Universidade do Minho
Escola de Ciências

Francisca Isabel Nunes Oliveira

Implementation and optimization of downstream strategies to manipulate *Microcystis aeruginosa* growth and toxicity

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Dissertação de Mestrado
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e Bioempreendedorismo em Plantas

Trabalho realizado sob orientação do
Professor Doutor Engenheiro José António Couto Teixeira
e do
Professor Doutor Rui Manuel Tavares

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Autor

Francisca Isabel Nunes Oliveira

E-mail: francisca.n.oliveira@gmail.com

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Título

Implementation and optimization of downstream strategies to manipulate *Microcystis aeruginosa* growth and toxicity

Desenvolvimento e optimização de técnicas de recolha de *Microcystis aeruginosa* com o intuito de manipular o seu crescimento e produtividade de toxina

Orientadores

Professor Doutor Engenheiro José António Couto Teixeira

(Departamento de Engenharia Biológica, Escola de Engenharia, Universidade do Minho)

Professor Doutor Rui Manuel Tavares

(Departamento de Biologia, Escola de Ciências, Universidade do Minho)

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Abstract

Microcystis aeruginosa is a microorganism that belongs to the group of cyanobacteria, which includes more than 2000 prokaryotic species. Despite being often called blue-green algae, cyanobacteria are classified as Gram negative bacteria. The microrganisms of this species possess an intracellular cyanotoxin– microcystin (MC) – that presents a great potential in therapeutic terms, both as an antimicrobial and as anticancer/antitumor agent. On the other hand, this toxin is dangerous to humans and animals, when present in high concentrations in water. These two factors, along with the high price of the cyanotoxin (above 28 000 €.mg^{-1}), make the study of *M. aeruginosa* extremely interesting, specifically when it comes to its production and removal processes. In order to contribute to a decrease in the cost of MC production, this work studied the downstream processing of *M. aeruginosa*, particularly its harvesting process, which is responsible for 20 – 30 % of the total costs at industrial scale. The focus of this work was the optimization and comparison of the harvesting efficiency of *M. aeruginosa* using flocculation induced by pH and by different flocculant agents, namely: aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$), and chitosan (Ch). The results obtained showed a harvesting efficiency (HE) above 90 % after 8 hours of treatment using pH 3 and 4 to induce flocculation of *M. aeruginosa*. Regarding the floculation induced by flocculant agents, it was possible to observe a HE of 92 % after just 4 hours using 3.75 mg.L^{-1} of $FeCl_3$. Among the methods tested, this was the highest HE and was achieved at a ZP value of -8.98 mV. The optimization performed with ZP alteration allowed not only to reach higher HEs for all flocculation types, but also to decrease the used dosage of flocculant agents. Moreover, the optimal conditions of flocculation were found for a ZP in the range of -6.7 and -20.7 mV.

Keywords: *Microcystis aeruginosa*, harvesting process, zeta potential, flocculation, flocculant agents

Resumo

A *Microcystis aeruginosa* é um microrganismo pertencente ao grupo das cianobactérias, que inclui mais de 2000 espécies procariontes. As cianobactérias, apesar de serem comummente designadas de algas verde-azuladas, são classificadas como sendo bactérias gram-negativas. Os microorganismos deste espécie produzem uma cianotoxina, a microcistina (MC), que apresenta um enorme potencial terapêutico, quer como agente anticancerígeno/antitumoral e antimicrobiano. Por outro lado, esta toxina apresenta um elevado risco para os humanos e animais quando presente em elevadas concentrações na água. Estes dois fatores, juntamente com o elevado preço desta cianotoxina (mais de 28000 € por mg), tornam o estudo da *M. aeruginosa* de extremo interesse, especialmente no que toca ao seu processo de produção e recolha. Com a finalidade de contribuir para a diminuição do custo de produção da MC, este trabalho foi realizado de forma a estudar o processo de recolha da *M. aeruginosa*, que é frequentemente responsável por 20 a 30 % dos custos totais de produção à escala industrial. Deu-se um especial enfoque à otimização e comparação da eficiência de recolha da *M. aeruginosa* para processos de floculação induzida por uma gama de pH de 2 a 12 e pela utilização de diferentes concentrações de três agentes floculantes: o cloreto de alumínio ($AlCl_3$) a 15, 7.5, 3.75 e 1.88 $mg.L^{-1}$, o cloreto de ferro ($FeCl_3$) a 30, 7.5, 3.75 e 1.88 $mg.L^{-1}$ e o quitosano (Ch) a 7.31, 2, 0.5 e 0.25 $mg.L^{-1}$. Os resultados revelaram que as maiores eficiências de recolha na floculação induzida por pH, acima de 90 %, foram obtidas após 8 horas de tratamento usando pH 3 e 4. Relativamente à floculação promovida por agentes floculantes, através da modificação do potencial zeta foi possível obter uma eficiência de 92 % após 4h utilizando 3.75 $mg.L^{-1}$ de $FeCl_3$. De entre os métodos testados, esta foi a melhor eficiência de recolha alcançada, sendo obtida para um valor de potencial zeta de -8.98 mV. A optimização dos processos, levada a cabo pela alteração do potencial zeta, permitiu verificar que as melhores eficiências foram obtidas para um intervalo de potenciais zeta entre -6.7 e -20.7 mV, e possibilitou também a utilização de menores dosagens de agentes floculantes.

Palavras-chave: *Microcystis aeruginosa,* recolha celular, potencial zeta, floculação, agentes floculantes

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List of Nomenclature

Abbreviations

CN – Charge neutralization

FL3 – Long band pass filter

FS – Forward-angle light scatter

HE – Harvesting efficiency

MC – Microcystin

OD – Optical density

OD_{750} – Optical density at 750 nm

OIVZP – Optimal interval of values for zeta potential

SF – Sweep flocculation

SS – Side-angle light scatter

WHO – World Health Organization

ZP – Zeta potential

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1. Introduction

1.1. Thesis motivation, outline and aims

Considering the threat posed by the formation of blooms by cyanobacteria populations of *M. aeruginosa* in water supplies and the potential negative impact of their intracellular cyanotoxin (microcystin, MC) in humans and animals, there is a need to perform the harvesting of cells under mild operational conditions in order to avoid the release of MC to the surrounding environment. Despite of its toxicity, MC is also a very promising product for biomedical applications, which makes this an interesting research theme especially given the high costs involved on its production. Also, it is very important that microalgal-based biofuel companies become able to find better ways to apply the process of harvesting, not only in order to preserve the quality of the primary product and of possible coproducts, but also to lower costs (Allnutt and Kessler, 2015).

Considered a step of the downstream processing which also includes cell debris removal and, in some cases, cell disruption, purification and polishing in order to ensure that the final product desired quality is obtained (Mirón *et al.*, 2003; Jungbauer, 2013), harvesting has a big impact in the final cost of biomass production. Gudin and Thepenier (1986) and Kim *et al.* (2013) referred that the harvesting of microalgae represents about 20 to 30 % of the total expenditure of the whole process.

Based in all these facts, the aim of this work was to find ways of reducing those costs through the study and optimization of *M. aeruginosa* LEGE 91094 flocculation induced both by pH and by three well-known flocculant agents: aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$) and chitosan (Ch). Particularly, the harvesting efficiency, optimal dosage, the zeta potential and membrane integrity of this cyanobacterium cells were assessed in several experiments.

This dissertation is divided into four sections:

- Introduction: Description of thesis framework and state of the art;

- Materials and methods: Methodologies used in this work are presented;
- Results and discussion: The results obtained are shown and discussed;
- Conclusions: Main conclusions of this work, as well as suggestions for future work, are presented.

1.2. State of the Art

1.2.1. Microalgae biology

Present in both marine and freshwater ecosystems, either in small colonies or individually and in a wide range of environmental conditions, microalgae are unicellular microorganisms that can be prokaryotic or eukaryotic (Kumar *et al.*, 2010). In the latter case, microalgae present organelles, while in the first (which is the case of cyanobacteria), there are no presence of flagella, nuclei, mitochondria, Golgi bodies and plastids (Lee, 2008).

These microorganisms, together with multicellular ones (such as kelps, a large brown macroalgae) constitute the group Algae. Members of this group can be both freshwater and marine organisms: the most complex subgroup of the first is a division of green algae, named Charophyta; and the latter include seaweeds, which present high complexity and large size (Ariede *et al.*, 2017).

It is estimated that more than 50 000 species of microalgae exist, even though only around 60 % of that number has been studied (Richmond, 2004). In order to classify such a broad diversity of species, several characteristics are considered, including the cell walls, the bioactive compounds, the pigments, the storage products and other cytological and morphological aspects (Tomaselli, 2004).

In terms of nutritional regimes, microalgae can also be classified as autotrophic, heterotrophic, or mixotrophic. If they are autotrophs, they produce complex organic compounds from simple substances available in their medium, using energy from light (photosynthesis) or inorganic chemical reactions (chemosynthesis) (Brennan and Owende, 2010). In the second case, they create energy out of the organic carbon they ingest or absorb from their medium, and they can also be divided in two

categories: photoheterotrophs, if they use light for energy; or chemoheterotrophs, if they oxidize organic compounds to obtain energy. If microalgae combine between autotrophy and heterotrophy, they are classified as mixotrophic (Lee, 2008).

Regarding photosynthesis, even though the mechanism used by these microorganisms is similar to that of the plants, microalgae are more efficient due to their simple structure and their ease of access to nutrients, water and CO_2 (Chisti, 2007). This, together with other promising characteristics of microalgae, contribute to their high potential in applications of many industrial sectors, as it can be seen in the next section.

1.2.1.1. Potential applications of microalgae

Microalgae have some interesting applications in a wide range of industrial sectors, and its biomass can be directly applied, or can be used to produce high-value compounds that are then used in other applications.

Due to their high photosynthetic efficiency, microalgae application to carbon sinking is very interesting, especially because they can fix CO_2 from several sources, which include industrial exhaust gases, the atmosphere or soluble carbonate salts (Brennan and Owende, 2010). This fixation is performed through the transfer of CO_2 from the air to their aquatic environment and is considered a basic method of carbon sinking. This application has big potential, since it can be used through the coupling of microalgae production systems with industrial facilities, diminishing pollution and increasing their growth, so they can be applied to other industries. In fact, Sobczuk *et al*. (2000) has proved that carbon counts for 40 % to 50 % of microalgae biomass, meaning that to produce 1.0 kg of microalgae, 1.5 to 2.5 kg of CO_2 is needed, which means that to associate polluting industrial facilities with microalgae cultivation can be benefic, as some studies proven (Lee *et al*., 2000; Doucha *et al*., 2005).

Another very promising application of microalgae is biofuel production, due to their ability to produce considerable amounts of biomass and oils that are suitable to be converted in biodiesel, bioethanol, biohydrogen and biogas. Besides,

microalgae present characteristics that make them more attractable to be used in biofuel production when in comparison to plant-based biofuel crops: they grow at a faster pace and present higher photosynthetic efficiency (Chiu *et al*., 2009; Dragone *et al*., 2011); there's no need of pesticides or herbicides in their production; they can grow in non-potable water; there's no need to use arable land, which means that microalgae do not compete with food or agricultural products; and their production is not affected by weather conditions (Benemann and Oswald, 1996; Brennan and Owende, 2010; Rajvanshi and Sharma, 2012).

The application of microalgae to biofuel has been used in a more significative way recently, due to the growing concern over the fossil fuels' price and climate changes (Chisti, 2007).

Another application of microalgae is their use as aquaculture feed for mollusks, shrimps and fish, due to their nutritional value (Muller, 2000; Sandnes *et al*., 2006).

Moreover, some biomolecules produced by microalgae are used for medical, nutritional or cosmetically applications (Rosenberg *et al*., 2008). Specifically, the production of polysaccharides, vitamins, pigments (like phycobiliproteins or carotenoids), β-carotene and polyunsaturated fatty acids are suitable for other application of microalgae (Abalde *et al*., 1991; Molina-Grima *et al*., 1994; Christaki *et al*., 2011). Besides, some of the biologically active compounds produced by microalgae presented anti-inflammatory, antioxidant, antifungal, antibacterial, anticancer and antiviral properties (Burja *et al.*, 2001; Singh *et al.*, 2005). Despite their potential, the study of their production control and their secondary metabolites is still under development.

1.2.2. Cyanobacteria

Formerly classified as algae and displaying common properties with microalgae (for example, in their applications), cyanobacteria are often referred to as blue-green algae (Wilmotte, 2004), but developments in molecular phylogeny studies demonstrated that they should be classified as bacteria.

These microorganisms played an important role in the emergence of aerobic environment, as well as in supporting the evolution of photosynthetic eukaryotes and they even contributed to the development of various oxygen-dependent organisms (Ramanan *et al.*, 2016).

Cyanobacteria are prokaryotic organisms that have a granular cytoplasm presenting several ribosomes. In contrast to green algae, their cell wall is not composed of cellulose: it is constituted by lipopolysaccharide and peptidoglycan. (Skulberg *et al*., 1993).

Present in a wide range of environmental conditions, but essentially in damp soils and water, this group of bacteria fix nitrogen, perform aerobic photosynthesis in their thylakoids (internal membranes) and possess two photosystems, types I and II (Liberton *et al*., 2013).

Morphologically they can be unicellular or filamentous and they do not possess flagella. Cyanobacteria include colonial species that form hollow spheres, sheets or filaments, and can be also found in symbiosis with lichen-forming fungi or plants (Whitton, 2012). Due to their diversity, some filamentous species possess cells that can be divided in three types: vegetative, akinetes or heterocysts. The first case corresponds to the most common photosynthetic cells, which are formed under propitious environments. Akinetes are spores that can form to resist severe climate conditions. The heterocysts are formed in anaerobic conditions or when nitrogen fixation is limited. This specialized type of cell presents a thick wall and are able to assimilate nitrogen into nitrates, nitrites or ammonia through the nitrogenase enzyme (Fay, 1992; Golden and Yoon, 1998).

As cyanobacteria do not possess flagella, they present several other characteristics that allow them to perform locomotion. For example, some are able to float in water columns due to their capability of forming of gas vesicles (Walsby, 1994). In other cases, they come together to form filaments called hormogonia, which are capable of waving back and forward, and moving along surfaces, away from the main biomass (Khayatan *et al*., 2017).

1.2.2.1. Potential applications of cyanobacteria

Over the past years, a wide variety of secondary metabolites produced by cyanobacteria like *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* have been studied, revealing several properties and potential to be applied in biotechnological industry (Hayashi *et al.*, 1994), just like microalgae. Many of these substances (of peptidic nature) are produced at the ribosomes, while others are supported by the complex gene clusters that may vary from species to species (Leão *et al*., 2012). These metabolites have also been assessed from the point of view of their importance for cyanobacteria as predation inhibitors, allelopathic, signal and helper in competition (Haque *et al*., 2017).

Compared with other marine organisms and other invertebrates, cyanobacteria have the advantage of being easily isolated and cultivated, avoiding an excessive collection of organisms in their natural environment and thus making these studies ecologically sustainable (Leão *et al*., 2012). Despite these advantages, a lot of research in the area of secondary metabolites production (namely which culture parameters need manipulation) is still needed, in order to enable cyanobacteria's full potential in a wide range of applications (Burja *et al.*, 2001; 2002).

Wackett (2011) argues that due to environmental and economic concerns, society is continuously seeking new alternatives to fossil fuels. Like in the case of microalgae, cyanobacteria can play an important role in reducing the costs of biofuel production, because their cultivation is considered low-cost and simple: they grow simply with air (nitrogen or carbon dioxide), water, light and specifically with phosphorous-containing salts (Berman-Frank *et al.,* 2003; Pepe-Ranney *et al.,* 2015). Cyanobacteria high rate of growth and elevated lipids' content, which occurs in the stationary phase of growth and under nutrient scarcity among other stress conditions, also contributes for reducing the costs associated with biofuel production (Hannon *et al*., 2010).

Moreover, secondary metabolites of cyanobacteria are also advantageous to the environment since they can fix carbon dioxide to use in the production of alcohols, alkanes, alkenes, terpenes or esters (Wackett, 2011; Connor and Atsumi, 2010; Haque *et al*., 2017).

Another very interesting application of cyanobacteria is in bio-H_2 production. Hydrogen usage is very promising for implementing important changes in the transportation sector and its demand is said to increase in the future (Balat, 2008). Despite being able to be produced in several ways, H_2 production is usually based in non-renewable sources (Madamwar *et al*., 2000). Here, cyanobacteria can play an important role, since they're able to produce hydrogen by hydrogenase in anaerobic and dark environments or by nitrogenase in the presence of light (Rao and Hall, 1996; Hansel and Lindblad, 1998).

Cyanobacteria can also increase the levels of oxygen in water and remove nitrogen and phosphorus at the same time. As result, they are largely used for effluent treatment (Sharma *et al.,* 2011; Haque *et al.,* 2017). Besides and regarding other environmental problems, several cyanobacterial allelopathic substances demonstrated great potential to be implemented to control toxic blooms (Leão *et al*., 2012).

Cyanobacteria are being studied for applications in pharmaceuticals as well, namely some of these organisms have displayed antibiotic, antiviral, antifungal, antiprotozoal anticancer and anti-inflammatory properties (Burja *et al.*, 2001; Singh *et al.*, 2005). These characteristics are due to cyanobacteria toxic activity and are possibly linked to defence strategies (Burja *et al.*, 2001).

1.2.2.2. Negative impacts of cyanobacteria

Like it was previously referred, an increased effort to study the secondary compounds that cyanobacteria produce has been made. This is also important considering the negative impacts that cyanobacteria may present, which can be severe in some cases since they can lead to water impairment, by bioaccumulation at higher trophic levels through biotic (for example, herbivorous fish and zooplankton) disturbance (Merel *et al*., 2013).

Moreover, cyanobacteria produce toxins that can cause dangerous effects in humans, namely on the skin (dermatotoxins), on the nervous system (neurotoxins), and in the organs like the liver (hepatotoxins) (Bajpai, *et al.,* 2011). The latter (for

example, microcystins and nodularins) are mainly produced by the *Anabaena, Microcystis, Nodullaria, Nostoc, Oscillatoria*, and *Cylindrospermopsis* genera and are responsible for the most common intoxications due to cyanobacteria, presenting themselves as destructive to the liver (Burja *et al.*, 2001).

1.2.3. Features of *M. aeruginosa*

M. aeruginosa is the unicellular cyanobacterium that is most commonly found in freshwater eutrophic environments and presents a colonial behavior. It belongs to the *Microcystis* genus (Guiry and Guiry, 2018), which contains more than 20 species and varieties. Table 1.1 represents the taxonomy of *M. aeruginosa*.

Table 1.1 Taxonomy of *M. aeruginosa* species.

Taxonomy	
Empire	*Prokaryota*
Kingdom	*Eubacteria*
Subkingdom	*Negibacteria*
Phylum	*Cyanobacteria*
Class	*Cyanophyceae*
Subclass	*Oscillatoriophycidae*
Order	*Chroococcales*
Family	*Microcystaceae*
Genus	*Microcystis*

M. aeruginosa occurs predominantly in ponds, reservoirs, lakes, and slowly flowing rivers (Edwards and Lawton, 2009). In spite of being prokaryotic and aerobic, *M. aeruginosa* has a great tolerance to anaerobic and obscure conditions in eutrophic lakes during some periods of time (Shi *et al*., 2007).

The cells have ovoid or circular shape and their diameter may vary from 2.61 μm to 5.40 μm (Kim *et al*., 1997). They also present gas vesicles (vacuoles) that give them buoyancy ability (Walsby, 1994; Shi *et al*., 2007). Figure 1.1 shows a microscope image of the species.

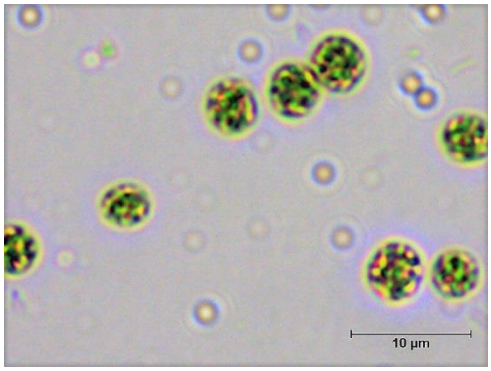

Figure 1.1 Microscopic view (60x) of the individual cells of *M. aeruginosa*.

Generally, this cyanobacterium is able to respond to several fluctuations in their environment since they can move and look for positions with higher irradiance or nutrient concentration in a water column and inside the blooms, by adjusting their buoyancy. This capability is regulated by the production of carbohydrates (through photosynthesis), which can be interrupted if the carbon dioxide levels drop abruptly (Potts and Whitton, 2000; Reynolds, 2006; Paerl *et al*., 2016). They remain viable through adverse periods of lack of nutrients due to several physiological adaptations such as fixing both gaseous carbon dioxide and dissolved inorganic carbon (Paerl and Millie, 1996), iron chelation (Wilhelm and Trick, 1994) and storing important nutrients like nitrogen and phosphorous (Healy, 1982; Reynolds, 2006; Paerl *et al*., 2016).

1.2.3.1. Potential applications of *M. aeruginosa*

Like other cyanobacteria, *M. aeruginosa* presents numerous medicinal and biocidal applications, and it can also be used in biofuel synthesis. For example, two bioactive compounds produced by *M. aeruginosa,* siatoxin and microviridin Toxin BE-4, have shown antibiotic and antitumoral functions, as reported by Arment and Carmichael (1996) and Vijayakumar and Menakha (2015).

Another secondary compound called kasumigamide, also produced by *M. aeruginosa*, was found to have antialgal properties against *Chlamydomonas neglecta* (Ishida *et al*., 2000; Berry *et al.,* 2008). Also, Rós *et al*. (2012) demonstrated that

lipids formed by this cyanobacterium can be utilized as a source for biodiesel production.

On the other hand, *M. aeruginosa* can form several different peptides, some of them presenting high toxicity, namely neurotoxins and hepatotoxins (Carmichael, 1986). In the next section MC, the most common toxin produced by this cyanobacterium, will be addressed.

1.2.4. Microcystin

M. aeruginosa is responsible for forming a peptide toxin called Microcystin-LR. The release of MC starts at the late exponential growth phase of *M. aeruginosa* culture and increases during the stationary phase (Watanabe and Oishi, 1985). This release is only possible after cellular lysis (Edwards and Lawton, 2009), and it may allow the entrance of this toxin into the food chain, through birds or fish, or the contact with humans via intoxicated water (Harada *et al*., 1996).

This toxin is known for resisting chemical oxidation and hydrolysis, and presents a very stable behaviour in water, being able to reach an half-life of 10 weeks in normal environments (WHO, 2003).

To protect the consumers from the effects of cyanotoxins, the World Health Organization (WHO) has set a provisional upper limit (intercellular and extracellular) of 1 $mg.L^{-1}$ of MC-LR in water for human consumption. Also, WHO has established a Tolerable Daily Intake by body weight of 0.04 $mg.kg^{-1}$ (WHO, 1998; WHO, 2003). The negative impacts of cyanobacteria, and specifically of cyanotoxins, on the quality of drinking water were also responsible for the inclusion of MC on the list of contaminants of the United States Environmental Protection Agency (Richardson and Ternes, 2005; Bajpai *et al.*, 2011). To ensure the cyanotoxins removal from drinking water supplies, greater importance has been given to the development of high efficient water treatment strategies (Edwards and Lawton, 2009).

The exposure to hepatotoxins like MC may lead to death after several hours or days. The worst event involving human deaths occurred in Brazil, in 1996, when 60

people died after a hemodialysis process where water from a reservoir contaminated with MC was used (Jochimsen *et al*., 1998; Bajpai *et al*., 2011). In addition, this cyanotoxin was also present in a water reservoir for human consumption in Amazonia (Vieira *et al.,* 2005; Bajpai, *et al.,* 2011).

1.2.4.1. Potential applications of MC

Like it was previously referred for other cyanotoxins, the MC extracted from *M. aeruginosa* also presents biocidal activity, specifically it can act as an herbicidal, larvicidal or algicidal (Nizan *et al.*, 1986; Ikawa *et al*., 1996; Jang *et al.*, 2007; Berry *et al.*, 2008).

Other studies like Braithwaite *et al.* (2012) and Zanchett and Oliveira-Filho (2013), also pointed MC as a very promising anticancer drug and as treatment of Parkinson's disease symptoms.

1.2.5. Influence of environmental factors on growth of *M. aeruginosa* and its toxin production

Due to certain factors such as human activity, climate change and high temperatures, the occurrence of *M. aeruginosa* blooms have been increasing worldwide. Because of the presence of cyanotoxins, some of the blooms are toxic to humans, and may put the public health at risk, if they occur in drinking water supplies and recreational areas (Edwards and Lawton, 2009; Merel *et al*., 2013; Geada *et al*., 2017).

The understanding of the environmental factors that influence *M. aeruginosa* is of utmost importance, not only to control or avoid these harmful blooms, but also to use these cyanobacteria in the potential applications mentioned before in this work. Several abiotic factors such as the age of the cultures, salinity, pH, and nutrients concentration have a strong impact on *M. aeruginosa* growth and on cyanotoxins' production. Freshwater environments are the most favorable ones, because this species grows better in waters with pH between 6 and 9, temperatures between 15

°C and 32 °C and high concentration of nutrients, namely nitrogen and phosphorus (Edwards and Lawton, 2009).

Van der Westhuizen and Eloff (1985) showed that temperature plays an important role in the growth of *M. aeruginosa* and that it also influences its toxicity. In this study, several experiments were performed, and it was concluded that there was no correlation between toxicity and growth rate, because high temperatures (32 ˚C) produced high growth rates but lowered the toxin production (which peaked at 20 ˚C).

Geada *et al.* (2017) is one of the few studies that compared the combined effect of temperature, pH, light intensity and CO_2 concentration in growth rates of *M. aeruginosa* and MC production and concluded that this cyanobacterium produced more MC under conditions that were different from those more favorable to *M. aeruginosa*'s growth.

1.2.6. Cyanobacteria's harvesting

As mentioned before, harvesting plays a key role in the potential application of cyanobacteria in several biotechnological areas (cf. section 1.2.2.1). Consequently, Allnutt and Kessler (2015) referred that it is of utmost importance to find more cost-effective ways for collecting biomass.

Despite of its relevance, the process of harvesting still needs further development aiming at the decrease of its costs and of the amount of energy used. These costs and energy expenditure are related to the difficulty of harvesting cyanobacteria cells, because of their intrinsic properties: they are small (smaller than 10 μm), possess a surface that is negatively charged, and have typically low densities (0.5 $kg.m^{-3}$) that are close to water (Molina-Grima *et al*., 2003; Kim *et al.*, 2013).

The harvesting process can involve two different phases. The first one depends on the technique used and on the initial biomass parameters, and consists of thickening the biomass (to obtain a solid mass of 2-7 %) in a way that makes possible their separation from the medium. The second phase is responsible for

dewatering in order to concentrate the slurry, which requires more energy than thickening (Barros *et al.*, 2015; Tan *et al.*, 2017), and can be preceded by it (Molina-Grima *et al.*, 2003).

1.2.7. Harvesting techniques

The decision between which harvesting technique to utilize is often defined by both the end use and the cyanobacteria characteristics (Molina-Grima *et al.*, 2003; Brennan and Owende, 2010; Chen *et al.*, 2011).

Among all the techniques, the most used are the following: flocculation, autoflocculation, bioflocculation, flotation, gravity sedimentation and electrical-based treatments for thickening; and filtration or centrifugation for dewatering step (Brennan and Owende, 2010; Barros *et al.*, 2015). A summary of these methods can be seen in Figure 1.2.

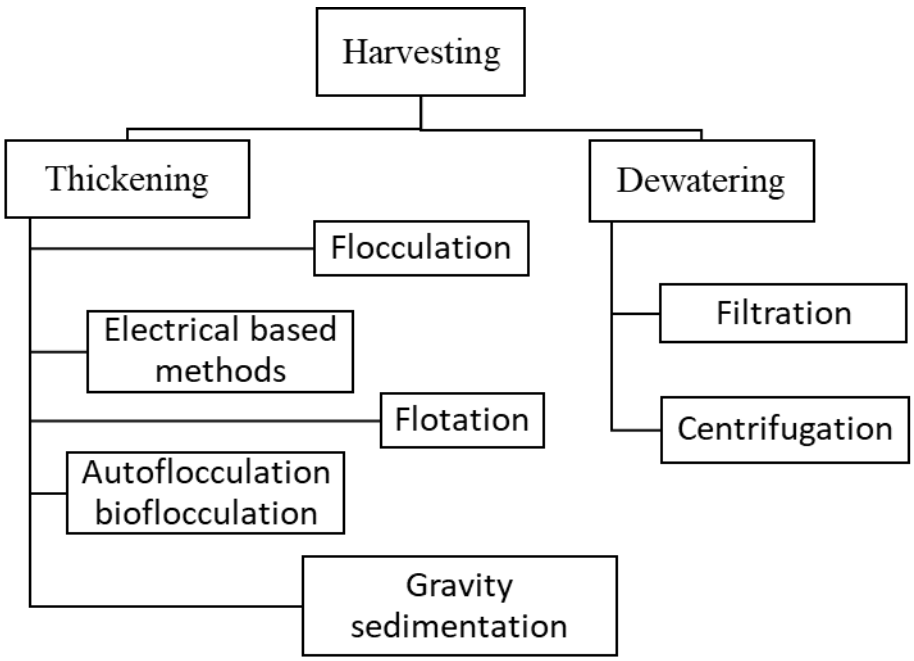

Figure 1.2 Schematic representation of several harvesting techniques.

It is important to pay attention to relevant points regarding harvesting techniques, such as the fact that cyanobacteria should not face contamination during the process, and it must not be toxic for them at any point. Furthermore, it would

also be positive to recycle the medium of the culture (Uduman *et al.*, 2010) after the harvesting step, and the synergy between techniques should be studied and took into consideration (Kim *et al*., 2013).

Moreover, the cell membrane integrity should be considered, because, in the case of *M. aeruginosa*, it keeps MC (an important end-product) intact inside the cells, which is not respected by some of the harvesting methods applied to the treatment of water with cyanobacteria (Sun *et al.*, 2012; Pei *et al.*, 2014).

The next sections describe in detail the different harvesting techniques available. The advantages and disadvantages of each of those methods are also presented and are often based on the time consumed and energy needed, since these are key factors in industrial processes.

1.2.7.1. Flocculation

This harvesting technique induces the formation of flocs of cells either through pH modification or by employing chemical agents. In the case of chemical agents, these can be organic, inorganic or a mix of both categories (Chen *et al.*, 2011).

Flocculation can be applied alone or together with centrifugation and it is considered one of the most promising and practical harvesting techniques (Hagström *et al.*, 2010).

In fact, Barros *et al.* (2015) and Uduman *et al.* (2010) refer that flocculation is simple, fast and achievable at a low energy expenditure.

1.2.7.1.1. pH-induced flocculation

The flocculation induced by pH is accomplished by changing the pH of the medium to values that lead to the neutralization of the negative surface charges of the cells, consequently resulting in cell aggregation (Molina-Grima *et al.*, 2003; Hadjoudja *et al.*, 2010).

The advantages of this method are the possibility of recycling the medium after the harvesting method, its low cost (due to the low energy needed and the fact that a flocculant agent is not required), and its non-toxicity to the cells (Horiuchi *et al.*, 2003).

A study focused on the harvesting of *Phaeodactylum tricornutum* by adjusting the pH to acidic-alkaline values using chloride acid showed that, for values below 9.12, no positive results were achieved. This result was justified by the disintegration of chloroplasts at low pH. In contrast, the increase of pH, by the addition of sodium hydroxide, revealed an efficiency of 98 % of cells removal at pH 11 (Şirin *et al.*, 2012).

Blanchemain and Grizeau (1999) tested the harvesting of *Skeletonema costatum* by pH induction and realized that a decrease of pH to 8 led to continuous suspension of the cells. However, with the addition of sodium hydroxide, the cells agglomerated at pH 10.2.

A pH decrease to 4.5 was performed in a mixed cell culture medium where the cells of *Chlorococcum nivale*, *Chlorococcum ellipsoideum* and *Scenedesmus sp.* aggregated and settled (>90 %). However, the cells of *Chlorella zofingiensis* and *Chlorella vulgaris* did not show the same behaviour (just 22 and 9 %, respectively, have agglomerated) (Liu *et al.*, 2014).

Besson and Guiraud (2013) were able to achieve a 90 % recovery efficiency of *Dunaliella salina* increasing the pH to 10 with the addition of sodium chloride. The same procedure was applied on *Chlorella vulgaris*, *Scenedesmus* sp., and *Chlorococcum* sp. and similar results were also obtained by Wang *et al.* (2013).

A work using *Desmodesmus communis* has obtained agglomeration through the variation of pH. With an initial pH of 7, more than 95 % of efficiency was achieved at pH 3, 4, 5, 6, 11, and 12 after 100 min (Pezzolesi *et al.*, 2015). In the case of *Skeletonema costatum* and *Chaetoceros gracilis*, a complete harvesting of biomass was obtained at pH 11 and 12 and a maximum of 60 % was achieved for pH between 2 and 6 (Pérez *et al.*, 2017).

1.2.7.1.2. Flocculant-induced flocculation

The negatively charged surface of cyanobacteria plays a crucial role in the flocculation process since it causes repulsion between the cells, which makes them to remain in suspension in the medium (Uduman *et al.*, 2010; Chen *et al.*, 2011). For that reason, in order to achieve the formation of flocs, the addition of a chemical coagulant (for example, multivalent cations or cationic polymers) is needed. These agents will be able to change the charge of the surface and, consequently, stop or reduce the repulsion and promote cell aggregation (Brennan and Owende, 2010).

Two types of flocculation can be induced by flocculants, highly influenced by the pH and the coagulant dosage: charge neutralization (CN) and sweep flocculation (SF). The first one is triggered by the minimization of the repulsion between particles due to the presence of cationic hydrolyzed products at relatively acidic pH values or typically low coagulant concentrations. SF, on the other hand, occurs when the pH is neutral or alkaline and the flocculant agent is added in higher dosages (Johnson and Amirtharajah, 1983). This results in the formation of metal coagulant precipitates (for example, $Al(OH)_3$ or $Fe(OH)_3$) which are heavy, sticky and larger than cells, sweeping them down to the bottom (Duan and Gregory, 2003).

The flocculant agents can be of two types: organic or inorganic. Chitosan (Ch) is the most widely used coagulant of the first type (Ahmad *et al.*, 2011; Lama *et al.*, 2016). Regarding inorganic agents, ferric (for example, $FeCl_3$ and $FeSO_4$) and aluminum coagulants are some of the most widely used to induce flocculation (Salama *et al.*, 2016; Abomohra *et al.*, 2018).

The success of the flocculation process depends on numerous aspects related to the agent itself, to the medium, and to the species utilized. Considering the flocculant itself, its concentration, charge density, molecular weight, and solubility play an important role on the efficiency of the process. In terms of the medium, the ionic strength and its pH are also relevant. For instance, in terms of the medium salinity, it was shown by Bilanovic *et al.* (1988) that, if its value was high, the interaction between the microorganism and the flocculant agents could be compromised. Also, Papazi *et al.* (2010) reported that higher flocculation rates were achieved if the flocculant was more electronegative. Besides this, the initial biomass concentration

also affects the performance of the flocculation (Molina-Grima *et al.*, 2003; Papazi *et al.*, 2010; Uduman *et al.*, 2010; Rashid *et al.*, 2013).

The flocculation induced by flocculant agents is a highly scalable process that can be applied a wide variety of species and large volumes.

On the other hand, flocculation also presents disadvantages, which can be related to the medium, the flocculants and the process itself (Chen *et al.*, 2011; Barros *et al.*, 2015). Regarding the first aspect, flocculation induced by inorganic agents contaminates the medium, which poses some limitations to its recycling. Besides, this harvesting method also produces large volumes of sludge since a high concentration of inorganic coagulant is needed to separate the cells from the medium. In terms of the flocculant agents, as well as the high dosage referred previously, their specificity regarding the species is also a disadvantage. Furthermore, the fact that the process is pH sensitive can also present difficulties. Lastly, the price of the flocculants can result in higher costs and they can also be toxic to the cells.

When thinking about scalability of the process, the cost plays an important role. In terms of economic analysis, it is important to note that, despite of the high costs related to the flocculant agents, chemical-induced flocculation is one of the cheapest harvesting methods. Specifically, inorganic flocculants present the best solution, even though the ferric coagulants change the color of the cells to brown (Barros *et al.*, 2015). Aluminum salts also keep the costs low but can cause cell lysis (Papazi *et al.*, 2010). Farooq *et al*. (2015) performed a comparison between $FeCl_3$ and alum, to verify that $FeCl_3$ was the best choice for *Chlorella vulgaris* removal, obtaining approximately 100 % of HE with 0.75 $g.mL^{-1}$ of this flocculant.

Ch presents a more expensive solution, though it has important advantages because it allows the reutilization of the medium, mainly due to its biodegradable and non-toxic characteristics (Schlesinger *et al.*, 2012). For instance, applying a concentration of 30 $mg.L^{-1}$ of Ch, Rashid *et al.* (2013) obtained a harvesting efficiency of 95 % on *Chlorella vulgaris* cultures. It was also proven that the coagulation was provoked by charge neutralization (Rashid *et al.* 2013). Ch's good performance was also demonstrated by Xu *et al.* (2013): a dosage of 10 mg per gram

of algal biomass resulted in 99 % of harvesting efficiency of *Chlorella sorokiniana* at pH 6.

Regarding aluminum-based flocculants, Şirin *et al.* (2012) verified that, using aluminum sulphate or polyaluminum chloride (PACl), it was possible to achieve a harvesting efficiency in the range of 60 to 80 % for *Phaeodactylum tricornutum*. Also, Papazi *et al.* (2010) used twelve different salts on the harvesting of *Chlorella minutissima* to prove that the most efficient were aluminum, ferric and zinc salts, and that the first one achieved the best results. The concentrations used in this study were 0.75 $g.L^{-1}$ of the sulphate and 0.5 $g.L^{-1}$ of the chloride salts.

1.2.7.1.3. Autoflocculation and bioflocculation

Autoflocculation can occur when the consumption of carbon dioxide forms inorganic precipitates, which have direct effects in the medium. These effects include the supersaturation of the medium with calcium and phosphate that cause the elevation of pH and consequent cellular agglomeration and flocculation (Uduman *et al.*, 2010).

The bioflocculation process is similar to autoflocculation but the agglomeration and flocculation of the cells is due to a bioflocculant (extracellular polymeric substances) produced by the microorganism (Barros *et al.*, 2015; Kim *et al.*, 2013). To study the process of *Chlorella vulgaris* bioflocculation by the selection of several bioflocculants, several microalgae were tested by Oh *et al.* (2001). They demonstrated that an increase in pH from 5 to 11 was due to the presence of a bioflocculant produced by *Paenibacillus* sp. AM49. In this test, an efficiency of 83 % was achieved, being this value higher than those obtained with the application of polyacrylamide (78 %) or aluminum sulfate (72 %).

These methods are low cost, low energy, do not need any flocculant, which allow the reuse of the medium, and are non-toxic to microalgae (Horiuchi *et al.*, 2003).

1.2.7.2. Flotation

Another harvesting method that can be used to remove the biomass is flotation. This process allows the accumulation of cells on the surface of the medium through the use of small air bubbles that lift up the microorganisms (Tan *et al.*, 2017).

The success of this technique depends on the size of the cells and a limit of 500 μm was recommended by Matis *et al.* (1993). The smaller the cells, the higher the efficiency of the process.

This methodology can be employed according to the following options: dissolved air flotation, if the bubbles are sized between 10 and 100 μm; and dispersed air flotation, if the bubbles' size ranges from 100 to 1000 μm (Tan *et al.*, 2017).

Works using dispersed air flotation in *Chlorella* sp. cultures presented a recovery of 90 % and the best pH values for cells harvesting were 4 and 5 (Liu *et al.*, 1999).

The advantages of this technique are the requirement of low space for operation and the short operation times, in spite of the use of coagulants (Rubio and Smith, 2002; Hanotu *et al.*, 2012).

Despite of the great potential exhibited, flotation still needs further improvement in order to make it more cost-effective and efficient, especially at an industrial scale. Additionally, due to the different characteristics microalgae and cyanobacteria species present, nowadays it is still not possible to obtain a generalized process to apply on a wide spectrum of species. Given this, the need for more research regarding the impact of these factors on flotation efficiency gains relevance (Zhang and Hu, 2012).

1.2.7.3. Filtration

In the filtration technique, the medium is forced to pass through a membrane so that the cells are retained (Barros *et al.*, 2015). Both pressure and vacuum can be used to promote the flow of the medium (Molina-Grima *et al.*, 2003).

The method of filtration is more efficient if applied to the separation of microorganisms sized above 70 μm (such as *Coelastrum* and *Spirulina*) than in the case of cells that are smaller than 30 μm, like *Chlorella* or *Scenedesmus* (Brennan and Owende, 2010; De Godos *et al.*, 2011). In the latter case, other methods such as membrane microfiltration and ultra-filtration are generally used, although these processes are expensive because of the frequent substitution of the membranes (Quirk and Woodrow, 1983).

The filtration process applied on the separation of *Coelastrum proboscideum*, was performed using both a vacuum filter and pressure filter, in a continuous and discontinuous way. The best results were achieved by the utilization of a discontinuous filtering process (Molina-Grima *et al.*, 2003).

The main advantages of filtration are the absence of chemicals in the process as well as its simplicity and high separation rates (Kim *et al.*, 2013). On the other hand, the main disadvantages are the following: the membrane needs to be constantly cleaned because the membrane pores become clogged; generally, the process is not used for large scale applications; and the pumping costs are relevant (Barros *et al.*, 2015; Molina-Grima *et al.*, 2003).

1.2.7.4. Centrifugation

By the application of centrifugal force, centrifugation separates the cells from the medium. This process is more propitious to be applied to large microalgae (above 70 μm) (Muñoz and Guieysse, 2006) and depends on several conditions such as the settling characteristics of the cells, the settling depth and their residence time during centrifugation (Molina-Grima *et al.*, 2003). Another parameter that influences the outcome of centrifugation is the species used, as Heasman *et al.* (2000) proved after achieving an efficiency of 95 % with 13 000 ×*g*.

Along with being fast, this method has several other advantages: it can be applied to treat large volumes; under the best conditions, it reaches an efficiency of 95 %; and no chemicals are needed (Molina-Grima *et al.*, 2003; Tan *et al.*, 2017). The main disadvantages are the high energy and maintenance costs when it is applied at large

scale (Bosma *et al.*, 2003). However, according to Dassey and Theegala (2013), the cost of treating a large volume of culture could be reduced by increasing the flow rates (bigger than 1 $L.min^{-1}$), even though the efficiency of this experiment decreased to values below 90 %.

1.2.7.5. Gravity sedimentation

Gravity sedimentation is a method based on the density and size of the cells. Cells are deposited on the bottom of the vessel or tank through the action of gravity.

The process is simple and more efficient with cultures containing high density and larger cells (Brennan and Owende, 2010). It can be used to separate biomass from their medium at low energy costs, which is interesting for low value end products (Rawat *et al.*, 2011). However, the process is not reliable and because of this the process is generally used with lamella separators and sedimentation tanks (Uduman *et al.*, 2010).

1.2.7.6. Electrical-based methods

Taking advantage of the negatively charged cyanobacteria cells and the electrodes that create an electrical field, electrical-based methods can form precipitates at the surface of the electrodes (electrophoresis) or at the bottom of the container (electro-flocculation) (Barros *et al.*, 2015).

This method can be used together with coagulation, in a process called electro-coagulation, which is based on the formation of the coagulant through electrolytic oxidation (Tan *et al.*, 2017). There's another example of the combined use of electric fields together with other harvesting method: the electro-flotation. This technology is based on bubbles that are created through water electrolysis and lead to cells displacement to the surface of the medium (Gao *et al.*, 2010).

The efficiency of these methods depends on several factors: pH; temperature; time; and the type of electrode, being the ones made of iron less efficient than those

made of aluminium (studies point to an efficiency of 78.9 % versus 100 %, respectively) (Barros *et al.*, 2015).

Regarding a comparison to other flocculation-inducing methods, electrical-based processes can be expensive in large scale applications. This disadvantage is directly related to the need of equipment and the cost of the energy used in the process. On the other hand, there's no need to apply chemicals during this process, which can be advantageous (Christenson and Sims, 2011).

1.2.7.7. Comparison of harvesting techniques

As it has been mentioned before, the harvesting process is responsible for 20 to 30 % of the total cost of the biomass production process. These costs are only acceptable for high value end products. Because of that, it is of utmost importance to find ways to significantly reduce the costs of the process to increase profit margins (Wijffels and Barbosa, 2010).

This necessity has promoted the appearance of several studies that aimed to optimize the process of harvesting through several techniques. Table 1.2 gathers information about the studies that were applied to *M. aeruginosa*.

Table 1.2 Several harvesting techniques applied to *M. aeruginosa* species.

Species	Technique	Flocculant Dosage	Initial biomass concentration (cell.mL^{-1})	Parameters	Results	Flocculant Costs	References
***M. aeruginosa* (MIC309)**	pH-induced flocculation	—	3.0x10^5	pH 3, 4, 5, 7, e **11*** ASM-1 medium	78 % (Cell viability)	—	Qian *et al.* (2014)
***M. aeruginosa* (FACHB-905)**	Flocculation induced by Ch and response surface method (RSM)	5.05, 6.34, 6.82, **7.31***, 7.4, 8.39, 8.63, and 9.26 mg.L^{-1}	2x10^6	BG11 medium Several values of agitation speed and time tested	99 % (HE)	—	Pei *et al.* (2014)
M. aeruginosa	Flocculation induced by Ch-modified soil	1, **2***, 3, 4, and 5 mg.L^{-1} Ch added to 10 mg.L^{-1} soil	~3.48x10^6	pH 8 BG11 medium	90 % (HE)	—	Li *et al.* (2015)
***M. aeruginosa* (FACHB-469)**	Flocculation induced by CFA (coal fly ash) combined with chitosan	0.5, 1.0, 1.5, 2.0, 2.5, **3.0***, **3.5***, **5.0***, and 6.0 mg.L^{-1}	4.15 - 4.23×10^6	pH 8 BG11 medium CFAL / Ch ratio (mL:g): **12:1***; 20:1; 40:1	98.2 ± 1.5 % (HE)	0.07 US $.m^{-3}	Yuan *et al.* (2016)
	Ch-induced flocculation	0.5, 1.0, 1.5, 2.0, 2.5, **3.0***, 3.5, 5.0, and 6.0 mg.L^{-1}		pH 8 BG11 medium	81.6 ± 1.9 % (HE)	10 US $.kg^{-1}	
***M. aeruginosa* (FACHB-905)**	Flocculation induced by $AlCl_3$	0, 5, 10, **15***, 20, 25, and 30 mg.L^{-1}	~2x10^6	BG11 medium	100 % (HE)	—	Sun *et al.* (2012)

* Optimal conditions found by the authors; HE – Harvesting efficiency.

Table 1.2 (Continued)

Species	Technique	Flocculant Dosage	Initial biomass concentration (cell.mL^{-1})	Parameters	Results	Flocculant Costs	References
***M. aeruginosa* (FACHB-905)**	Flocculation induced by polyaluminum chloride (PACl)	1, 2, 3, **4***, and 5 mg.L^{-1}	~10^{6}	pH 8.4 BG11 medium	94.3 % (HE)	0.23 US $.m^{-3}	Sun *et al.* (2013)
***M. aeruginosa* (FACHB-905)**	Flocculation induced by $AlCl_3.6H_2O$ pre-treated with NaClO	2.0, 2.4, 2.7, 3.4, and **4.1***mg.L^{-1}Al; 0, 1, 2, 3, and **4***mg.L^{-1} NaClO	2.2x10^{6}	pH 8.4 BG11 medium	~ 99 % (HE)	—	Ma *et al.* (2012)
***M. aeruginosa* (FACHB-905)**	Flocculation induced by $Al_2(SO_4)_3$ pre-treated with $KMnO_4$-Fe(II)	**$KMNO_4$**: 0, 5, 10, 15, **20***µM **(=3.16 mg.L^{-1})**; **Fe(II)**: 0, 15, 30, 45, **60***µM; **$Al_2(SO_4)_3$**: **20*** - 50 µM	1.0x10^{6}	pH 7.9 BG11 medium Several values of agitation speed and time tested	91 % (HE)	—	Qi *et al.* (2016)
	Flocculation induced by $Al_2(SO_4)_3$	50 µM			75 % (HE)	—	
M. aeruginosa	Flocculation induced by $FeCl_3$	15 and **30*** mg.L^{-1}	3.0x10^{5}	ASM-1 medium pH 8, 8.1, **8.2***, 9, and 9.1	74 % (HE)	—	Chow *et al.* (1998)
***M. aeruginosa* (FACHB-905)**	Flocculation induced by cationic starch	5, **10***, and 80 mg.L^{-1}	3.15 - 3.25X10^{6}	ΔpH= 0.5 - 11.8 pH **8.60 ± 0.1*** BG11 medium	86 % (HE)	1-3 US $.kg^{-1}	Shi *et al.* (2015)

* Optimal conditions found by the authors; HE – Harvesting efficiency.

Table 1.2 (Continued)

Species	Technique	Flocculant Dosage	Initial biomass concentration (cell.mL^{-1})	Parameters	Results	Flocculant Costs	References
M. aeruginosa	Bioflocculation with *Paenibacillus* (AM49)	**7 - 8** g.L^{-1}*	0.062 g.L^{-1}	Chu 13 modified medium pH 5, 7, 9, and **11***	49 % (HE)	—	Oh *et al.* (2001)
***M. aeruginosa* (PCC7820)**	Flotation with flocculation induced by WAC (aluminum polyhydroxichlorosulphate)	2 - 12 mg Al_2O_3.L^{-1} of WAC (**8 mg.L^{-1})***	5.0 - 8.0x10^4	pH **7.42 ± 0,09*** BG11 medium **5 bar*** **8 % recycle ratio***	> 92 % (HE)	—	Teixeira and Rosa (2007)
***M. aeruginosa* (PCC7820)**	Flotation with flocculation induced by $Al_2(SO_4)_3$	0.7 - 1.36 mg.L^{-1} Al (**1.1 pg.cell^{-1})***	6.0x10^5±1.5x10^4	pH 5 and **7***	97.3 % (HE)	—	Henderson *et al.* (2010)
***M. aeruginosa* (CS-564/01)**	Flotation with flocculation induced by $Al_2(SO_4)_3$	0 - 5 mg.L^{-1} Al **1 mg.L^{-1}*** Al	7.5×10^5	MLA medium **pH 7*** recycle ratio: **10 %***	> 95 % (HE)	—	Yap *et al.* (2014)
	Flotation with PolyDADMAC (polydiallyldimethylammonium chloride)	0.0 - 0.8 mg. L^{-1} (**0.3 mg.L^{-1})***		**pH 7*** MLA medium recycle ratio: **20 %***	99 % (HE)	—	

* Optimal conditions found by the authors; HE – Harvesting efficiency.

Table 1.2 (Continued)

Species	Technique	Flocculant Dosage	Initial biomass concentration (cell.mL^{-1})	Parameters	Results	Flocculant Costs	References
***M. aeruginosa* (1450/3)**	Flotation with CTAB (Cetyl-trimethylammonium bromide)	0.017 and **0.0034***mequiv.L^{-1}	$7.5\times10^{5}\pm1.5\times10^{4}$	**pH 7*** Recycle ratio: 20 - **60*** %	87% (HE)	—	Henderson *et al.* (2008a)
	Coagulation induced by CTAB without flotation	**0.0022 - 0.004 mequiv.L**$^{-1*}$		**pH 7***	64 ± 5 % (HE)	—	
M. aeruginosa	ECF (Electro coagulation flotation) with aluminum electrode	0.5, **1.0***, 2.0, 3.0, and **5.0*** mA.cm^{-2}	$1.2\text{x}10^{9}$ - $1.4\text{x}10^{6}$	pH 4, 5, 6, **7***, 8, 9, and 10 18, 27 e **36 ºC***	100 % (HE)	—	Gao *et al.* (2010)
	ECF with iron electrode	0.5, **1.0***, 2.0, 3.0, and 5.0 mA.cm^{-2}			78.9 % (HE)	—	

* Optimal conditions found by the authors; HE – Harvesting efficiency.

As mentioned in section 1.2.7.1.1, flocculation can be achieved by a change in the pH of the culture medium. However, flocculation by pH change has only been tested in a reduced number of microalgal species (Wu *et al*., 2012; Qian *et al.*, 2014 and Ummalyma *et al*., 2016). Also, analyzing Table 1.2 it is possible to conclude that little is known about the effect of a wide range of pH values in the harvesting of *M. aeruginosa*. In fact, Qian *et al.* (2014) was one of the few exceptions that performed a study of flocculation induced by pH for this cyanobacteria species. This study assessed *M. aeruginosa* cell viability at pH 3, 4, 5, 7 and 11, and verified that the most alkaline value tested was the one with the best results (cf. Table 1.2).

Several studies of optimization of Ch-induced flocculation in several strains of *M. aeruginosa* were found in the literature (Pei *et al*., 2014; Li *et al*., 2015 and Yuan *et al*., 2016) and the conditions applied by the authors are summarized in Table 1.2. After the comparison, it was possible to verify that Pei *et al.* (2014) achieved the best results in terms of HE (99 %) for a concentration of 7.31 $mg.L^{-1}$ of Ch. Also, this comparison showed that not only species variation (as previously mentioned on secção 1.2.7.1.2), but also the cyanobacteria strain used can influence HE.

Regarding organic flocculants, Sun *et al*. (2012) obtained 100 % of cells removal with $AlCl_3$ at 15 $mg.L^{-1}$. As it can be verified in Table 1.2, this was the work that achieved better results in terms of HE of *M. aeruginosa* for this flocculant. Besides this study, there's another one that achieved around 99 % of HE, using 4.1 $mg.L^{-1}$ of Al combined with a pre-treatment of 4 $mg.L^{-1}$ of NaClO (Ma *et al*., 2012). Furthermore, other authors like Sun *et al*. (2013) and Qi *et al.* (2016) obtained 94.3 % of HE with 4 $mg.L^{-1}$ of PACl and 75 % with 50 µM of $Al_2(SO_4)_3$. Concerning $FeCl_3$, Chow *et al*. (1998) performed a study in which a comparison of different dosages of this flocculant (15 and 30 $mg.L^{-1}$) and different pH conditions was performed. The best result was of 74 % of HE, obtained with 30 $mg.L^{-1}$ of $FeCl_3$ and with the pH of the medium adjusted for 8.2 (cf. Table 1.2).

Oh *et al.* (2001) achieved values of just 49 % of HE of *M. aerugionosa*, applying bioflocculation using the bioflocculant produced by *Paenibacillus* (AM49), however, using the same bioflocculant dosage and an initial optimal pH (cf. Table 1.2), this experiment obtained 83 % of HE of another species (*Chlorella vulgaris*).

There are also several studies that used flotation for an effective removal of M. aeruginosa, as it can be seen in Table 1.2, but those performed by Yap *et al*. (2014) e Henderson *et al*. (2010) were the ones which obtained 99 % and 97.3 % of HE, respectively. In the first case, flotation was combined with flocculation induced by 0.3 $mg.L^{-1}$ of a high performance organic compound (polyDADMAC), and in the second, flotation was combined with flocculation induced by 1.1 $pg.cell^{-1}$ of Aluminum.

Lastly, Gao *et al.* (2010) achieved maximum HE (100 %) using the electro coagulation flotation technique referred before in section 1.2.7.6, with an aluminum electrode, versus an HE of 78.9 % obtained with the same technique, but with a ferric electrode (cf. Table 1.2).

1.2.8. Cell viability and flow cytometry

Flow cytometry is a technique that allows the analysis, count or sorting of single cells in a solution, by measuring their fluorescence using a cytometer. This equipment uses the quantification of scattered and fluorescent light signals to obtain the results, that do not depend on the features or physiological states of the cells (Hyka *et al.,* 2013).

This technique can be applied to analyze cyanobacteria, especially because of their autofluorescence which is result of the presence of photosynthetic pigments such as carotenoids and chlorophylls. In the case of chlorophyll a content, it is possible to differentiate these microorganisms from others and from particles without the application of a dye, which is often required for the success of flow cytometry and that should always be carefully selected (Hyka *et al.*, 2013).

In this work, flow cytometry was used to assess cellular viability.

1.2.9. Zeta potencial

Zeta potential (ZP) is a measure of the magnitude of the surface charge of microalgae cells (Lananan *et al.*, 2016), which can change according to the water

condition (e.g. temperature or pH) (Zou *et al.*, 2005) or different growth phases (Henderson *et al.*, 2008b).

Renault *et al.* (2009) pointed that flocculation using Ch was influenced by ZP and Li *et al*. (2015) presented an optimal interval of ZP values (OIZVP) for the flocculation of cyanobacteria, achieved through the application of Ch modified soil. Bearing this in mind and the fact that pH is one of the several known factors that affect ZP (Malvern, 2018), it was decided to investigate the relationship between ZP of *M. aeruginosa* cells and the harvesting efficiency.

2. Materials and Methods

2.1. *M. aeruginosa* culture and suspension preparation

The Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) generously supplied *M. aeruginosa* LEGE 91094, which was grown in Z8 medium (Kotai, 1972), at 20 °C, and under 10 μmol.m^{-2}.s^{-1} of light intensity, using a 12:12 hour light/dark cycle. The pH of the medium was adjusted every day to 8.7 and the cultures were aerated at 0.38 vvm and renewed on a monthly basis.

To prepare the cyanobacterial suspension used on the flocculation assays and to ensure equal initial conditions, the cells were centrifuged at 8000 rpm for 15 min and re-suspended in distilled water using a vortex. After this, the biomass concentration was adjusted to 0.5 g.L^{-1}.

2.2. Analytical methods

All the HE experiments were carried out in duplicate. The concentration of biomass was monitored in terms of OD_{750} using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA) and the HE was calculated applying Eq. 1:

$$HE\% = \frac{OD_0 - OD_t}{OD_0} \times 100\% \quad \text{[Eq. 1]}$$

where OD_0 and OD_t represent the optical density (750 nm) at the beginning of the process and after t (h) time, respectively.

To assess HE of the assays, 0.5 mL aliquots from the midpoint of the culture surface and the bottom of the test tube were taken for analysis at the beginning of the process and after 2, 4, 6, 8, and 24 hours. Additionally, at 24 hours and after the withdrawal of the HE samples, the tubes were vigorously agitated, and an extra 1 mL aliquot was taken to measure ZP and cell viability.

2.3. Flocculation optimization

2.3.1. pH-induced flocculation

The pH values used to induce flocculation were in the pH range of 2 to 12. The culture was transferred into 11 glass test tubes presenting a volume of 42 mL each. All the experiments were performed in duplicate (n=2).

NaOH (0.5 and 1 $mol.L^{-1}$) and HCl (1 and 2 $mol.L^{-1}$) solutions were utilized to adjust the pH of the assays.

The pH of the samples was measured using a portable Hanna Instruments 2210 pH meter with a combined pH electrode.

2.3.2. Flocculation induced by flocculant agents

2.3.2.1. Preparation of stock solutions

An $AlCl_3$ stock solution of 3 $g.L^{-1}$ was prepared by stirring continuously this flocculant in 10 mL of ultrapure water, until it was fully dissolved, according to Sun *et al*. (2012) procedure. $AlCl_3$ (anhydrous) was purchased from Fluka Analytical (USA).

Following the instructions of Chow *et al.* (1998), a 20 $g.L^{-1}$ $FeCl_3$ stock solution was prepared with 10 mL of distilled water and the ACS reagent $FeCl_3$ ($FeCl_3 \cdot 6\ H_2O$), purchased from Sigma-Aldrich (USA). The solution was stirred continuously with a magnetic stirrer until the flocculant was dissolved.

As described in Pei *et al.* (2014), a stock solution of 1 $mg.L^{-1}$ of Ch was prepared by dissolving it in a 1 % acetic acid solution. After that, the solution was stirred during 30 min at 100 rpm by a magnetic stirrer. The Ch with low molecular weight used was purchased from Aldrich Chemistry (USA).

All the stock solutions were kept in a dark and dry environment to prevent degradation for a maximum of 10 days. After this period, they were discarded, and fresh solutions were made.

The starting volume of $AlCl_3$ applied was selected based on Sun *et al.* (2012), where an optimal concentration of 15 $mg.L^{-1}$ was found. The flocculant was added to 42 mL of *M. aeruginosa* culture and shaken vigorously for homogenization. After that, an aliquot was withdrawn from the intermediate point between the bottom of the test tube and the culture surface. Blank control was established as the assay running without any addition of flocculant.

The $FeCl_3$ coagulant was added in a concentration of 30 $mg.L^{-1}$. This value was described by Chow *et al*. (1998) as being the optimal dose to induce flocculation of *M. aeruginosa* cells with this agent.

Before the addition of the optimal concentration of this flocculant, the pH of the assay was adjusted to 8.2, as referred by Chow *et al.* (1998). The control test was set as the assay running without the initial pH adjusted and with no addition of flocculant. An aliquot of the supernatant was taken as described above, for $AlCl_3$.

The optimal initial Ch dosage of 7.31 $mg.L^{-1}$ described by Pei *et al.* (2014) was applied and the pH of the medium was adjusted to 8 using 0.5 $mol.L^{-1}$ NaOH and 1 $mol.L^{-1}$ HCl solutions. The adjustment of pH was made before the coagulant addition to the assays, according to the methodology of the referred article.

The rest of the procedure was performed using the same methods applied before to $FeCl_3$ and to $AlCl_3$.

2.4. Comparison of harvesting methods

After concluding which pH value was the best-performer in terms of flocculation efficiency, a study comparing its performance with the three well-known flocculants ($AlCl_3$, $FeCl_3$, and Ch) was made.

Similarly to the pH experiments, these tests were conducted in duplicate and studied for 24 hours.

2.5. Optimization of harvesting methods using ZP as an indicative tool

By analyzing the ZP, it is possible to understand if the particles in a solution are repelling or attracting each other. In the first case, the ZP will be largely negative or positive, meaning the particles will present themselves dispersed in the solution. In the second case, the ZP will present low values and it will be possible to conclude that the particles are mutually attracted, and flocculation is possible (Malvern, 2018).

Following the conclusions of Li *et al.* (2015), where chitosan modified soil-induced flocculation pointed at higher HEs when the ZP was within the range of -6.7 to -20.7 mV (optimal interval of values for the ZP - OIVZP) it was decided to study the influence of ZP on HE by varying the concentrations of $FeCl_3$, $AlCl_3$ and Ch or the pH of the culture medium.

Regarding concentration, it was decided to begin with the value of the three most well-known flocculant agents considered to be optimal by the literature for *M. aeruginosa*. Afterwards, an optimization of these methods was done, lowering their concentration in a proportional way.

A 0.8 mL sample withdrawn at 24 hours was measured, which was performed in triplicate at room temperature using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). The mean of the measured values was then considered.

2.6. Cell viability

To distinguish cells from debris, the cytometer evaluates the chlorophyll a content (the long band pass filter, FL3, detecting as autofluorescence at >650 nm) and overlaps two scattering signals: the forward-angle light scatter (FS) which measures size and the side-angle light scatter (SS) which measures the complexity of cell aggregates, to set a threshold.

After 24 hours of treatment, a 0.2 mL sample was used to assess cell viability. These samples were then vortexed to re-suspend the cells. After this, the

cyanobacterial cells were analyzed in triplicate through flow cytometry using the EC800 Flow Cytometer Analyzer (Sony Biotechnology Inc., USA), to confirm if the concentration of the three flocculant agents and the range of pH used did not affect the membrane integrity of the cells, through the viability indicator (FL3) analysis.

The flux cytometry statistical analysis was performed using cytometer software V. 1.3.6 (Sony Biotechnology Inc, Champaign, IL, USA) and graphs were drawn using GraphPad Prism 6 software for Windows.

2.7. Statistical analysis

Harvesting efficiency experiments were performed in duplicate and zeta potential tests in triplicate. The samples were taken independently from each other and the statistical analysis was assessed using the software STATISTICA version 12.0 (StatSoft Inc. 1984-2014). A factorial ANOVA test was done for all the HE and ZP experiments. For the cell viability experiments an One-Way ANOVA followed by a Tukey post-hoc test with significance at $p<0.05$ were performed.

3. Results and Discussion

3.1. Optimization of pH-induced flocculation

The harvesting efficiency (HE) of *M. aeruginosa* was studied through pH-induced flocculation assays, in a range values between 2 and 12. These assays were performed in duplicate and the results can be observed in Figure 3.1.

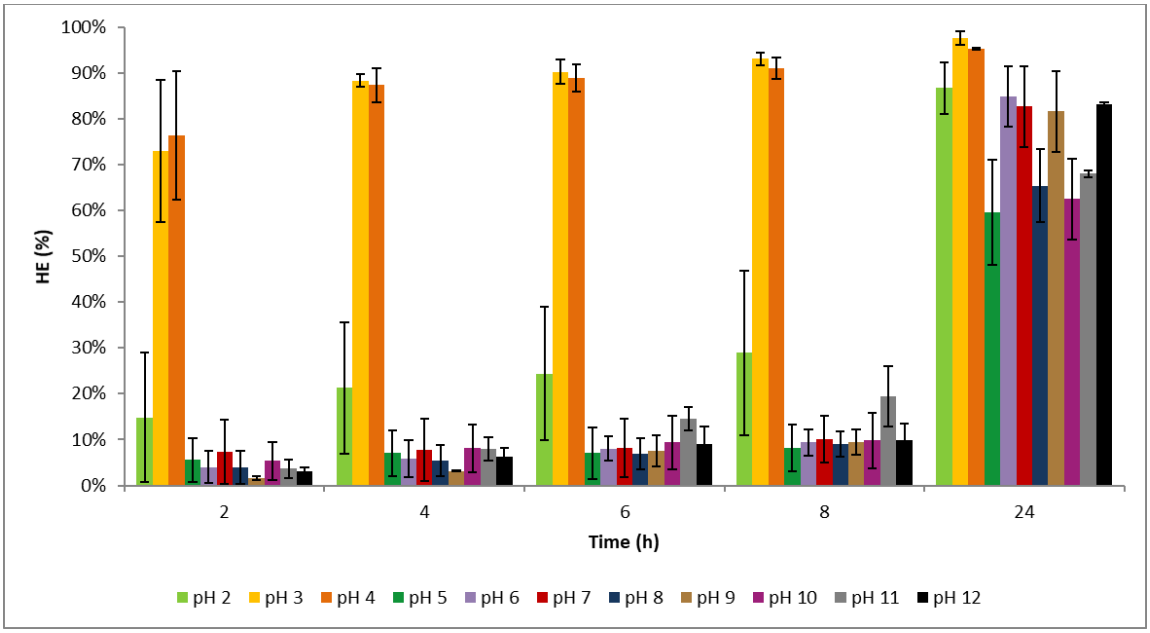

Figure 3.1 Assessment of HE on pH-induced flocculation experiments for 24 hours (*n*=2). Error bars correspond to minimum and maximum values.

As it can be seen in Figure 3.1, by adjusting the pH values to 3 and 4 higher HE were attained: more than 87 % of *M. aeruginosa* cells, after 4 hours, and around 90 % after 6 hours of treatment. Liu *et al.* (2013) also reported that pH 4 achieved the highest HE (as high as 90 %) when studying the harvesting processing of three different freshwater microalgae – *Chlorococcum nivale*, *Chlorococcum ellipsoideum* and *Scenedesmus* sp..

From Figure 3.1, it is also possible to conclude that the time needed by pH values 3 and 4 to obtain high efficiencies was shorter. Two hours after the treatment started, the HE was already 73 % for pH 3 and 76 % for the best case (pH 4). These values, at an early stage of the treatment, are greater than those obtained for all the other pH values studied after 8 hours, which show a clear difference between the two best-performers and the rest.

The flocculation mechanism that occurred in this case was charge neutralization (CN). This mechanism consists on the neutralization of the negatively charged cyanobacteria cells' surface by the dissociation of their carboxylic acid groups when the pH is lowered, as demonstrated in several studies (Hadjoudja *et al.*, 2010; Liu *et al.*, 2013).

Furthermore, although the optimum pH interval mentioned by Liu *et al.* (2013) is in line with the results presented in Figure 3.1, other studies point to different outcomes. Wu *et al.* (2012), for instance, proposed the use of alkaline environments with pH above 9 to achieve the highest efficiencies after testing the procedure with three freshwater microalgae (*Chlorococcum* sp., *Scenedesmus* sp. and *Chlorella vulgaris*) and other two marine species. These differences show that the strain studied strongly influences the success of the pH-induced flocculation methods.

Knowing that there are consequences on the environment (namely on the ZP) when the pH is modified, it was decided to study those alterations. Figure 3.2 shows the values of *M. aeruginosa*'s ZP obtained when pH of the medium ranged from pH 2 to 12.

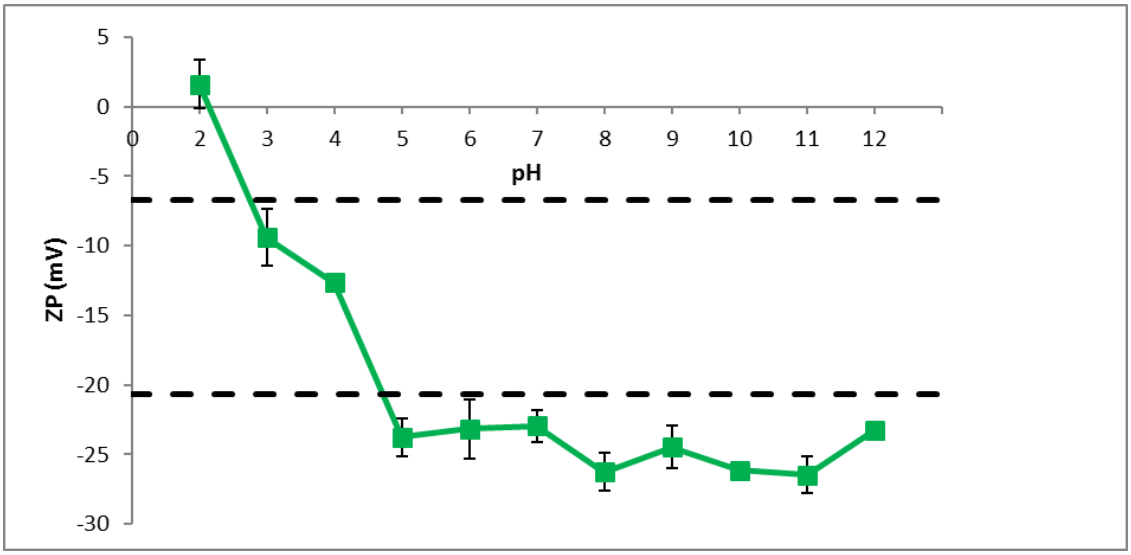

Figure 3.2 Variation of ZP of *M. aeruginosa* at different pH values (n=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

It is possible to conclude that the assays with the highest HEs (pH 3 and 4, as presented before) correspond to ZP values that are included in the optimal interval

of values for the zeta potential (OIVZP). Therefore, considering the fact that after 2 hours of treatment the values of HE obtained with pH 4 were higher than those obtained with pH 3, and the difference between the two over time was not very significant, the first one was chosen as the optimal pH value to induce *M. aeruginosa* flocculation.

Figure 3.3 – A, B and C shows the image representation of the pH-induced flocculation assay.

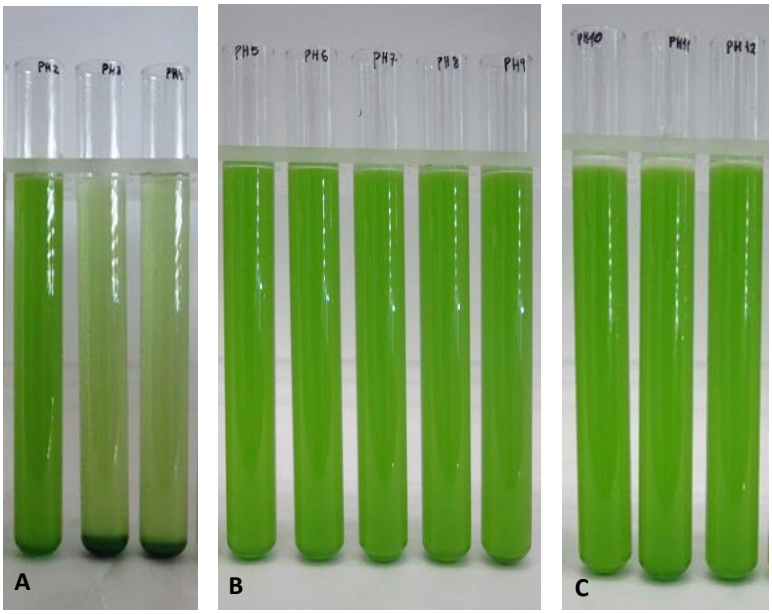

Figure 3.3 pH-induced flocculation experiments after 2 hour of treatment: A – pH 2, 3, and 4; B – pH 5, 6, 7, 8, and 9; and C – pH 10, 11, and 12.

As it can be observed in Figure 3.3-A, after 2 hours of treatment the supernatant of the pH 3 and pH 4 was clearer and their tubes presented more deposited cells than the others (including the assays of Figure 3.3-B and Figure 3.3-C).

3.1.1. Cells' viability tests

Current literature focuses on the study of higher values of pH. However, Liu *et al.* (2013) was one of the few exceptions that proved cellular viability at pH 3.5 for other three freshwater microalgae.

To ensure cellular membrane integrity of *M. aeruginosa* during the pH-induced flocculation assays carried out in the previous section it was performed a cell viability analysis by flow cytometry. The results can be seen in Figure 3.4.

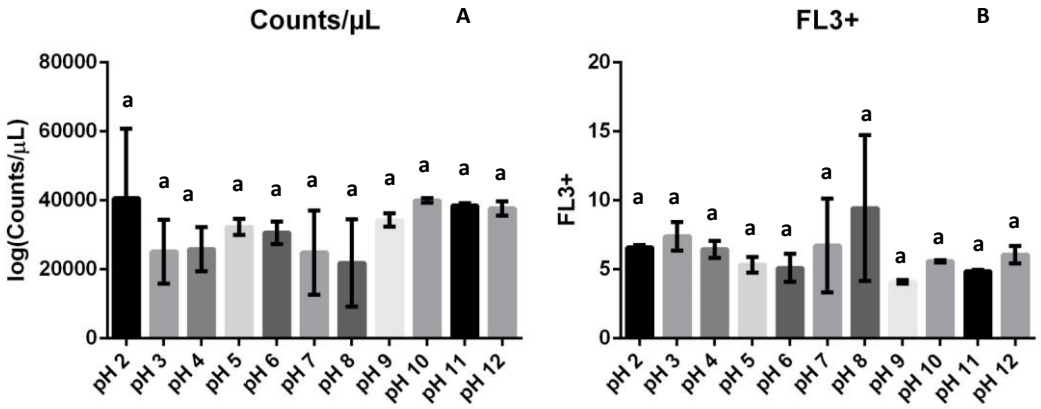

Figure 3.4 Flow cytometry results (n=3) for *M. aeruginosa* cells after 24 hours of exposure to a range of pH values in terms of: A – counts of flocculated cells per µL and B – auto-fluorescence of chlorophyll-a. Different lower case letters represent statistically significant difference (Tukey HSD test, $p < 0.05$).

After the statistical analysis of the flux cytometry results of the assays flocculated by pH in the range of 2 to 12, it was possible to conclude that there were no statistically significant differences between different pH assays, in terms of counts of flocculated cells per µL and viability (auto-fluorescence), as it can be seen in Figure 3.4 – A and B, respectively. Therefore, cell viability was ensured for all the pH values tested in this work.

3.2. Comparison of HE on different harvesting methods

Besides the experiment presented on the previous section, a comparison of the HE of four methods was performed to understand which was the most efficient process.

These four techniques included pH 4 (chosen in the previous section) and three other well-known flocculants ($FeCl_3$, $AlCl_3$, and Ch) whose conditions were retrieved from literature. The concentrations applied for $FeCl_3$, $AlCl_3$, and Ch were 30 (Chow *et al.,* 1998), 15 (Sun *et al*., 2012) and 7.31 (Pei *et al*., 2014) $mg.L^{-1}$, respectively.

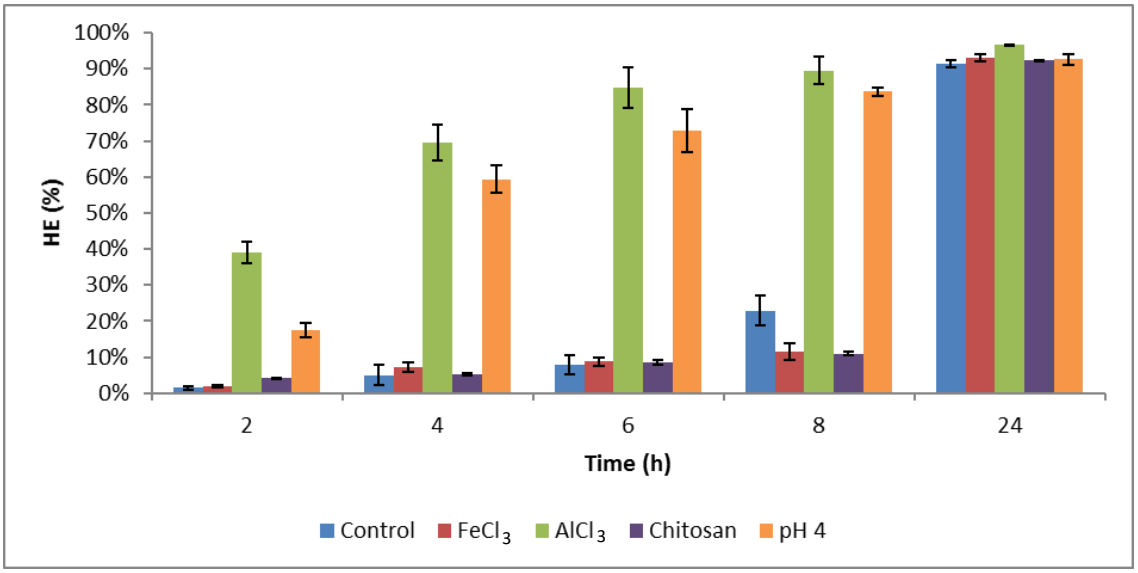

Figure 3.5 Comparison of HE of flocculation of *M. aeruginosa* induced by three different flocculant agents and control on a 24 hour treatment (n=2). Error bars correspond to minimum and maximum values.

$FeCl_3$ and Ch behaved similarly to control tests throughout the 24 h experiment, as presented in Figure 3.5. In terms HE, $AlCl_3$ and pH 4 showed the most successful results when comparing all the treatments. These two experiments presented a HE of approximately 85 and 73 %, respectively, after 6 hours of treatment. Additionally, after just 4 hours, these two treatments achieved better performances than the other two after 8 hours.

Similarly to the analysis of the previous section, the variation of ZP of *M. aeruginosa* was also studied. The assays were performed in triplicate and the results can be seen in Figure 3.6.

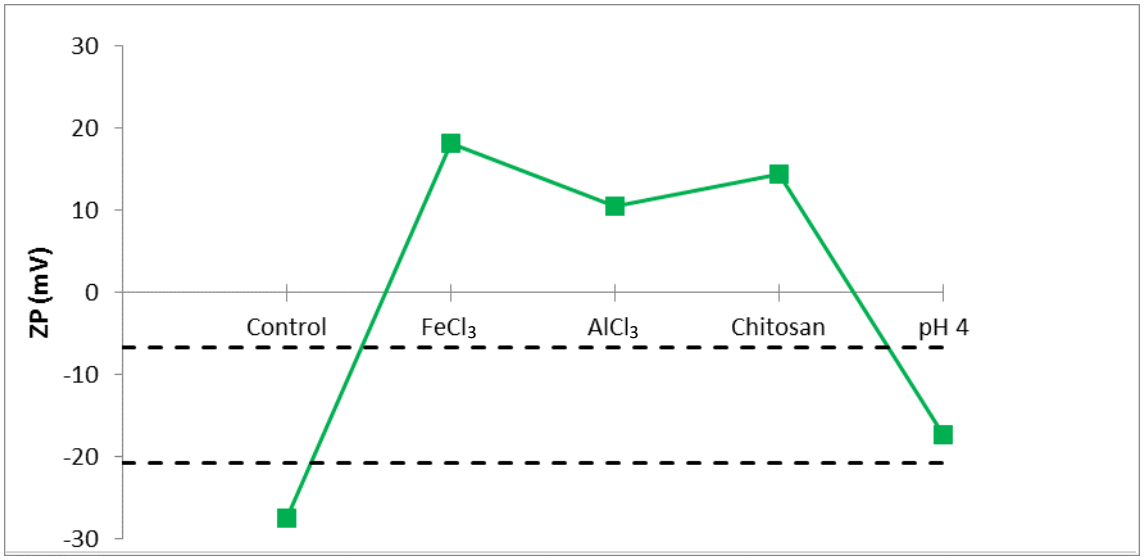

Figure 3.6 ZP values of the assays performed to compare the HE of all methods tested (*n*=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

The ZP of the cyanobacteria treated with $AlCl_3$ was expected to be inside the OIVZP, given the performance of this coagulant on *M. aeruginosa* sedimentation and the effect of ZP on the HE. One possible explanation is that sweep flocculation (SF) might have taken place in this case. Meaning, the large, sticky and heavy metal precipitates could have been formed due to the excessive dosage of coagulant and cells were dragged to the bottom of the tubes (Wu *et al.*, 2012; Duan and Gregory, 2003).

Other aspects such as biomass concentration, strain of cyanobacteria used, sampling point or agitation could have influenced the low values of efficiency of *M. aeruginosa* removal achieved using $FeCl_3$ and Ch as well as the high values of ZP, as presented in Figure 3.6.

While the work of Pei *et al.* (2014) shows results of 99 % of HE after one hour of treatment with Ch, the data presented here reveal much lower values (just 11 % after 8 hours). There are significant differences that can explain this gap. First, the biomass concentration used in this work was 10 times higher than that of Pei *et al.* (2014). Secondly, there was no use of agitation in this study, while in the latter an optimization of the agitation time and speed was performed. Finally, Pei *et al.* (2014) used a sampling point of just 1 cm below the culture surface, which is much closer

to the surface than the one used in this work. All these differences could have led to better results in terms of HE.

Regarding the ZP values observed for pH 4 assays, as expected, they were inside the OIVZP.

Figure 3.7 Comparison of four different flocculation methods for *M. aeruginosa* after 4 hours of treatment: control; 30 mg.L^{-1} of $FeCl_3$; 15 mg.L^{-1} of $AlCl_3$; 7.31 mg.L^{-1} of Ch, and pH 4 (from left side to the right).

Figure 3.7 shows that the results of the assays performed were in agreement with those presented in Figure 3.5. After 4 hours of treatment, it is possible to notice that the culture where the $AlCl_3$ was applied is the clearer of all the tubes followed by the pH 4 assay (Figure 3.7). This conclusion can also be applied to the observation of cells settled at the bottom of the tubes.

3.2.1. Cells' viability tests

The assays carried out in the previous section were analysed by flow cytometry to ensure that no damage was inflicted to the cells during the harvesting methods. The results can be seen in Figure 3.8.

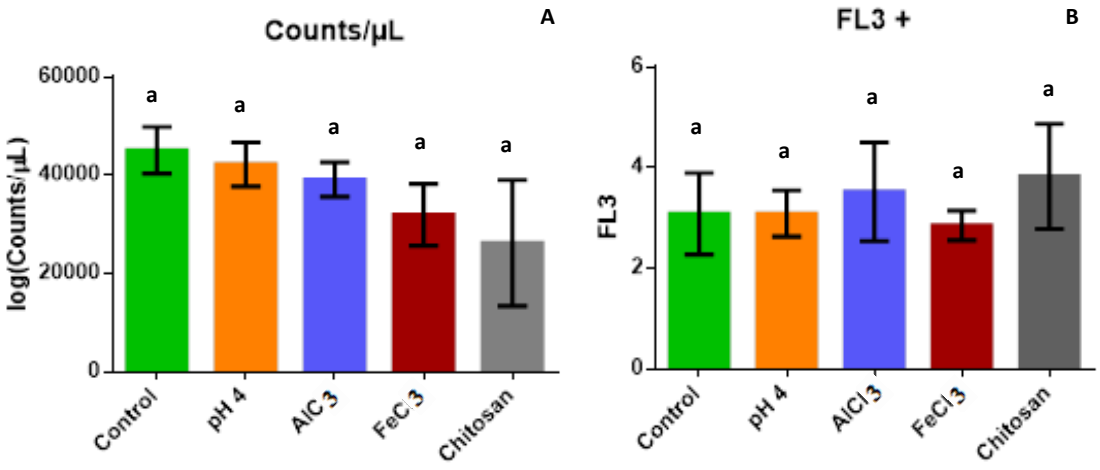

Figure 3.8 Flow cytometry results (n=3) for *M. aeruginosa* cells after 24 hours of exposure to harvesting treatments in terms of: A – counts of flocculated cells per µL and B – auto-fluorescence of chlorophyll-a. Different lower case letters represent statistically significant difference (Tukey HSD test, $p < 0.05$).

No statistically significant changes in terms of counts of flocculated cells per µL and viability (auto-fluorescence) were observed between the different treatments and control, as it can be seen in Figure 3.8 – A and Figure 3.8 – B. Therefore, cellular viability is ensured for pH 4, $AlCl_3$ at 15 $mg.L^{-1}$, $FeCl_3$ at 30 $mg.L^{-1}$ and Ch at 7.31 $mg.L^{-1}$.

Qian *et al.* (2014) tested the cells' viability of *M. aeruginosa* and two other cyanobacteria species (*Anabaena circinalis* and *Cylindrospermopsis raciborskii*) for pH 3, 4, 5, 7, and 11 and showed that *M. aeruginosa* presented high tolerance for pH values below 5. This is in line with the results obtained in the work presented here for pH 4.

3.3. HE optimization – assessment of the effect of ZP

After analysing the results showed in (Figure 3.5) and realizing that the values of ZP were not in the OIVZP (Figure 3.6), it was decided to perform a deeper study in order to get a better understanding of these processes. Therefore, a series of experiments were done to assess the impact of ZP in HE of *M. aeruginosa*. To proceed with the study, different concentrations of the three well-known flocculant agents were tested so that the ZP values remained within, below and above the OIVZP.

The results are presented on the following sections by this order: $AlCl_3$ experiments, $FeCl_3$ experiments, and Ch experiments.

3.3.1. $AlCl_3$ experiments

Regarding $AlCl_3$ flocculation optimization, its concentration was reduced when compared to the original one (15 $mg.L^{-1}$). The assays tested were conducted in duplicate during 24 hours and had the following dosages: 15, 7.5, 3.75 and 1.88 $mg.L^{-1}$. The results are shown in Figure 3.9.

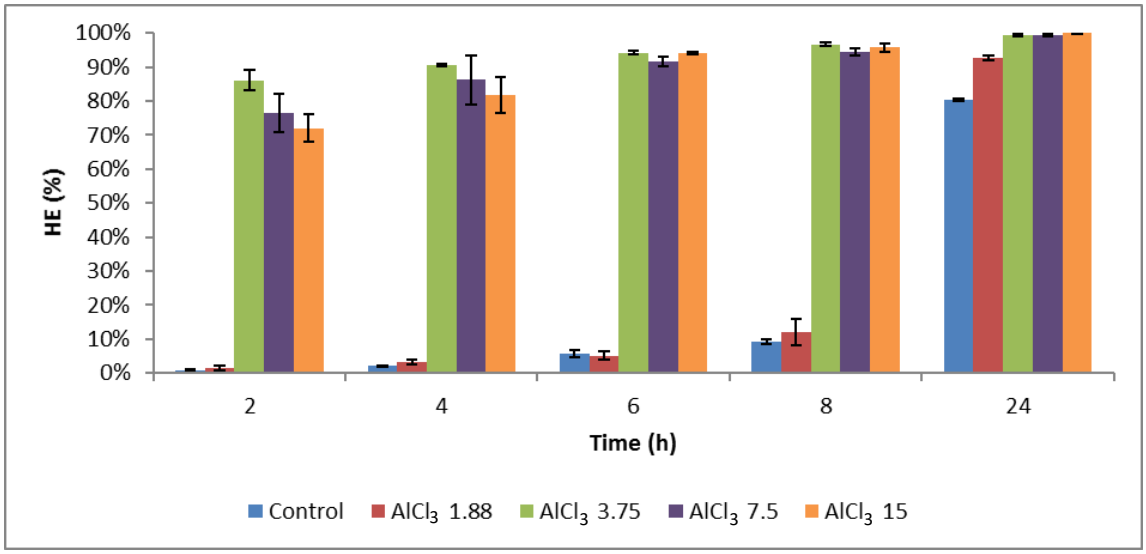

Figure 3.9 HEs obtained after 2, 4, 6, 8, and 24 hours using different concentrations of $AlCl_3$ and the control (n=2). Error bars correspond to minimum and maximum values.

Analysing Figure 3.9, it is possible to conclude that the results of $AlCl_3$ at 1.88 $mg.L^{-1}$ point to low efficiency after 2, 4, 6, and 8 hours of treatment. In fact, in comparison to the other concentrations tested, this is by far the worst case. After 8 hours of treatment, it achieved an HE of only 12 %. On the other hand, intermediate concentrations of $AlCl_3$ achieved better results, not only after 8 hours but also at earlier stages of the treatment. In particular, the concentration of 3.75 $mg.L^{-1}$ reached 86 % and 91 % of HE, after 2 and 4 hours of treatment, respectively. Comparing with the concentration tested initially (15 $mg.L^{-1}$), the HE obtained with 3.75 $mg.L^{-1}$ of $AlCl_3$ was 14 and 9 % higher after 2 and 4 hours, respectively.

As it was done in the previous sections, the ZP of *M. aeruginosa* was measured and its values can be observed in Figure 3.10.

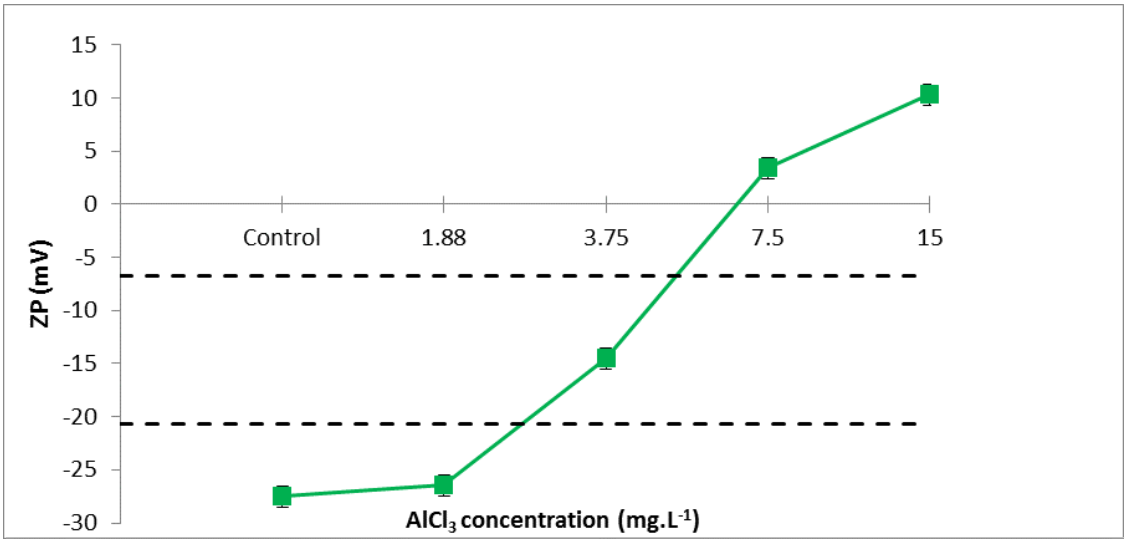

Figure 3.10 Variation of ZP of *M. aeruginosa* at different $AlCl_3$ concentrations (n=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

Through Figure 3.10, it is possible to see that the optimal concentration of $AlCl_3$ was the only one to present a value within the range of the OIVZP, which reinforces the connection between HE and ZP.

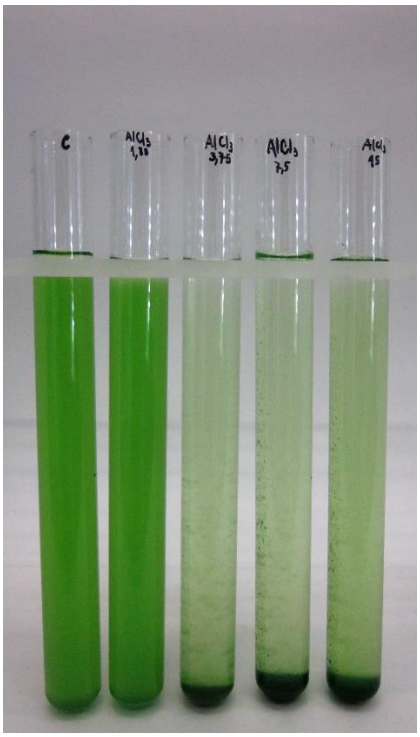

Figure 3.11 Comparison of the results of *M. aeruginosa* harvesting using four different dosages of $AlCl_3$ after 2 hours of treatment: control; 1.88 $mg.L^{-1}$; 3.75 $mg.L^{-1}$; 7.5 $mg.L^{-1}$ and 15 $mg.L^{-1}$ (from the left side to the right).

The analysis of Figure 3.11 indicates that the supernatants' transparency increases as concentration diminishes, meaning that 7.5 $mg.L^{-1}$ assay presents a clearer culture than 15 $mg.L^{-1}$ and so on. However, this does not apply to the 1.88 $mg.L^{-1}$ assay, which behavior is similar to the one found in the control test.

3.3.2. $FeCl_3$ experiments

After the study of different concentrations of $AlCl_3$, the same was done to the $FeCl_3$. For this agent, the concentrations used on the assays were the following: 1.88, 3.75, 7.5 and 30 $mg.L^{-1}$. Similarly to $AlCl_3$, the latest value refers to the concentration applied initially and it was decided to test lower concentrations. The HEs of these tests can be seen in Figure 3.12.

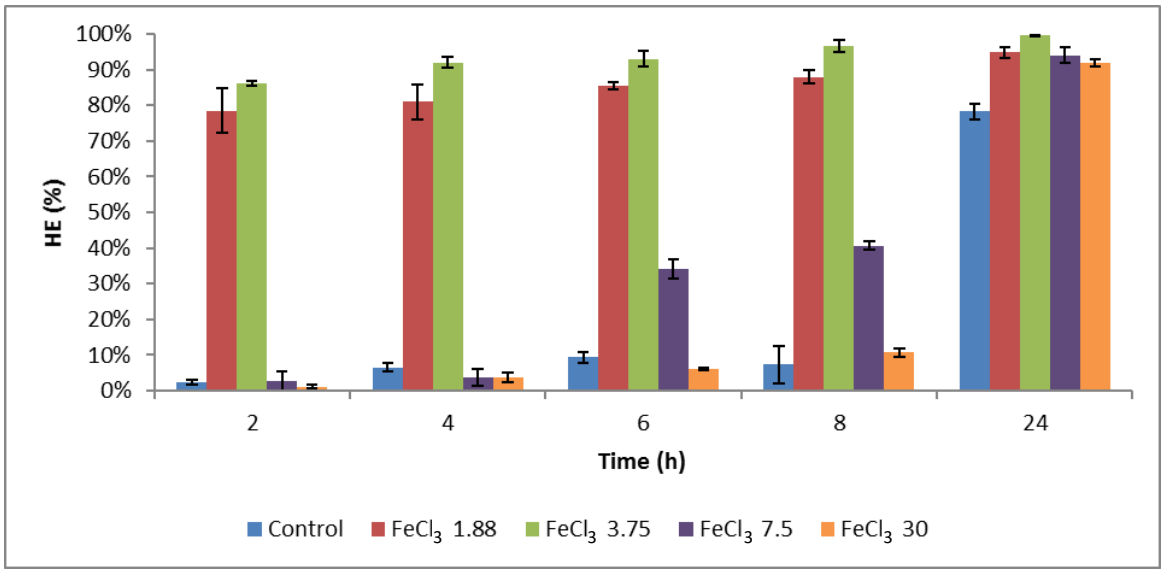

Figure 3.12 Comparison of HEs using different concentrations of $FeCl_3$ and the control, after 2, 4, 6, 8 and 24 hours (n=2). Error bars correspond to minimum and maximum values.

According to these data, it is possible to confirm that there are two better performers: $FeCl_3$ at 1.88 and at 3.75 $mg.L^{-1}$, with the second being the best in terms of HE.

The HE achieved by the 3.75 $mg.L^{-1}$ assay corresponds to more than 90 % after 4 hours. Considering the use of 1.88 $mg.L^{-1}$, the HE was higher than 80 % after the same treatment duration. These results are better than the ones obtained with the

7.5 mg.L^{-1} concentration but the most significant difference is the increase of the efficiency when the comparison is made between the results of the 3.75 mg.L^{-1} assay and those obtained by the initial concentration of 30 mg.L^{-1}. In this case, an increase of 85, 88, 87, and 86 % of the HE after 2, 4, 6, and 8 hours of treatment, respectively, was observed.

The next Figure (Figure 3.13) shows the impact of different concentrations of $FeCl_3$ on *M. aeruginosa*'s ZP.

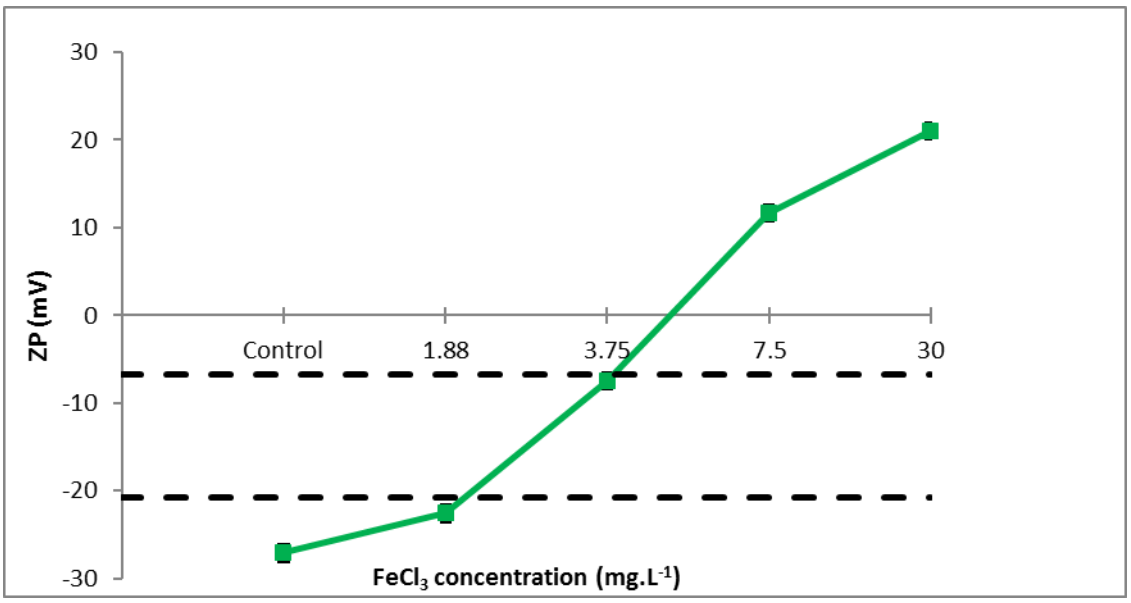

Figure 3.13 ZP values of the assays performed to compare the HE of different $FeCl_3$ concentrations (n=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

Similarly to what happened with $AlCl_3$, the concentration of $FeCl_3$ that resulted on a higher HE (3.75 mg.L^{-1}) was the only one with a ZP within the range of the OIVZP (Figure 3.13).

Figure 3.14 Comparison of the results of *M. aeruginosa* harvesting using four different $FeCl_3$ concentrations after 2 hours of treatment: control; 1.88 $mg.L^{-1}$; 3.75 $mg.L^{-1}$; 7.5 $mg.L^{-1}$, and 15 $mg.L^{-1}$.

The results shown in Figure 3.14 demonstrate that, after just 2 hours of treatment, the assay with 3.75 $mg.L^{-1}$ of $FeCl_3$ was the one with the most transparent culture and more deposited cells, followed by the 1.88 $mg.L^{-1}$ test. Regarding the two highest concentrations (7.5 $mg.L^{-1}$ and 15 $mg.L^{-1}$), the results were similar to the control test.

3.3.3. Ch experiments

Following the same procedure of the other flocculant agents, the concentrations of Ch tested were lower than the initial concentration used in this study (7.31 $mg.L^{-1}$): 2, 0.5 and 0.25 $mg.L^{-1}$. Figure 3.15 presents the results obtained.

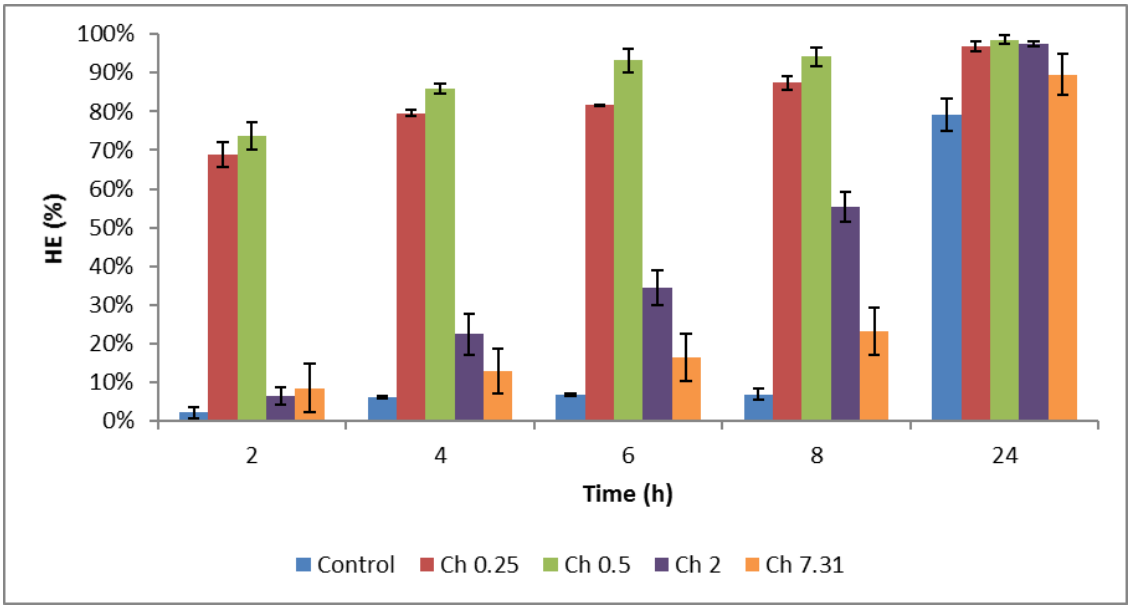

Figure 3.15 HEs of the assays performed with different concentrations of Ch and the control after 2, 4, 6, 8, and 24 hours (*n*=2). Error bars correspond to minimum and maximum values.

In general terms, all the assays showed better results than those obtained for the initial concentration (7.31 $mg.L^{-1}$). It is noteworthy the fact that, when comparing the optimal concentration found (0.5 $mg.L^{-1}$) assay with 7.31 $mg.L^{-1}$ just after 2 and 4 hours of treatment, increments in HE of around 65 and 73 % were observed. Interestingly, the concentration of 3 $mg.L^{-1}$ pointed as optimal by Yuan *et al.* (2016) and the dosage of 1 $mg.L^{-1}$ were tested in preliminary experiments but did not present significant differences in terms of HE and ZP when compared to those obtained with 2 $mg.L^{-1}$. As consequence, we didn't proceed with the assays using these two concentrations of Ch.

As it occurred with the $AlCl_3$ and the $FeCl_3$ experiments, the improvement in the HE achieved with the same flocculants was influenced by their concentration, which provoked changes in the ZP of the cells. These changes are displayed in Figure 3.16.

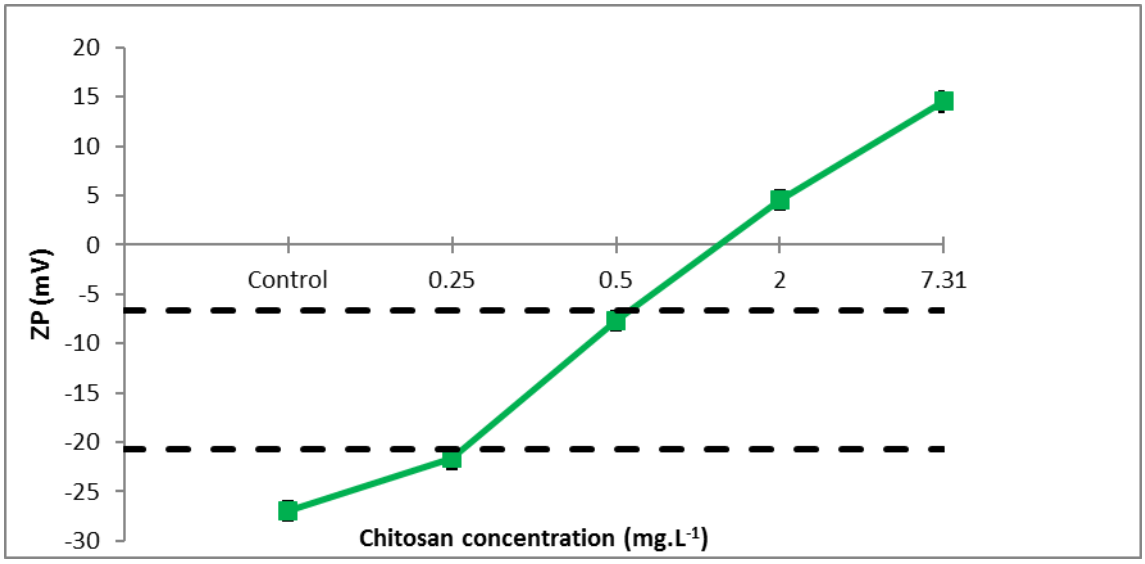

Figure 3.16 Variation of ZP of *M. aeruginosa* at different Ch concentrations (*n*=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

The value of ZP obtained for the concentration of 0.5 mg.L^{-1} was within the range of the OIVZP, as it can be seen in Figure 3.17.

Figure 3.17 Comparison of the results of *M. aeruginosa* harvesting induced by four different Ch dosages after 2 hours of treatment: control: 0.25 mg.L^{-1}; 0.5 mg.L^{-1}; 2 mg.L^{-1}, and 7.31 mg.L^{-1}.

Figure 3.17 presents the results obtained after 2 hours of treatment. Through its observation, it is possible to conclude that the concentration of 0.5 mg.L^{-1} was the

one in which the culture was the clearest and the amount of deposited cells was the greatest. The second-best assay was the experiment carried out with 0.25 mg.L^{-1} of Ch, while the two higher concentrations were similar to the control test.

Taking into account what has been presented before, the analysis of the impact of different Ch concentrations in ZP also showed that whether the flocculation is induced by a pH change or by a flocculant agent, the HE of *M. aeruginosa* flocculation is strongly dependent on ZP.

3.3.4. Comparison of optimal conditions

After the analysis presented for each flocculant agent on the previous sections, it was performed one last experiment to compare the optimal conditions obtained for all the different methods (Figure 3.18), guaranteeing that the cultures' ZP would be comprised between -6.7 and -20.7 mV. The concentrations applied for $FeCl_3$, $AlCl_3$, and Ch were 3.75, 3.75 and 0.5 mg.L^{-1}, respectively.

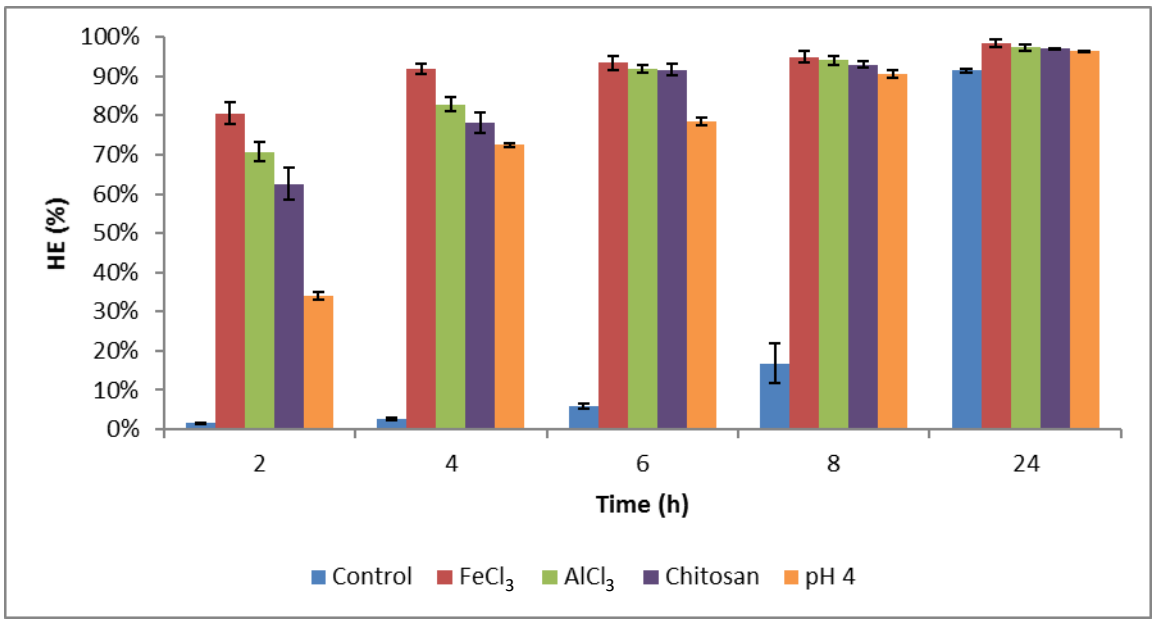

Figure 3.18 Comparison of HE under optimal conditions of flocculation of *M. aeruginosa* induced by pH 4, $FeCl_3$, $AlCl_3$ and Ch and the control after 24 hours of treatment (n=2). Error bars correspond to minimum and maximum values.

As it was expected, all the assays presented very good performances in terms of HE, making it possible to conclude that using ZP to increase the removal efficiency of cells seems to be extremely advantageous. This is particularly evident when comparing the HEs of Figure 3.18 with those obtained when the initial concentrations of $FeCl_3$ and Ch were used (Figure 3.5). In fact, the use of these optimal concentrations reflects in HEs above 91 % for all flocculants after 8 hours of treatment.

Through the observation of Figure 3.18, it is possible to confirm that $FeCl_3$ is the most efficient method achieving 92 % after just 4 hours. $AlCl_3$ is the second-best treatment studied, reaching 83 % after 4 hours of treatment.

This result is in line with Gonzalez-Torres *et al*. (2014) that have demonstrated that, with low concentrations of $FeCl_3$ and at pH 7, this flocculant was more efficient than $AlCl_3$ for the harvesting of *M. aeruginosa*. In addition, the main mechanisms thought to be responsible for improving flocculation are both CN and SF (Johnson and Amirtharajah, 1983; Duan and Gregory, 2003; Gonzalez-Torres *et al*. 2014).

A final word related to Ch. In Riaño *et al*. (2012), it is possible to find information pointing to the need of the use of higher dosage of this flocculant (214 $mg.L^{-1}$) to obtain an HE of 92 % on a group of microalgae in which *Microcystis* sp. was included. In fact, the concentrations studied in Riaño *et al.* (2012) were often higher than 10 $mg.L^{-1}$, which can cause serious damage to cell membrane. This damage allows the release of MC in the case of *M. aeruginosa*, which is unacceptable due to the danger it presents. Also, Mucci *et al.* (2017) showed that a Ch concentration of 0.5 $mg.L^{-1}$ do not pose relevant alterations to the structure and behaviour of *M. aeruginosa*. However, significant changes (such as the decrease in the flocs size and complexity) might be found if the dosage is above 1 $mg.L^{-1}$.

In this work, we demonstrated that with low concentrations of Ch, it is possible to achieve very good results in terms of HE if the flocculation occurs under conditions corresponding to the adequate value of ZP.

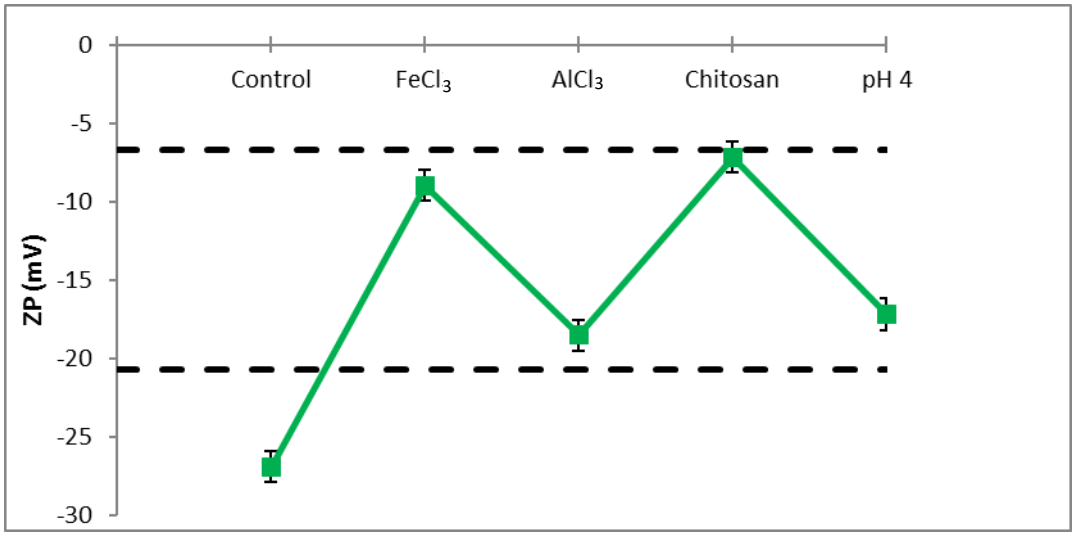

Figure 3.19 ZP values of the assays performed to compare the HE of all methods tested (*n*=3). Black dashed lines (–) represent the OIVZP for flocculation according to Li *et al.* (2015). Error bars correspond to the standard deviation of sample dispersion determined for triplicates.

Following the same procedure of the previous sections, Figure 3.19 proves that the values of ZP obtained in this last study are all within the OIVZP.

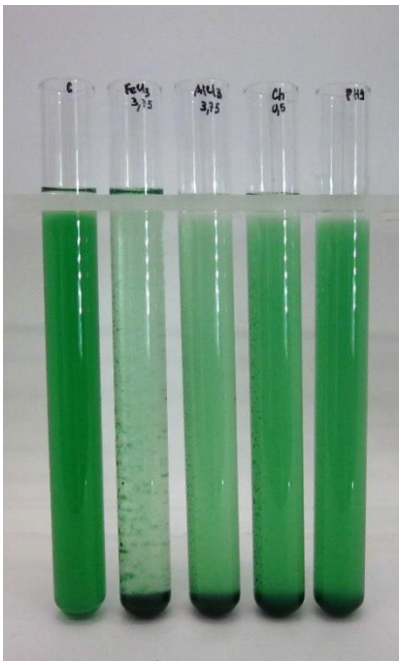

Figure 3.20 Comparison of the optimal conditions of the four flocculation methods of *M. aeruginosa* used in this study after 2 hours of treatment: control, 3.75 mg.L^{-1} of $FeCl_3$, 3.75 mg.L^{-1} of $AlCl_3$, 0.5 mg.L^{-1} of Ch, and pH 4.

As it can be seen in Figure 3.20, after 2 hours of treatment the culture of $FeCl_3$ assay presents itself clearer and with a greater amount of cells settled at the bottom than the rest of the flocculants, being followed by $AlCl_3$, Ch and pH 4 tests.

4. Conclusions and future work

The HE of pH-induced flocculation of *M. aeruginosa* was studied and the results pointed to more than 90 % of cells removal after 8 hours of treatment, if the pH value was 3 and 4. This proved that pH-induced flocculation is a very promising technique, also because its costs are low.

Between these two values of pH and because it presented higher HE, pH 4 was the chosen for a comparison with other flocculation-inducing agents: $AlCl_3$, $FeCl_3$ and Ch. These flocculants' concentrations were found in the literature (Sun *et al*., 2012; Chow *et al*., 1998 and Pei *et al*., 2014) and the results showed that both pH 4 and $AlCl_3$ were good performers in terms of HE. However, a deeper study indicated that all assays excluding pH 4 presented values of ZP outside of the range of the optimal interval of values for the ZP (OIVZP).

This work clearly demonstrated that alterations in ZP directly affected the efficiencies of cells removal. Values of more than 91 % of HE were achieved for all the experiments after 8 hours of treatment. The results also showed that $FeCl_3$ at a concentration of 3.75 $mg.L^{-1}$ was the best method achieving 92 % of HE of *M.* aeruginosa after just 4 hours of treatment.

Process optimization based on ZP allowed not only increases of 65 % and 88 % in the HE (Ch and $FeCl_3$, respectively), but also a reduction of the dosage applied initially. The ZP values for which the various methods were found to be optimal were in the range of -6.7 to -20.7 mV. However, in these two cases in terms of scalability and minimizing the environmental impact since the differences in HE between the two lowest concentrations (1.88 and 3.75 $mg.L^{-1}$ for $FeCl_3$ and 0.25 and 0.5 $mg.L^{-1}$ for Ch) were so minimal we could also have considered the lowest concentration tested as the optimal dosage.

Thereby, the monitoring of concentration of the chemical agents through ZP measurements can decrease the time and the costs of harvesting by the optimization of dose control.

Regarding cells' viability, it was verified that, for the optimal concentrations found in this work and for a wide range of pH from 2 to 12, there was no damage to *M. aeruginosa* cells and, therefore, it is possible to conclude that there was no release of intracellular MC. This could point to a decrease in MC cost production, which consequently would cause its commercialization more feasible.

As future perspective, it would be interesting to proceed to the economic evaluation of the process, in terms of costs, ecological footprint and scale-up viability. Also, evaluate if there is no degradation of extracellular MC during the flocculation process. Besides, the isolation and purification of new metabolites guided by bioactivity assays would provide new substances, that might lead to the discovery of new drugs (Leão *et al*., 2012). Another interesting area of study is the OIVZP. Namely, it would be important to assess if it produces similar results both using other coagulants and harvesting techniques.

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