1999; Hakenkamp et al. 2001) drives important changes in the benthic-pelagic organic matter dynamics in invaded ecosystems (Sousa et al. 2008b; Sousa et al. 2012). The second mechanism materializes in shell accumulation in the bottom substrata, which constrains availability of refuges and changes the substrate characteristics through the addition of physical complexity or the input of additional resources (Gutiérrez et al. 2003; Sousa et al. 2009; Ilarri and Sousa 2012; Ilarri et al. 2012; Ilarri et al. 2014).

The impressive filtration capabilities (see table 1 for a revision) of the clam have been claimed to be a major driver of the first above mechanism (Sousa et al. 2008a). However, filtration rates for the clam have been recorded using different units, contexts and filtered items (table 1), which may constrain a more immediate view of the clam's capacity. Still, the studies by Cohen et al. (1984) note that the clams can clear water at rates as high as 33 mL h^{-1} g^{-1} under realistic and favorable conditions (e.g. no contamination, favorable size and shape of filtered items), denoting a powerful filtration capability – this interpretation can be facilitated by the records by Buttner and Heidinger (1981), which allow a view on the comparison between dry weight and clam-based measurements that may facilitate this interpretation.

Some attributes of bivalve pests (the Asian clam or the zebra mussel in particular), such as its accumulation capabilities and high environmental tolerance/resilience, can be used to compensate for the damage they cause (Karatayev et al. 2007; Elliott et al. 2008; Rosa et al. 2014a; Binelli et al. 2014; Silva et al. 2016). Such exploitation of the species abilities can be understood as another branch within pest management programs, allowing the offsetting of the nuisance's negative impacts to a certain extent. Both Rosa et al. (2014a) and Silva et al. (2016) showed that the powerful filtering abilities of the Asian clam, combined to its remarkable capacity to accumulate metals, can be tuned to integrate bioremediation settings or simply assist water purification in invaded, but also polluted ecosystems. Apart from these pollution-driven scenarios, the possibility of using the Asian

clam as a bioremediator in eutrophic waters was suggested for the first time long ago by Cohen et al. (1984), who demonstrated a direct relationship between clam's density and water quality improvement in the Potomac River (USA).

Table 1: Literature records on the filtration rate of *C. fluminea* on different food itens. T stands for temperature; FW stands for fresh weight; DW stands for dry weight

Filtration activity	Filtered item	Notes	Reference
˚20 - 150 mL h^{-1}	Water column of Delta-Mendota canal	T = 20 - 24˚C	Prokopovich 1969
+200 - 800 mL $clam^{-1}$ h^{-1}	*Melosira sp.*	T = 18 - 27˚C	Mattice 1979
+ 347mL h^{-1} $clam^{-1}$; 177 mL h^{-1} g FW^{-1}; 1.561 mL h^{-1} g DW $^{-1}$	*Scenedesmus sp.*	T = 21 - 24˚C	Buttner and Heidinger 1981
*24.1 mL h^{-1} g DW $clam^{-1}$	phytoplankton of Potomac river	T = 26.5˚C; Different ages of clams	Cohen et al. 1984
+109 - 1370 ml hr^{-1}	*Chlorella vulgaris*	T = 20ºC; Shell length= 22.4 mm (SE= 0.24)	Lauritsen 1986
+30 - 140; 10 - 220; 25 - 610 mL $clam^{-1}$	*Chlorella vulgaris*; Microspheres (ø=1; 2; 5; 16 µm)	T = 15ºC	Way et al. 1990
+7173.7 - 10971.1 mL $clam^{-1}$ h^{-1}	PolyVinylToluene beads. ø = 2.020 nm	T= 20˚C; Shell length= 12.1 mm (±0.3)	Leff et al. 1990

*4.4 ± 0.6 mL g DW^{-1} min^{-1}	*Escherichia coli* strain JM83		Silverman et al. 1995
+100% removal of a oocyst suspension (1 x 10^6 L^{-1}) after 24h	*Cryptosporidium parvum* oocysts	Shell length= 1.5 - 2.5 cm	Graczyk et al. 1998

˚Pumping rate; +Filtration rate; *Clearance rate

A major component within eutrophication scenarios is the cyanobacteria assemblage that frequently develops and dominates the growing phytoplanktonic mass. Usually, cyanobacteria blooms are related with particular conditions such as a low N:P levels, water stability, reduced transparency, increase of water temperature, pH and conductivity (Codd 2000; De Figueiredo et al. 2004; Macário 2013). Due to the ecophysiological abilities that cyanobacteria present, they are generally a superior competitor when compared to microalgae (Merel et al. 2013). Since they can perform aerobic and/or anaerobic photosynthesis, a rapid development can occur in different habitats, and their ability atmospheric dinitrogen fixation as assisted by nitrogenase (Paerl et al. 2001; Wiegand and Pflugmacher 2005) leads to an enhanced range of tolerance to dissolved nutrient scarcity. Besides their capacity to outcompete microalgae, rapidly dominating the phytoplankton community, some cyanobacteria can produce toxins that can harm the aquatic biota via ingestion, surface contact or ingestion of contaminated organisms, ultimately presenting a risk to human health (Carmichael 1992; De Figueiredo et al. 2004; Metcalf and Codd 2012). Some cyanobacteria genera with the ability to produce toxins are *Anabaena*, *Aphanizomenon*, *Cylindrospermospis*, *Microcystis* and *Oscillatoria*.

Although the use of bivalves as ecotechnological control tools targeted at cyanobacterial blooms has been suggested (Pires et al. 2007; Gulati et al. 2008; Triest et al. 2015; Waajen et al. 2016), experimental studies directly supporting the possibility are very scarce. Vanderploeg *et al.* (2001) examined the filtering activity over *Microcystis aeruginosa* by *Dreissena polymorpha* and observed that, although the mussels kept a high and continuous

filtering activity, they did not ingest the cyanobacteria and large amounts of pseudofaeces were produced leading to a promotion and maintenance of the cyanobacteria bloom. Waajen et al. (2016) showed that quagga mussels (*Dreissena rostriformis bugensis*) can reduce phytoplankton biomass in hypertrophic ponds, including cyanobacteria, this inducing clear water states. Regarding the Asian clam in particular, Cohen et al. (1984) confirmed its role in improving water quality but not specifically addressing cyanobacteria, and Hardenbicker et al. (2015) showed that the clam substantially supressed coccoid cyanobacteria but not filamentous strains. Thus, not much is known on the capacity of the Asian clam to filter on cyanobacteria and consequently on its ability to assist the mitigation of cyanobacteria blooms and its effects. Although *C. fluminea* is generally considered a nonselective filter feeder (Way et al. 1990; Boltovskoy et al. 1995), some factors have been recently shown to influence food selection in freshwater clams such as the quality of food, cell size and particle structure (Gazulha et al. 2012; Frau et al. 2016), and the Asian clam was already found able of selective feeding based on plankton size-range (Atkinson et al. 2011).

In fact, cyanobacteria are structurally and compositionally distinct from green microalgae, thus it is likely that consumption patterns by the Asian clam can be driven by these differences (Von Elert et al. 2003). Cyanobacteria are gram-negative photosynthetic prokaryotes (Paerl et al. 2001), amongst the most diverse gram-negative prokaryote groups in morphology, physiology and metabolism (Wiegand and Pflugmacher 2005). Still, cyanobacteria typically present low sterol content, which makes them a poor quality food item, via toxicity of some strains, poor digestibility, feeding interference by mechanical interference with the filtering process for filamentous cyanobacteria and biochemical deficiencies for filter-feeding zooplankters (Porter and Mcdonough 1984; Ahlgren et al. 1990; Von Elert et al. 2003) and clams (Basen et al. 2012), compared to green microalgae. In general, microalgae are also a richer phosphorous source (Brett et al. 2000), as well as are typically better sources of poly-unsaturated fatty acids than cyanobacteria for filter-feeder zooplankters (Brett and Müller-Navarra 1997) and clams (Basen et al. 2011). In addition, endotoxic lipopolysaccharides (LPS) are present in the outer cell layer cyanobacteria that induce or modulate diverse toxic effects in the aquatic biota, including

osmoregulatory imbalance and water ingestion stimulation in fish (Best et al. 2003; Wiegand and Pflugmacher 2005).

Considering all the above, we designed the present study a first approach to assess the ability of the Asian clam as a biofilter in eutrophication scenarios dominated by cyanobacteria. Both the filtration and the production of pseudofaeces were assessed using a fully controlled laboratory experiment, aiming at getting an insight on the likelihood of the clam to become an agent assisting the remediation of such scenarios. Different cyanobacteria species and strains were tested, all theoretically fitting the size range of particles that can be removed from water by freshwater clams (Wallace et al. 1977) to better capture the effects of different sizes and morphological features in this putative capacity of the clam.

III.2. Material and Methods

III.2.1. Cyanobacteria and microalgae laboratory culturing

Nine cyanobacteria strains were used during the assays (see chapter II). These were cultured from cyanobacteria isolates collected in several Portuguese lakes and watercourses: #22, #24 and #26 *Anabena and* #10, #21 Nostocales strains were collected in Baixo Mondego; #27 Oscillatoriales, #11 *Microcystis* and #13 *Cylindrospermopsis* strains were collected in the Vela lake (Figueira da Foz, Western Central Portugal). The reasoning for selecting these cyanobacteria for this study included different morphology presented (see chapter II); the possibility of them to form and reach bloom typical densities when in favorable conditions (Codd 2000; De Figueiredo et al. 2004; Macário 2013); and the possibility of some of the strains to become toxic (Carmichael 1992; Carmichael et al. 2001; De Figueiredo et al. 2004; Macário 2013).

Long-term maintenance in the laboratory of cyanobacterial stock cultures was made in 100-mL Erlenmeyer vessels filled with 50 mL MBL-Woods Hole culture medium (Nichols 1973). These stock cultures were kept at 20 ± 2ºC, under a $16h^{L}$:$8h^{D}$ photoperiod (Fig. 1). Renewal

was done fortnightly by harvesting about 10 mL from the old inoculum and inoculating in fresh, sterilized (autoclaved; 60-90 min, 120 ºC, 1 atm) MBL-Woods Hole culture medium. As required by the testing protocol, cultures were grown under the above conditions in aerated 4 L glass vessels until reaching the stationary growth stage (Fig. 2). This required a 10-14-day growth period, depending on the species and strain.

The green microalgae *Raphidocelis subcapitata* was used throughout the study as a control treatment. Concentrated suspensions of this microalga have been used successfully in the laboratory to maintain healthy *C. fluminea* cultures in the long-term, as well as adequate food for long-term culturing and testing with the clam (Gomes et al. 2014; Rosa et al. 2015). Furthermore, filtration tests were already conducted with *R. subcapitata*, where appreciable filtration rates were observed and easy ingestion was confirmed (Pereira et al., pers. comm.). The microalgae were cultured as described above for cyanobacteria, also in MBL-Woods Hole culture medium, but respecting shorter renewing cycles (weekly renewal).

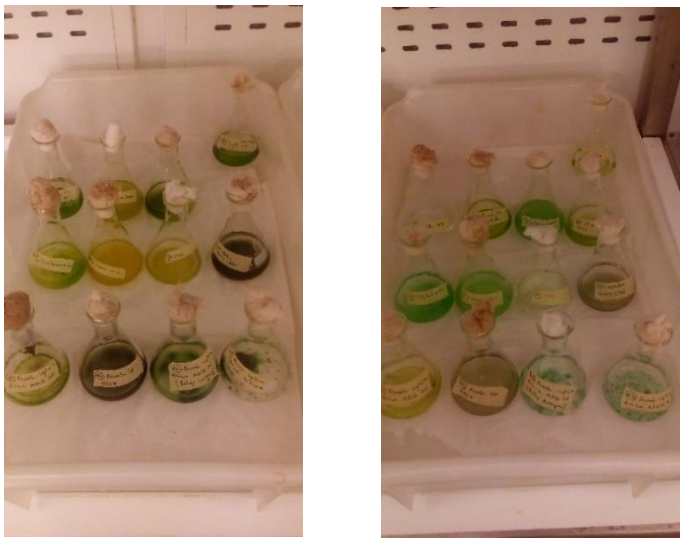

Figure 1: Old (left) and recent (right) cyanobacteria stock cultures.

Figure 2: Grown cyanobacteria culture (#21 Nostocales) in a 4L vessel.

III.2.2. Clam collection and laboratory maintenance

Clams were collected in a shallow creek in Mira, Coimbra, Centre Portugal (Vala dos Moínhos, Casal de S. Tomé; N 40º 24' 56'', W 8º 45' 05''). This site holds a well-established, stable clam population with mean densities reaching density peaks of over 6000 clams m^{-2} in the summer (Rosa et al. 2014b). By using a shovel, the sediment was collected into a 5-mm-mesh bag for separation of clams from sediment. The retained material after thorough washing was sorted out in a tray. Clams with shell length ranging 20 - 25mm were separated and transported in plastic buckets (20 L) filled with ca. 10 L water from the watercourse; one thousand clams per bucket was the reference density used for transport.

In the laboratory, field water was gradually replaced by dechlorinated tap water for acclimation; similar buckets were used for laboratory maintenance of organism stocks, but the density was adjusted to ca. 500 individuals per bucket (Fig. 3). The animals were maintained under continuous aeration at constant temperature (20 ± 2°C) and photoperiod (16 h^{L}: 8 h^{D}). The stock cultures were inspected for and cleared from dead animals on a daily basis, and were renewed twice every week by transferring all living clams to clean buckets with fresh dechlorinated tap water; before the transference, clams were washed

under the tap water flow while rubbing them against each other carefully. Once the clams were transferred, each stock was fed with 30 mL of a concentrated suspension of *R. subcapitata* cultured as described above; this was optimised previously to ensure that food was provided *ad libitum* to the clams. These suspensions were prepared by concentrating through centrifugation from grown cultures.

Figure 3: *Corbicula fluminea* maintenance under laboratory conditions.

III.2.3. Pre-testing calibration

Given the experimental requirements of filtration tests (time frame, number of measurements, need to return the samples to the test vessels; see below) the decrease in absorbance at 440 nm (Schimadzu UV-VIS 1800 spectrophotometer) of the tested cyanobacteria/microalgae suspension was used to estimate % of removal from water column by clams and filtration rates throughout the test period, as a surrogate measure of cell density and chlorophyll *a* (Chl *a*). The corresponding calibration curves were established previously, as detailed in Chapter II, with cell counting and Chl *a* determination being carried out following standard procedures. In brief, microalgae cell counting was carried out under a microscope (Olympus CKX41) in a Neubauer improved counting chamber, while cyanobacteria density was determined also under the microscope using a Sedgwick-Rafter chamber (Lawton et al. 1999). Chl *a* concentration was determined after

vacuum filtering the sample through a GF/C filter (1.2 µm pore) following frozen storage at -20ºC. Chlorophyll was then extracted with a solution of 90% acetone and store at 4ºC overnight in a foil-wrapped tube. Then, it was centrifuged at 3500 rpm for 10 min and the absorbance of the supernatant was measured at 630, 647, 664 and 730 nm. The values obtained were used for the determination of Chl *a* using the trichromatic equations detailed in APHA (1995).

III.2.4. Filtration tests

To standardize initial conditions of tested clams, and based on Way *et al.* (1990), the animals undergoing each testing trial were transferred from the bulk culture into a flask filled with 900 mL dechlorinated tap water 48 h prior the starting of the experiments. They were fed with *R. subcapitata* after the transference and then left with no further food supply until they were used in tests.

The capacity of the Asian clam to filter each cyanobacterium was tested following previous optimisation trials (Pereira et al. person. commun.). Each trial was composed by two treatments, corresponding to the exposure to a cyanobacteria strain and to microalgae (*R. subcapitata*), the latter used as a positive control to confirm that healthy animals were tested. The standard criterion for this later confirmation, hence to validate the assay, was that all clams should be able to remove 20% of the total green microalgae biomass provided, at the end of a 120 min test period, provided that no stressful conditions are established, i.e. under control conditions (filtration tests - Pereira *et al*. person. comm.). Since the initial microalgae density used in optimization trials (clearance after 120 min was aimed in the study where these trials were made) was much lower than that used in the present study (mimicking bloom conditions was the goal here), the percent reduction was converted into a cell density benchmark defined by the 95% confidence interval of the mean (n = 162) cell number removed by clams (1.15×10^8 - 2.51×10^8 cells removed). Clams were individually tested in 120-mL glass vessels filled with 100 mL of final microalgae/cyanobacteria suspension, harvested directly from fully grown cultures into the

test vessels (Abs_{440nm} of 0.492 – 0.475 for microalgae and 0.951 - 0.302, for cyanobacteria). Ten replicates were assigned to the microalgae control and 25 replicates were used to complete the cyanobacteria treatment. Each 5 replicates were added a sixth equivalent vessel with no clam to control for the growth of algae or cyanobacteria during assay and, therefore, cancelling the possible induced error during the assay by the growth of the cyanobacteria and algae. The experimental setup used in each trial is illustrated by Fig. 4. All vessels were kept under continuous aeration to avoid algae/cyanobacteria sedimentation in the bottom of the vessel and hence ensuring the conditions for stable filtration activity. The tests were run at room temperature and illumination. The clams were left to acclimate to the test conditions for one hour prior to the beginning of the test. For the purposes, the clams were transferred into and kept for 1 h in the test vessels filled with 10 mL dechlorinated tap water. Only after this acclimation period, 90 mL of microalgae/cyanobacteria suspension was added to each replicate to complete the final test volume of 100 mL, and onset the testing period. This procedure allowed acclimation with no relevant dilution of the final algae/cyanobacteria concentration set for testing.

Figure 4: Experimental setup used to test the filtration of cyanobacteria by the Asian clam. The two left-hand light green rows correspond to microalgae and the remaining rows contain a cyanobacteria suspension. One clam was placed in each of five vessels in a row for filtering during the 120 min test period, while the sixth was clam-free to control for microalgae or cyanobacteria growth.

The initial cell density and Chl *a* content in the test vessels were determined following measuring on the respective culture and considering the 10:1 dilution applied to allow acclimation (see above). Cell density was estimated on the basis of 3 replicate $Abs_{440\ nm}$ measurements following integration with previously established calibration curves (see chapter II). Absorbance (440 nm) of the testing suspensions was read at the beginning (T_0), after 30 (T_{30}), 60 (T_{60}), 90 (T_{90}), and 120 minutes (T_{120}) exposure, this allowing a time-dependent percent overview on the microalgae/cyanobacteria removal from the water column. The sample taken for Abs readings was always returned to the correspondent vessel, except from T_{60} onwards. After 1 h, pseudofaeces production was almost always remarkable and the return of the sample to the vessel would re-suspend them and prevent the settling necessary at the end of the assay. Chl *a* was measured in three replicate samples harvested from the bulk microalgae/cyanobacteria culture (see above for details on the method). At the end of the assay (T_{120}), structured Chl *a* measurements were made to allow assigning Chl *a* content to different system compartments: that still suspended in the water, thus not filtered by the clams (Chl_{medium}), which was analysed following vacuum-filtering 15-25 mL of undisturbed test medium; that suspended in the test medium plus the fraction entrapped in pseudofaeces, which was analysed following filtering 10-20 mL of homogenised medium (through vigorous agitation) to re-suspend pseudofaeces (Chl_{total}). The Chl *a* content of pseudofaeces ($Chl_{Pseudofaeces}$) was calculated assuming the mass balance between the system's compartments at T_{120}, as expressed in equation 1. The Chl *a* actually ingested ($Chl_{ingested}$) by the clams was calculated considering that this compartment is composed by all Chl *a* that was filtered ($Chl_{medium,\ T0}$ - $Chl_{medium,\ T120}$) and not transferred into pseudofaeces ($Chl_{Pseudofaeces}$), according to equation 2.

(Equation 1) $\mathbf{Chl_{total} = Chl_{Pseudofaeces} + Chl_{medium}}$

(Equation 2) $\mathbf{Chl_{ingested} = (Chl_{medium,\ T0} - Chl_{medium,\ T120}) - Chl_{Pseudofaeces}}$

For better comparability with the literature, we planned to deliver filtering rates considering the clam unit and a dry weight unit. In order to achieve the latter, the shell length of all clams was measured right before the beginning of the acclimation part of the tests (mean of 21.8 ± 1.08 SD mm), and converted into dry weight (mean of 35.7 ± 4.49 SD mg DW) through the predictive models by Rosa *et al.* (2014b): DTW = $a\ L^b$, where DTW and *L* stands for clams' dry tissue weight (mg) and shell length (mm), respectively, while *a* and *b* stand for two constants, 0.0165 and 2.49 respectively, from the regression obtained for January, which was the month when the clam collection took place. Filtration rates were then calculated considering the whole test period on a dry weight (mL min^{-1} g^{-1}; equation 3) or a *per capita* (mL min^{-1} ind^{-1}; equation 4) basis.

$$\text{(Equation 3) } F_{120\ min} = \frac{V \times \ln\frac{C_i}{C_f}}{DW \times \Delta t}$$

$$\text{(Equation 4) } F_{120\ min} = \frac{V \times \ln\frac{C_i}{C_f}}{\Delta t}$$

In equations 3 and 4, *V* corresponds to test volume and Δ*t* to the established assessment period (120 min); C_i and C_f stand for initial and final Chl *a* concentration, respectively.

III.2.5. Statistics

Measured Chl *a* mass was used as a feasible endpoint for the statistical analyses. In order to check the significance of clam's filtering activity within each test, initial and final Chl *a* content of the test water were compared using paired samples *t* tests. The distribution of the difference between each pair of measurements was checked for normality by graphic inspection of frequency distribution and using the Anderson-Darling test. A single case was

found deviating from the normality assumption, where we ran the non-parametric *Wilcoxon paired-sample test* after verifying that data transformation could not improve the distribution (Zar 2010). Chl *a* mass removed from the water column by *C. fluminea* during 120 min was used to compare the green microalgae with each cyanobacterium regarding their quality as filtering items, through t-tests. The normality assumption, assessed in each sample as described above, was met either using the raw data or transformed datasets (*log* Chl *a* removed + 1). Homocedasticity was assessed through graphical inspection of each paired 95% confidence intervals for standard deviation, and using the F-test for equal variances. Data transformation (*log* Chl *a* removed + 1) was generally successful in homogenising variances between each two samples, except for two cases. Since the Mann-Whitney non-parametric alternative is at least as sensitive as the t-test to heterocedastic deviations, and considering that the t-test is robust enough to cope with non-severe deviations from homocedasticity especially for large sample sizes (Zar 2010), we still ran the parametric t-test in these cases following conservatively adjusting the alpha level to 0.01 (Zar 2010).

III.3. Results and Discussion

Although microscopically counted cell density and its relationship with absorbance have been explored as feasible methods to address cell density changes in microalgae suspensions (see for example widely accepted guidelines for testing with microalgae; OECD 2013), the same does not apply to cyanobacteria. Their variable and specific morphology, as well as trend for agglomeration or colony habit make the feasible counting under the microscope very difficult to achieve (Lawton et al. 1999). On the other hand, these morphological features may bias absorbance readings as discussed below. Therefore, the most feasible method for following variation of populations of cyanobacteria is still chlorophyll extraction and quantification (see also chapter II for an overview of this rationale in the context of the present study).

In spite of these difficulties in feasibly relating biomass surrogate parameters, we obtained a good agreement between Abs and Chl *a* content that supported the following of cyanobacteria fluctuation during filtration tests. Table 2 shows the biomass removed by the clams after allowed filtering on every cyanobacterium and on the microalgae during 120 min, as determined by absorbance readings with further estimation of Chl *a* or directly through Chl *a* quantification. There are differences on the values obtained between the two methods, but for some cases, the values denoting percent removal of cyanobacteria from water were similar, suggesting that for this specific set of species/strains absorbance can be a fair surrogate of chlorophyll concentration. This supported the use of absorbance for a general view on the filtration dynamics through time as discussed in sub-section III.3.1.

Table 2: Biomass of cyanobacteria and microalgae removed from the water column following filtration by the Asian clam during 120 min (ug Chl *a*). The central column shows Chl *a* removal estimated on the basis of absorbance readings at 440 nm through previously established calibration curves (see chapter II). The right-hand column denotes Chl *a* removal directly determined through Chl *a* quantification. Mean (± standard deviation) values are shown. Species/strains where the endpoints should definitively not be used as surrogates were marked bold.

Cyanobacteria /Alga	**Chl *a* estimated based on Abs_{440}**	**Quantified Chl *a***
#10 Nostocales	**37.45 (±25.01)**	**0^{+}**
#11 *Microcystis*	8.77 (±6.11)	1.68 (±2.47)
#13 *Cylindrospermopsis*	7.21 (±2.15)	3.87 (±1.59)
#15 Synochococcales	88.29 (±39.72)	79.43 (±31.72)
#21 Nostocales	**10.77 (±3.44)**	**31.51 (±2.89)**
#22 *Anabaena cylindrica*	**55.44 (±35.69)**	**105.04 (66.81)**
#24 *Anabaena cylindrica*	**55.65 (±17.70)**	**133.27 (±47.42)**
#26 *Anabaena sp.*	45.65 (±21.70)	41.83 (±32.60)

#27 Oscillatoriales	17.55 (±8.92)	22.44 (±13.93)
R. subcapitata	91.17 (±34.40)	60.00 (±21.21)

[+]Negative removal rates were found, hence 0% removal was conceptually assumed.

Still, for several cyanobacteria (#24 *Anabaena cylindrica*; #22 *Anabaena cylindrica*; #21 Nostocales; #10 Nostocales), estimated Chl *a* removal was distinct from measured Chl *a* removal (Table 2). The most reasonable explanation for this disagreement between the endpoints is the interference of non-homogeneous distribution of suspended particles with absorbance readings (Kirk 1994). This results from morphology features of the cyanobacteria and the concurrent ability to aggregate, since all the referred species/strains are filamentous, some with relatively long filaments that promote a packaging effect (Morel and Bricaud 1981; Sathyendranath et al. 1987; Mitchell and Kiefer 1988; Sosik and Mitchell 1991; Kirk 1994); such an effect is visible in the microscopic imagery obtained for Chapter II, and should reflect in the present absorbance readings since the tendency for aggregation increases with high densities as used at the beginning of filtration tests. There are also errors in absorbance measurement that may derive from cells physiology, morphology (Kirk 1994; Morel 2001; Simis et al. 2005; Randolph et al. 2008). Finally, it is worth noting the possible interference of pseudofaeces in absorbance readings. The longer the test period, the higher the expected production of pseudofaeces that may not settle immediately at the bottom of the test vessel and become collected in the sample taken for absorbance readings.

III.3.1. Filtration activity of C. fluminea*: microalgae vs cyanobacteria*

Absorbance readings on the microalgae controls, and its correspondence to cell number, were used to validate the assays, i.e. mostly to assess whether the clams used in each trial were in good health status (Table 3). The data confirm that *C. fluminea* individuals were in

good health status, validating all the assays. In all the assays, the control clams filtered above the lower reference value of 1.15×10^8 cells.

Table 3. Biomass removed (number of cells) through filtration of microalgae (control treatment) by clams for 120 min. The records obtained in the control treatment of all cyanobacteria tests are given for comparison with the reference values obtained in optimization trials with the microalgae *R. subcapitata* (see Material and Methods for details). Lower and upper 95% Confidence Limits (CL) of the standard deviation obtained for each test are shown.

Test	**Lower 95% CL (nr cells)**	**Upper 95% CL (nr cells)**
Reference (*R. subcapitata*)	1.15×10^8	2.51×10^8
#10 Nostocales	2.86×10^8	4.95×10^8
#11 *Microcystis*	1.42×10^8	3.66×10^8
#13 *Cylindrospermopsis*	3.15×10^8	4.13×10^8
#15 Synochococcales	2.15×10^8	4.22×10^8
#21 Nostocales	2.21×10^8	4.39×10^8
#22 *Anabaena cylindrica*	2.72×10^8	4.99×10^8
#24 *Anabaena cylindrica*	5.13×10^8	5.80×10^8
#26 *Anabaena sp.*	1.70×10^8	3.26×10^8
#27 Oscillatoriales	2.30×10^8	5.63×10^8

Figure 5 shows the filtration dynamics of *C. fluminea* through time as different cyanobacteria were offered compared to that exhibited over the corresponding control microalgae; the records reflect Chl *a* estimates using absorbance readings. With the exception of #15 Synochococcales species, biomass removed throughout the testing period did not reach records above 50 µg Chl *a*. Maximum Chl *a* removal was generally reached after the first 60 min test period and from this point onwards there was no appreciable

filtration activity of the clams. Fluctuation or decrease in Chl *a* removed through longer filtration periods was rather observed in some cases (see e.g. Fig. 5 b, f, g, i). Since the plotted values constitute estimates based on absorbance readings, this is likely to reflect the unfeasibility of absorbance as a surrogate for Chl *a* in filtration tests as discussed above. Therefore, further discussion will be centered in biomass removed at the end of the test period considering direct Chl *a* quantification.

In general, quantified Chl *a* content changed significantly in the test water. The comparison between initial and final Chl *a* content showed that, in 120 min, clams were able to significantly remove #27 Oscillatoriales (t = 7.21, p <0.001); #24 *Anabaena cylindrica* (t = 11.80, p <0.001); #26 *Anabaena* sp. (t = 4.43, p <0.001); #21 Nostocales (t = 5.74, p <0.001); #22 *Anabaena cylindrica* (t = 53.50, p <0.001); #15 Synochococcales (t = 12.52, p <0.001); #11 *Microcystis* (t = -1.46, p =0.158) and, expectedly, *R. subcapitata* (t = 11.39, p <0.001); #13 *Cylindrospermopsis* was poorly, but significantly filtered (Z = 258, p <0.011). Conversely, #10 Nostocales was not filtered at all, with Chl *a* content rather increasing in the test water (t = -4.17, p <0.001) as denoted in Figure 6. Marked differences were found in the magnitude of cyanobacteria filtration by the Asian clam, which are more easily tracked by comparing with green microalgae filtration as a reference (Fig. 6).

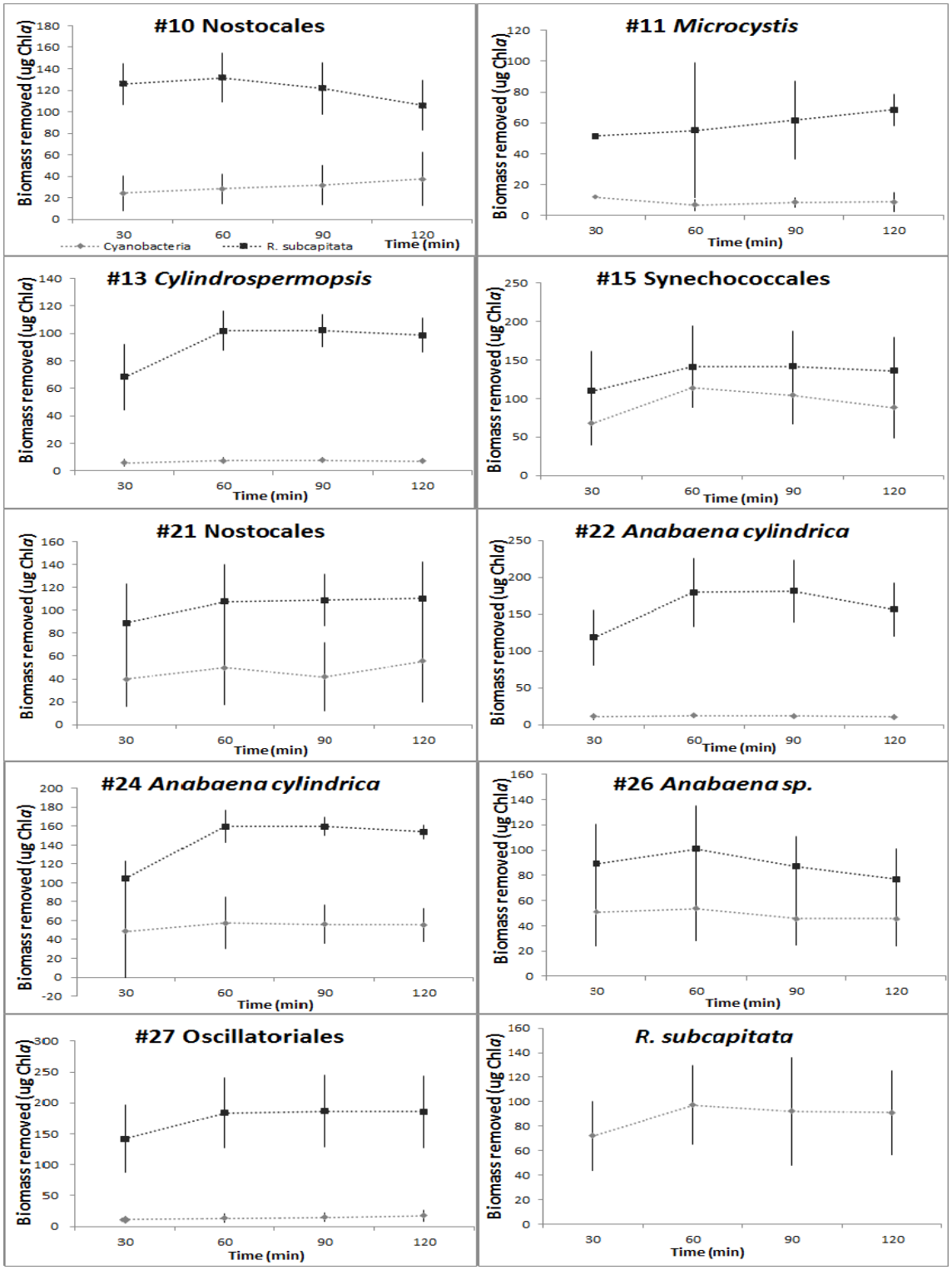

Figure 5: Chl *a* removed by the clams during the assay, sourced by each cyanobacteria (mean of 25 replicates) and the corresponding control microalgae *R. subcapitata* (mean of 10 replicates). The filtration dynamics over the green microalgae *R. subcapitata* was also tested (mean of 25 replicates) as a reference and is shown in the bottom-right graph. The Chl *a* records were estimated on the basis of absorbance readings at 440 nm in

samples taken from the test water at 30, 60, 90 and 120 min following the start of the assay. The lines were added to connect the marks for clarity purposes and error bars represent standard deviation.

Low Chl *a* removal records (below 50 µg) were found, as #27 Oscillatoriales and #21 Nostocales were tested (Fig. 6). These filamentous morphotypes tend to aggregate or the colonial strains can become bigger (Codd 2000; De Figueiredo et al. 2004; Macário 2013), at least in one dimension, than the maximum particle size of 16 µm that *C. fluminea* can ingest (Way et al. 1990), which may have limited the clam's filtration. However, the three *Anabaena* strains are very well filtered (#22 *Anabaena cylindrica*; #26 *Anabaena sp.*; #24 *Anabaena cylindrica*), with two being significantly better filtered than the green microalgae reference (Fig. 6). Thus, palatability may have also played a role in such noticed variation. Small sized cyanobacteria such as #11 *Microcystis* and #13 *Cylindrospermopsis* were not or were poorly filtered. The strain #11 *Microcystis* is a colonial cyanobacteria (see chapter II), therefore possibly limiting the filtering capacity of the clam. Also, the setting of test conditions to bloom-typical concentrations may have affected the test outcome. In fact, Way et al. (1990) found that the *C. fluminea* gills became saturated after 15 to 45 min, this variation being due to clam size and particle concentration. The Chl *a* removal records corresponding to #15 Synochococcales, which were significantly higher than those corresponding to the green microalgae reference (Fig. 6), can possibly be explained by its smaller size (generally unicellular and nonheterocystous) when compared to the rest of the cyanobacteria, and coccoid or rod-shaped form (Stanier et al. 1971; Brito et al. 2012).

Some strains of the least filtered cyanobacteria can produce harmful toxins (Carmichael 1992; Carmichael et al. 2001; De Figueiredo et al. 2004; Macário 2013). While cyanotoxin production may have contributed to a reduced filtration by the clams, we did not confirm such production and further discussion on this topic would be speculative. However, it is worth noticing that the literature shows that cyanotoxins can be filtered and accumulated by bivalves, including *Corbicula* sp. (Contardo-Jara et al. 2008; Martins et al. 2009; Puerto et al. 2011; Paldavičiene et al. 2015; Pham et al. 2015), suggesting that cyanotoxin production may not impair the filtration capacity of the clams.

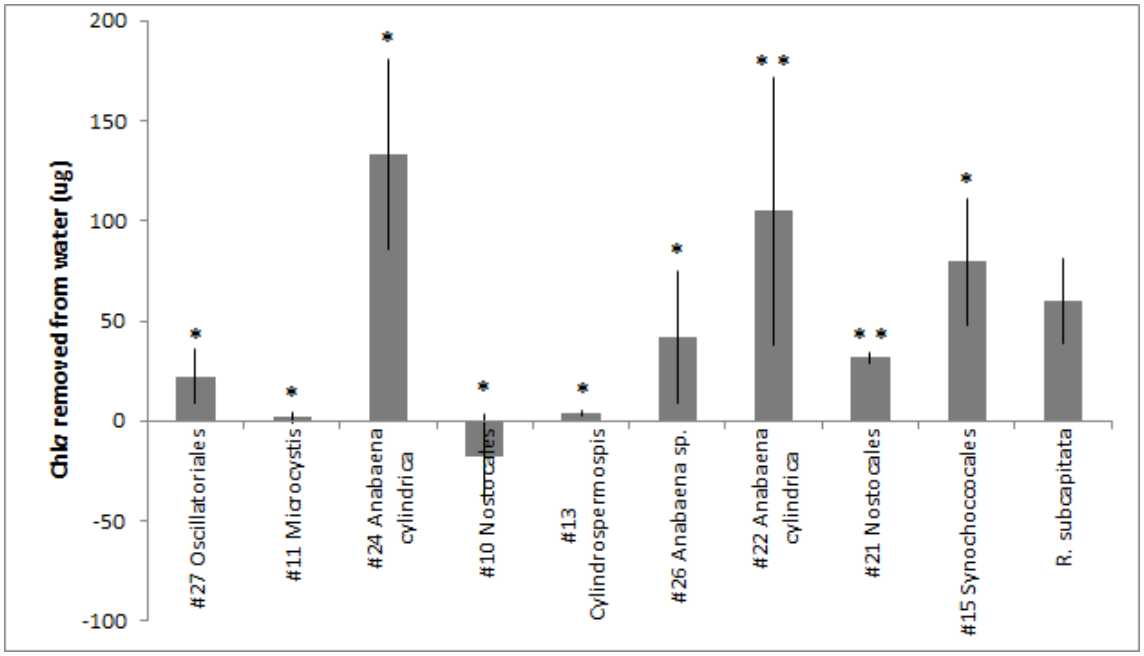

Figure 6: Comparison between the biomass removed from the water column corresponding to all cyanobacteria and the microalgae used as a reference for good filtration ability of *C. fluminea*. Error bars stand for the standard deviation. Asterisks denote significant differences between the removal of microalgae Chl *a* and each cyanobacterium's Chl *a*; one or two asterisks mark the use of an alpha level of 0.05 or 0.01 (*t*-test for independent samples), respectively, and the statistical summary for these tests is as follows: #27 Oscillatoriales – t-value = 7.25, p <0.001; #11 *Microcystis* – t-value = 17.04, p <0.001; #24 *Anabaena cylindrica* – t-value = -6.26, p <0.001; #10 Nostocales – t-value = 12.77, p <0.001; #13 *Cylindrospermopsis* – t-value = 21.44, p <0.001; #26 *Anabaena sp.* – t-value = 2.25, p <0.030; #22 *Anabena cylindrica* – t-value = 6.52, p <0.001; #21 Nostocales – t-value = 12.77, p <0.001; #15 Synechococcales – t-value = -2.53, p = 0.015

Per capita and dry-weight based filtration rates were calculated and are presented in table Table 4. Since the filtration rates were calculated on the basis of Chl *a* removal records, they generally reflect the patterns depicted in Fig. 6 and discussed above. Although filtration rates integrated the variation in Chl *a* content observed in the blank controls (no clams), a negative value was still found for the filtration rate upon #10 Nostocales, reflecting the trend noticed in Fig. 6. The negative filtration rate record hence suggests minor procedure errors, probably during sampling for Chl *a* quantification by collecting water contaminated with re-suspended pseudofaeces that could contain interfering Chl *a* amounts.

As denoted above (table 1), *C. fluminea* shows high plasticity in his filtering ability. The *per capita* filtering rates found for *R. subcapitata* in this study (16.57 ±6.84 mL h^{-1} clam $^{-1}$) are remarkably below the ones obtained by Mattice (1979), Buttner and Heidinger (1981) and even Leff et al. (1990), who recorded filtration rates of 200 - 800 mL $clam^{-1}$ h^{-1}; 347mL h^{-1} $clam^{-1}$; 7173.7 - 10971.1 mL $clam^{-1}$ h^{-1}, respectively. Even the highest per capita filtration rate obtained in the present study, for #21 Nostocales (61.69 ±10.83 mL $clam^{-1}$ h^{-1}), is still way below the 200 mL $clam^{-1}$ h^{-1} by Mattice (1979). However, Mattice (1979) used *Melosira sp.*, a diatom, while Buttner and Heidinger (1981) used *Scenedesmus sp.*, a green algae, both commonly present in the diet of *C. fluminea* and easily filtered. Leff et al. (1990) tested the filtration rate with PolyVinylToluene beads, which should not represent any limitation for the gill's filtering apparatus, to demonstrate the non-selectivity of *C. fluminea* feeding.

On the contrary, dry weight based filtering rate upon *R. subcapitata* found in the present study (464.3 ±191.7 mL h^{-1} g $^{-1}$) was markedly above that of 24.1 mL h^{-1} g DW $clam^{-1}$ by Cohen et al. (1984), and that of 1.561 mL h^{-1} g DW $^{-1}$ by Buttner and Heidinger (1981).

Table 4. *Per capita* and dry-weight based filtration rates exhibited by clams upon different cyanobacteria strains and the green microalgae *R. subcapitata* (given using minutes and hours as the time unit for better comparison with the literature). The values represent the mean of 25 replicate records and the standard deviation is given within brackets. Each replicate record was corrected for the Chl *a* variation monitored in the blank controls (no clam), thus eliminating the interference of a putative growth of the cyanobacteria/microalgae in the filtration rates records.

	Filtration rate			
	Per capita		**Dry Weight**	
Test	**mL min^{-1} $clam^{-1}$**	**mL h^{-1} clam $^{-1}$**	**mL min^{-1} g^{-1}**	**mL h^{-1} g $^{-1}$**
R. subcapitata	0.28 (±0.11)	16.57 (±6.84)	7.74 (±3.19)	464.3 (±191.7)
#10 Nostocales	-0.13 (±0.18)	-7.91 (±10.5)	-3.69 (±4.90)	-221 (±294.2)
#11 *Microcystis*	0.05 (±0.08)	3.26 (±4.88)	1.52 (±2.28)	91.38 (±136.7)

#13 *Cylindrospermopsis*	0.18 (±0.08)	11.03 (±4.94)	5.15 (±2.30)	309 (±138.3)
#15 *Synechococcales*	0.58 (±0.32)	34.9 (±19.4)	16.30 (±9.06)	977.9 (±543.7)
#21 Nostocales	1.03 (±0.18)	61.69 (±10.83)	28.81 (±5.06)	1728 (±303.5)
#22 *Anabaena cylindrica*	0.34 (±0.27)	20.59 (±15.4)	9.61 (±7.19)	576.9 (±431.6)
#24 *Anabaena cylindrica*	0.65 (±0.36)	38.98 (±21.83)	18.20 (±10.19)	1092 (±611.6)
#26 *Anabaena sp.*	0.13 (±0.12)	8.06 (±7.11)	3.74 (±3.32)	225.9 (±119.2)
#27 Oscillatoriales	0.30 (± 0.23)	18.19 (±13.71)	8.49 (±6.40)	509.7 (±384.3)

III.3.2. Chl a mass dynamics within the system: filtration vs ingestion

Figure 7 presents mass balance analysis regarding the percent allocation of Chl *a* to different compartments involved in the filtration test system. This analysis was generally feasible, although some cases where total Chl *a* measured did not fully coincide with the sum of Chl *a* measured in the test water with calculated Chl *a* content of pseudofaeces. The slight differences noticed should mostly result from natural variation in sampling at the end of the test. An extreme case where technical constraints should have occurred is that regarding #10 Nostocales, which were apparently not filtered by the clams (section III.3.1; Figure 6) but then were found ingested and entrapped in pseudofaeces (Figure 7). As offered #11 *Microcystis*, *C. fluminea* produced pseudofaeces (low quantity as directly observed and interpreted from the low filtration rates exposed in Table 4), but these could not be feasibly separated from the water column, preventing mass balance analysis for this cyanobacteria (note the absence of the allocation pie in Figure 7). Vanderploeg *et al.* (2001) observed that *Dreissena polymorpha* filters *Microcystis*, at rates comparable to the desirable food items, and highlighted that, similarly to our observations, pseudofaeces formed tended to easily break.

The presence of Chl *a* in pseudofaeces was clear in and common to all test systems where cyanobacteria were filtered (#13 *Cylindrospermopsis* was barely filtered *C. fluminea* (see III.3.1) hence did not produce pseudofaeces), contrarily to an almost imperceptible Chl *a* load of pseudofaeces in the *R. subcapitata* test system. This can be a direct indication of the lower palatability of the tested cyanobacteria compared to the green microalgae or it can relate to cyanobacteria cell size. Previous studies showed that 20 μm should be the upper size limit for effective processing by the Asian clam gills (Way et al. 1990); those particles of larger size suspended in the inhalant current should not be properly processed by the gill ciliary apparatus, which drives the production of pseudofaeces (Winter 1978; Russell-Hunter 1979). The cells of the non-filamentous cyanobacteria tested, #11 *Microcystis* and #15 Synochococcales, were both below the established upper size limit (see Chapter II or Olenina et al. (2006) for an overview on the dimensions within these taxa). In spite of this, #11 *Microcystis* was not filtered while #15 Synochococcales was better filtered that the reference microalgae and actually ingested - approximately half of the filtered mass was ingested with the other half being found in pseudofaeces (Fig. 7). This suggests that size was not the single constraint to ingestion and differential palatability may also play a role. Mass balance regarding the tested filamentous cyanobacteria also supports this argument. All have at least one dimension (diameter) that is below upper-size limits of *C. fluminea* gill apparatus (Chapter II; Olenina et al. 2006). However, while #13 *Cylindrospermopsis* were poorly filtered (Figure 6) with most of the initial Chl *a* load remaining in the water after allowing *C. fluminea* to filter for 120 min, #24 *Anabaena cylindrica* was filtered but mostly transferred into pseudofaeces, and remaining filamentous cyanobacteria were filtered and ingested, suggesting their suitability as a food resource for the clam (Fig. 7).

The cyanobacteria that were better ingested by *C. fluminea* were #27 Oscillatoriales, with half of the filtered Chl *a* being directed to the pseudofaeces and the other half being ingested (Fig. 7). The *Anabaena* strains were more easily filtered than #27 Oscillatoriales (Fig. 6) but a relative lower ingestion was achieved compared to the Chl *a* that was directed to pseudofaeces.

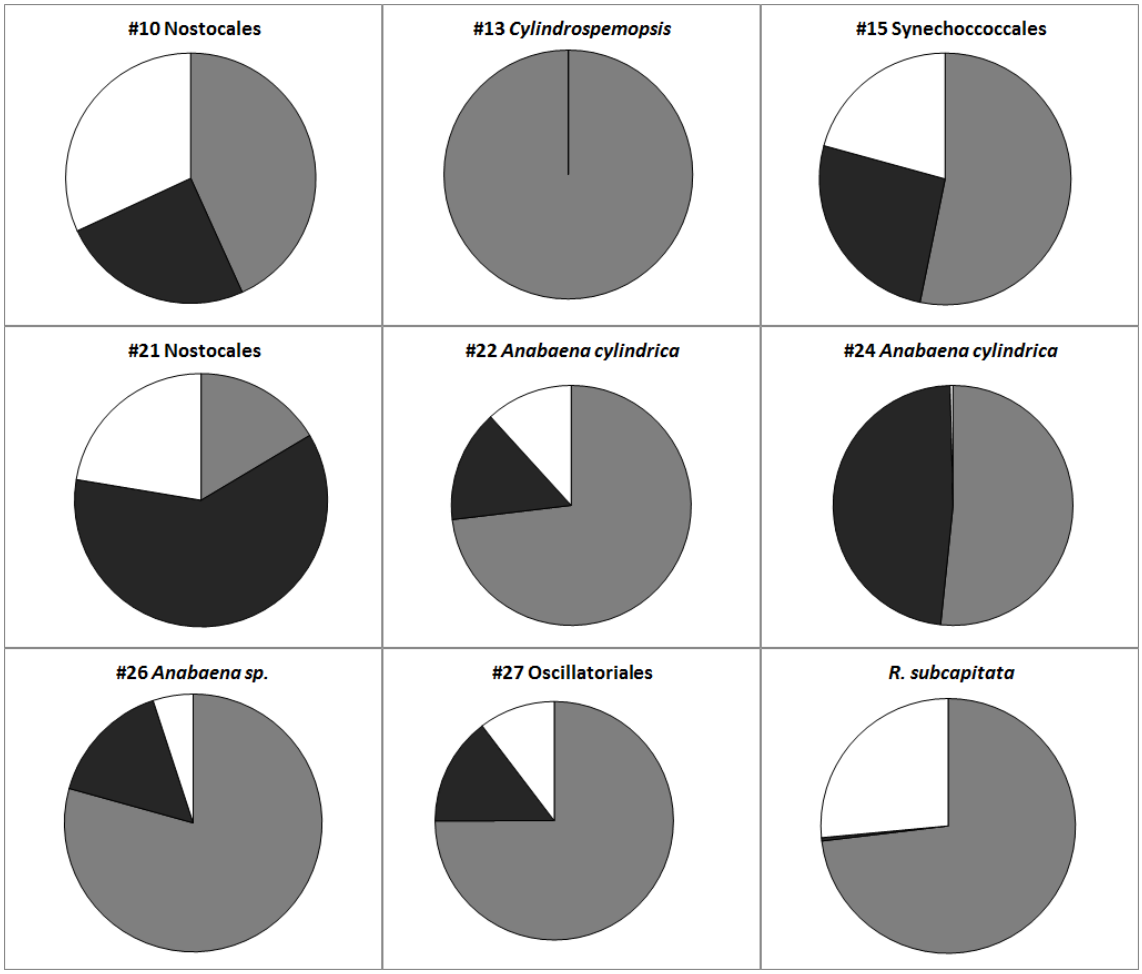

Figure 7: Mass balance (% of Chl *a*) in the test system after allowing *C. fluminea* to filter upon different cyanobacteria and the microalgae *R. subcapitata* for 120 min. Chl *a* measured in the water column is represented by grey pie portions and calculated Chl *a* content of pseudofaeces by black pie portions; calculated Chl *a* consumed by the clams is shown by white pie portions.

III.4. Conclusions

Although *C. fluminea* was not able of a high biomass removal for all cyanobacteria tested, the ability of the bivalve as a biofilter was generally proven. Some *Anabaena* strains and a Synochococcales strain were even better filtered than the reference green microalgae, which highlights the potential of the clam to act as a remediation agent in blooms dominated by these cyanobacteria. Part of the filtered cells was ingested and another part was directed to pseudofaeces. Although limited ingestion can be seen as a shortcoming at

a first glance, it may actually be beneficial for the use of the clam as a remediation agent. First, slower accumulation of undesirable material decreases the rate of renewal of remediator batches. Contaminated accumulators must be removed from the remediation setting and replaced by fresh ones to avoid the return of the contaminants to the system as the organisms die, and the disposal of this contaminated material is a debated concern (see e.g. Gomes 2012). Second, pseudofaeces production translates, in this case, on the concentration of the nuisance mass in heavier mucilaginous clots, which sediment and accumulates at the bottom of the system. This much resembles the activity of flocculants used to clean water from organic particles, for example in water treatment plants (Bolto 1995), but also in natural systems (e.g. Furukawa et al. 2014). It is then reasonable to suggest that a putative use of *C. fluminea* as a cyanobacteria bloom remediation agent may consider the species' filtration capacities added its ability as a natural flocculant. Still, the invasive character of *C. fluminea* is noteworthy when equating its application in such a suggested bloom remediation system.

III.5. References

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Chapter IV. Final remarks

This dissertation was developed as a preliminary assessment of the suitability of using the Asian clam *Corbicula fluminea* as a control agent or a remediation agent for cyanobacteria blooms. Laboratory cultures of cyanobacteria were used in filtration experiments that were collected and isolated from natural blooms in Portuguese water bodies. Chapter II describe the first stage of the work, where a comprehensive characterization of the cyanobacteria cultures was made. The morphological characterization and genetic analysis were quite conclusive, resulting in a relatively detailed taxonomic affiliation of the cyanobacteria, including at the strain level in some cases. Still, there were cases where genetic typing was unsuccessful and others where it disagreed with the morphological features observed under the microscope. Different productivity was observed among the cyanobacteria, as well as different relationships between surrogate measures of biomass yield (namely between optical density and chlorophyll *a* content). Data gathered supported the studies described in Chapter III, where an insight was gained on the ability of the Asian clam to filter and/or ingest the cyanobacteria.

It is worth mentioning that the results regarding the Asian clam filtration ability on cyanobacteria were not as expected considering the enthusiastic touting of the bivalve as a powerful filtering agent in the literature review. In fact, some cyanobacteria were hardly filtered by *C. fluminea*, weakly linking to morphological constraints, and an important component of the cyanobacteria filtered was not ingested, denoting the difficulties that other organisms may have to process the cyanobacteria composition or highlighting that cyanobacteria may be a poor food resource to the clam. Importantly, the non-ingested fraction of filtered cyanobacteria was concentrated in pseudofaeces that are rejected by the clams and returned to the external medium. Pseudofaeces constitute mucilaginous clots that settle consistently in the bottom, a picture resembling the mechanisms underlying chemical coagulation methods widely used in water treatment. In future studies, under carefully designed experimental settings, further light can be shed on the putative advantage of this concentration-and-settling activity in assisting the remediation of cyanobacterial blooms. Still, it is very important to remember that, although the use of

C. fluminea in this kind of applications may be proven suitable and practical in the future, this clam is highly invasive species capable of severe economic impairment. Therefore, and from a precautionary point of view, preventing the invasion in uninvaded systems is critical although the wide coverage of the Asian clam distribution worldwide shortens the scope of these concerns.

This study opened perspectives for further experiments to better explore the ability of the Asian clam as a biofilter and possibly a bioremediation agent in systems affected by cyanobacterial blooms. One of such follow-ups should involve the spiking of the tested cyanobacteria suspension with highly edible green microalgae to promote filtration and ingestion rates by biasing the sensorial capability of the clam. Also important is testing with actively toxic strains of cyanobacteria or just the cyanotoxins to evaluate their interference in filtration and ingestion rates. Also relevant would be to examine the filtration response of the clam to mixtures of different cyanobacteria taxa mimicking the natural cyanobacterial bloom composition. This can be a support for further testing of actual bloom samples, which should provide a more defined view on the actual suitability of the eco-technological solution proposed.