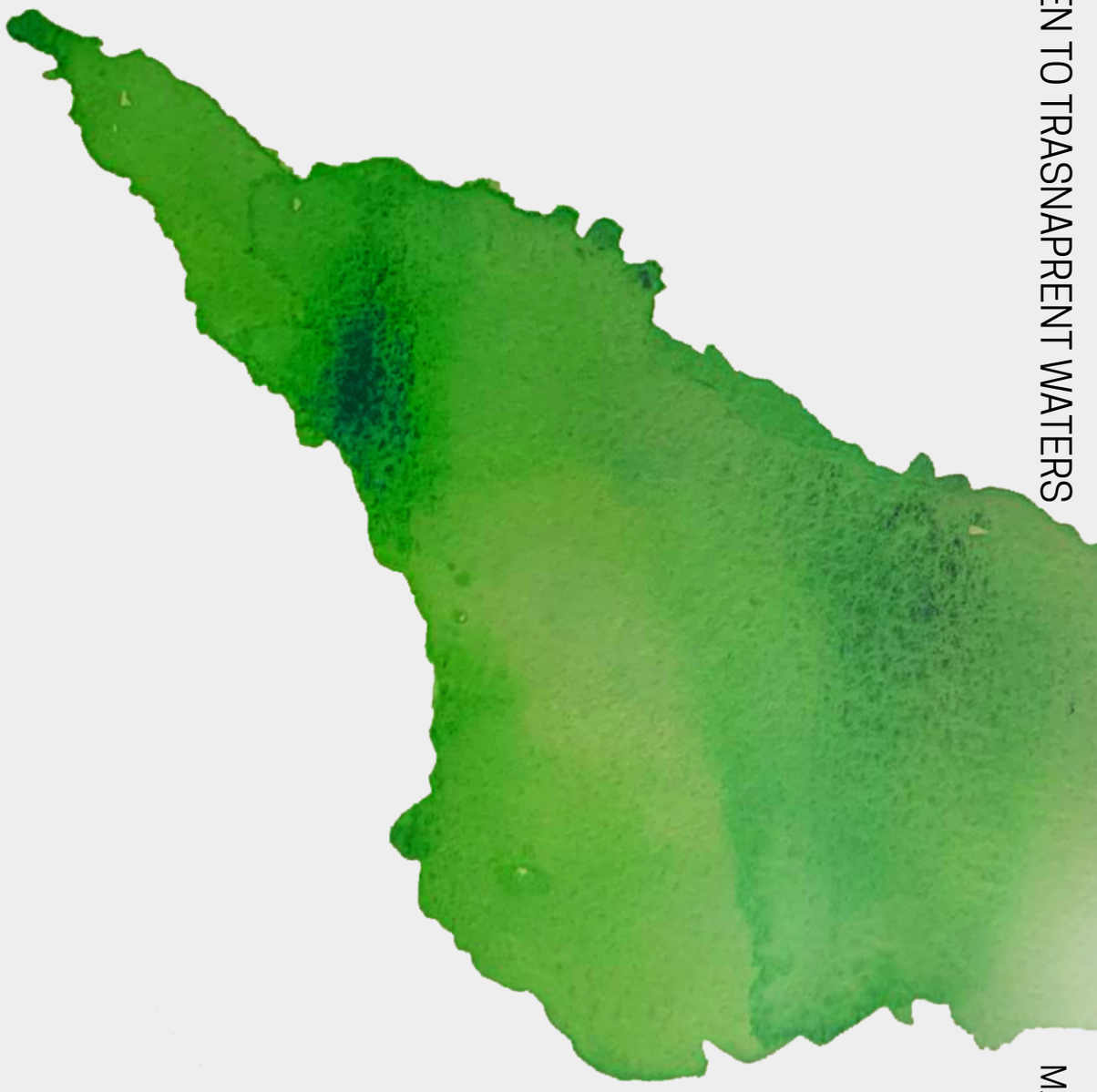

FROM GREEN TO TRASNAPRENT WATERS

MAÍRA N. T. MUCCI

FROM GREEN TO TRANSPARENT WATERS

Managing eutrophication and cyanobacterial blooms by geo-engineering

MAÍRA N.T. MUCCI

Invitation

You are kindly invited to attend
the public defense of
my PhD thesis entitled:

FROM GREEN TO TRANSPARENT WATERS

Managing eutrophication
and cyanobacterial blooms
by geo-engineering

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Thursday 18 April 2019

11:00 | Aula of Wageningen University

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Propositions

1. Managing eutrophication is a continuous process.
(this thesis)

2. Copy and paste of eutrophication management techniques will generally not lead to successful lake restoration.
(this thesis)

3. Keeping scientific publications behind paywalls leads to restricted access of knowledge and money leakage from education to profitable publishers.

4. Competition within science hampers scientific progress.

5. Social injustice is fed by silence.

6. One's level of freedom is related to economic and cultural factors.

Propositions belonging to the thesis, entitled:

'From green to transparent waters - Managing eutrophication and cyanobacterial blooms by geo-engineering'.

Maíra Mucci, 18 April 2019.

From green to transparent waters

Managing eutrophication and cyanobacterial blooms by geo-engineering

Maíra N. T. Mucci

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From green to transparent waters

Managing eutrophication and cyanobacterial blooms by geo-engineering

Maíra N. T. Mucci

Thesis

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at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Thursday 18 April 2019
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Maíra N.T. Mucci

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1

General introduction and thesis outline

This chapter contains parts from Mucci, M. 2017. *Mitigating algal blooms and eutrophication - the importance of system analysis and in-lake measures*. LakeLine magazine – Student Corner.

Eutrophication – causes and consequences

Earth was not always this magnificent blue marble with white sand beaches, colourful trees, delightful waterfalls, and fascinating creatures. Actually, if we could travel back on time approximately 4.5 billion years ago we would not be able to breathe. There was no dioxygen (O_2) in the atmosphere until the Great Oxidation Event. The key organisms behind the oxygen evolution that made complex life to flourish were cyanobacteria. Also nowadays, they play an important role in the carbon cycle, being responsible for half of the primary production in the oceans (Gadd and Raven, 2010). However, as advised by Aristotle, moderation is crucial, and their excessive growth has been turning water bodies into smelling green soups (Fig 1.1).

Figure 1.1: Green lagoon (Rio de Janeiro), pond (Wageningen), and canal (Groenlo) suffering from cyanobacterial blooms

The main reason behind an overgrowth of cyanobacteria is the over-enrichment of water bodies with nutrients, mainly nitrogen (N) and phosphorus (P), a phenomenon called eutrophication. Eutrophication is a natural aging process; over time, some aquatic systems accumulate more nutrients. However, anthropogenic activities are turning eutrophication one of the major worldwide water quality issues (Downing, 2014).

Agriculture, aquaculture, and livestock activities together with residential/industrial sewage discharge are the main outflows from human activities that cause eutrophication (Carpenter et al., 1998; Smith, 2003). As previously mentioned, the main consequence of eutrophication is the overgrowth of primary producers (macrophytes and phytoplankton), mainly cyanobacteria, that can proliferate to extremely high densities (Fig 1.1). Consequently, cyanobacterial blooms

result in turbid and malodorous water, as well as oxygen deficiency that may lead to fish kills (Paerl and Huisman, 2009; Smith and Schindler, 2009). Some cyanobacteria are also able to produce toxins which can be a threat to human and wildlife health (Azevedo et al., 2002; Faassen et al., 2012; Lürling and Faassen, 2013). Therefore, cyanobacterial blooms pose a major threat to the use of aquatic bodies for fishing, irrigation, recreational and as sources of drinking water all of which may result in severe economic impact (Dodds et al., 2009; Hamilton et al., 2013). Eutrophication and phytoplankton overgrowth are not an exclusive problem of freshwater systems. In fact, in coastal environments, eutrophication is considered the second most important stress on estuaries worldwide after habitat loss and alteration (Kennish, 2002 and references therein). Despite their global expansion, to mitigate eutrophication and cyanobacterial blooms remain a significant challenge to water managers.

Solutions

As simple as it is, managing eutrophication demands reduction of the available nutrients. The most straightforward step is to reduce the external nutrient input as much as possible (Cooke, 2005). There are authors who advocate that external nutrient load reduction is the only sensible measure (Hamilton et al., 2016; Paerl et al., 2016b), while others emphasize that also in-lake actions are often absolute necessity to mitigate eutrophication (Lürling et al., 2016; Osgood, 2017). As only external load control, might not bring any changes or it may take decades to centuries until the system recovers (e.g. Fastner et al., 2016). The delay in recovery relies on the legacy of nutrients (mainly P) stored in the sediment (Søndergaard et al., 2001). Besides, external nutrient load control is not always possible (Huser et al., 2016b) or it cannot be economically feasible. For instance globally, around 80% of the wastewater released to the environment is not adequately treated, and the high upfront investment costs imply that it will take many years before effective treatment and circular economies will be implemented in underdeveloped countries and countries in transition (van Loosdrecht and Brdjanovic, 2014; WWAP - United Nations World Water Assessment Programme, 2017). This does not, however, mean citizens and users of the receiving waters should wait until catchment and point source reductions lead to improved water quality. Several in-site interventions can be applied to immediately mitigate eutrophication nuisance, which might require repeated interventions until the needed catchment/external control has become effective.

N and P controversy

As eutrophication is the over-enrichment of surface water with nutrients, there is a continued discussion whether both nitrogen and phosphorus should be reduced or only phosphorus (Paerl et al., 2016b; Schindler et al., 2016). Those in favour of only P removal provide numerous field data in which P reductions were effective (Douglas et al., 2016b; Epe et al., 2017; Huser et al., 2016a; Lürling and Van Oosterhout, 2013; Nürnberg and Lazerte, 2016; Schindler et al., 2008; Waajen et al., 2016a). Those in favour of a joint N and P reduction show that an addition of both nutrients results in more phytoplankton biomass (Chen et al., 2013; Conley et al., 2009; Paerl et al., 2016; Paerl et al., 2011a, 2011b). However, further enrichment experiments to decipher the element that is limiting (Lewis et al., 2011) have limited predictive value to determine which element should be reduced. They only indicate which element should not be increased. The Law of the Minimum developed by Carl Sprengel (Sprengel, 1831) was illustrated using a barrel metaphor for the first time in 1903 by Hans Arnold (Gröger, 2010). The barrel represents that as well as the retention capacity is limited by the shorter staves, the algal growth is also limited by the element with lower supply. Thus, once the limitation in one element is lifted, another element will become limiting. Therefore, it is evident that the excess of both N and P will increase algal growth. However, to limit algal growth the reduction of only one element ("stave") is required (Fig 1.2). Based on the capability to form insoluble salts with aluminium, lanthanum, iron, and other metals, P-control is the single most logical target to manage eutrophication (e.g., Bishop et al., 2014; Carpenter, 2008; Douglas et al., 2016b; Epe et al., 2017; Huser et al., 2016a; Lürling et al., 2016; Lürling and Van Oosterhout, 2013; Mehner et al., 2008; Meis, 2012; Nürnberg and Lazerte, 2016;Schindler et al., 2008; Waajen et al., 2016a). Hence, the work presented in this thesis focuses on P control.

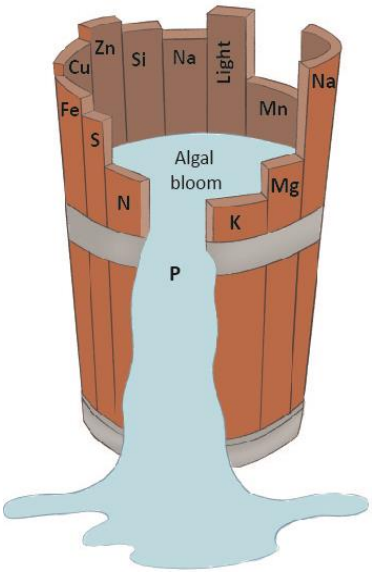

Figure 1.2: Barrel metaphor showing that all sources are needed for an algal bloom to occur, however only one source needs to be limited to control algal bloom formation. Drawn by G. Sant`Anna

Understanding the problem: system analysis

Cyanobacterial blooms are a global problem and the solution seems simple: to limit P. However, each aquatic system is unique and sources of P may differ, which may require different solutions for each system. Thus, to mitigate cyanobacterial blooms in a successful way, the first step is to understand the problem through a system analysis (SA) consisting of a nutrient balance, biological composition, and a cost-benefit analysis (Cooke, 2005; Lürling et al., 2016). It is important to understand from where (e.g. urban runoff, groundwater, sediment) and how much phosphorus enters the system to comprehend which source are the main driver of cyanobacteria blooms (Fig. 1.3). Then, it should also be noted that cyanobacterial blooms are not always a result of eutrophication as they are also observed in oligotrophic lakes (Nimptsch et al., 2016). These local accumulations of cyanobacteria are caused by the positive buoyancy of the cyanobacteria leading to surface accumulation and further concentration on leeside shores. Since this can happen when very low densities in oligotrophic water, during calm weather, aggregate at the surface, not all cyanobacterial problems are driven by over-enrichment with nutrients and thus not all require actions directed against reducing nutrients. This underpins the importance of a SA.

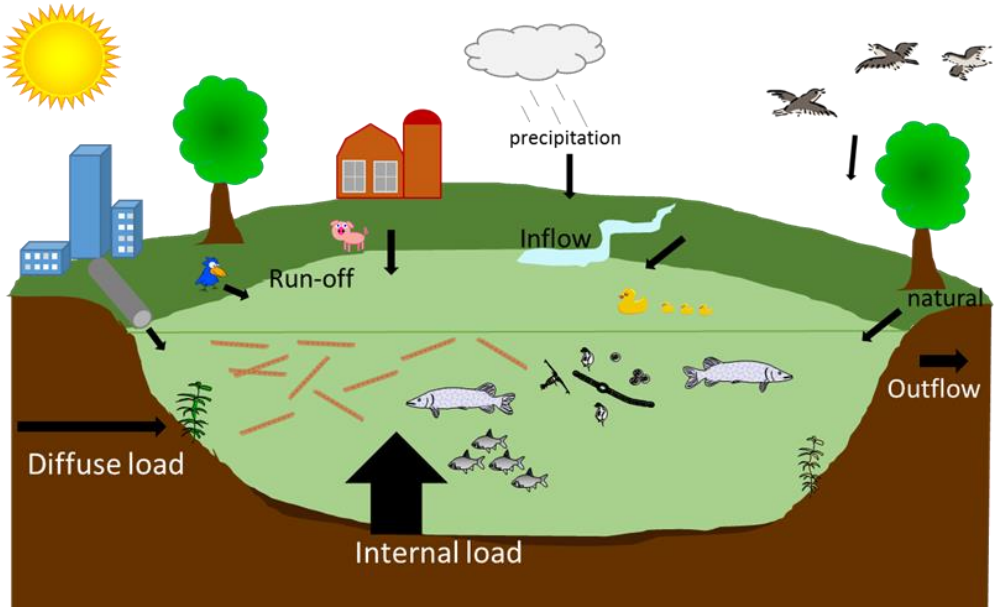

Figure 1.3: Scheme illustrating possible phosphorus sources (arrows) entering and leaving a lake that must be quantified as a first step to manage eutrophication. Modified from M. Lürling.

The SA must also determine biological composition of the water body and, for instance, include the fish stock and composition/abundance of macrophytes. Finally, the SA should include a cost-benefit analysis including the severity of the problem. For example, a contaminated lake in low-income countries which provides the only source of drinking water is more critical compared to contamination in recreational water bodies in high-income countries, where drinking water is supplied from safe sources. A proper system analysis will guide to the most promising set of measures to mitigate blooms, however the outcome can also be maintaining a *status quo*, "do nothing" when the measures are far too expensive or virtually impossible to implement.

Geo-engineering approach to managing eutrophication

Many tools have been used to manage eutrophication and cyanobacteria blooms: such as biomanipulation, algaecides, coagulants with clays, oxygenation, artificial circulation, catchment control, dredging and P-inactivation. Decisions on which tool(s) to use from this toolbox should be based on the outcome of a proper SA and on experimental tests on various scales (Lürling et al., 2016). From this set of tools, geo-engineering materials can be a powerful measure. Geo-engineering is defined here as "the manipulation of biogeochemical processes using P-removing materials" and it has been used worldwide as an effective tool to manage eutrophication and cyanobacterial blooms (Lürling et al., 2016; Mackay et al., 2014; Spears et al., 2014).

In this context, solid-phase P sorbents (SPS) have gained attention (Douglas et al., 2016a; Spears et al., 2013c). SPS can be natural soils, clays, and minerals or modified/synthesized materials (Douglas et al., 2004); that when applied in an aquatic system are able to adsorb the phosphate in the water column and when settled on the lake bed they will reduce the P-release from the sediment (Fig. 1.4A). Thus, the mode of action is preventing cyanobacteria overgrowth by strongly reducing the amount of available P. The most used P-sorbents in lake restoration have been lanthanum (La) and aluminium (Al) compounds (Cooke, 2005; Copetti et al., 2016), for instance, aluminium salts such as Alum and Lanthanum modified bentonite, commercially called Phoslock®.

To be considered as a potentially useful SPS, the material needs to be effective, relatively cheap, safe, and easy to manufacture and to apply (Lürling et al.,

2016). Thus, the first step to assess the feasibility of a SPS is through a series of laboratory studies.

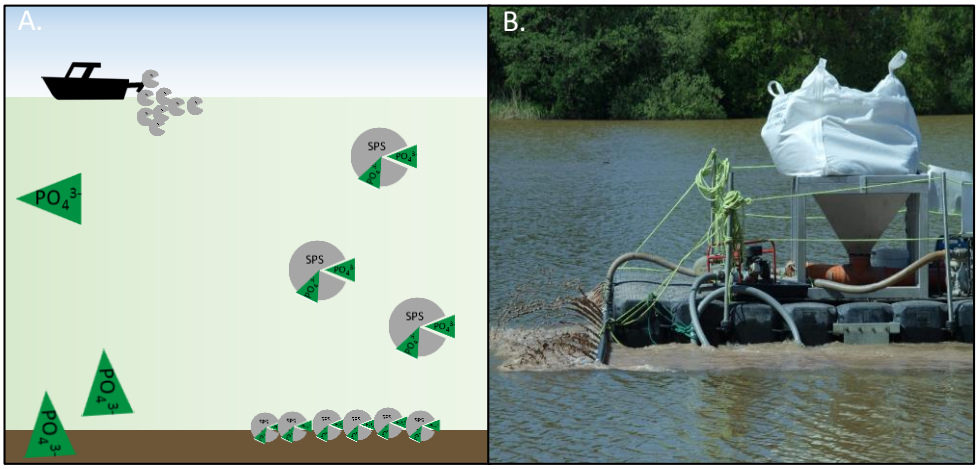

Figure 1.4*:* Panel A: principle of solid-phase P sorbent adsorbing phosphate (PO_4^{3-}) and reducing the PO_4^{3-}-release from the sediment. Panel B: a solid phase P sorbent, lanthanum modified bentonite being applied at the surface of Lake De Kuil - The Netherlands (picture from G. Waajen).

Inasmuch as SPS will only reduce phosphate and will not target P already used in cyanobacterial biomass directly, application of a SPS when a system is already suffering from a bloom will not bring a quick relief. Hence, the SPS is preferably added when the dissolved P concentration in the water column is higher than particulate P, which is mostly during winter in temperate systems. In case of perennial blooms, or application during already developed bloom, a low dose of flocculants (e.g., Polyaluminium chloride – PAC, and chitosan) can be combined with the SPS to flock the cyanobacteria and sink them out of the water column; so they can decay on the lake bed, whilst simultaneously released phosphate from the decaying biomass and from the sediment is bound – Technique called Flock and Lock (Fig. 1.5).

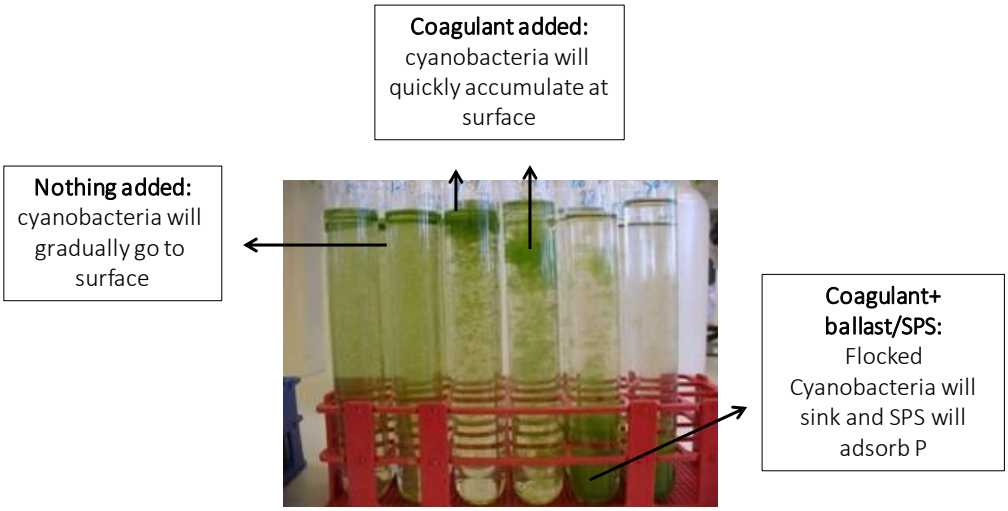

Figure 1.5: Demonstration of the Flock and Lock technique

This approach has been implemented effectively a few times using lanthanum modified bentonite (LMB) as a ballast/SPS and PAC or iron chloride as coagulant (Fig 1.4B) (Lürling and Van Oosterhout, 2013; Waajen et al., 2016a). Recently, an organic and biodegradable coagulant, chitosan, has gained attention as a possible alternative for inorganic and non-biodegradable metal based coagulants (Li and Pan, 2013).

Thesis outline and aims

The aim of this thesis was to study potential materials and in-situ techniques to manage eutrophication and control cyanobacterial blooms. For this, controlled laboratory experiments were performed and whole-lake intervention closely monitored.

As phosphorus control is essential to manage eutrophication, the thesis starts evaluating ten possible clays/soils in their ability to adsorb phosphate. As water bodies can have different chemical conditions, the compounds were tested in a realistic pH range and under anoxic conditions (chapter 2).

Since eutrophication also occurs in coastal systems; in chapter 3, the modified clay (LMB) that performed best according to chapter 2 was tested under different salinities looking at its P-removing efficiency in conditions with high amounts of potential competing salts.

Organic coagulants have been proposed as an alternative for inorganic coagulants to remove cyanobacteria biomass in lake restoration. Thus, in chapter 4 the effect of the, so-called "eco-friendly" coagulant, chitosan, was tested on five different cyanobacterial species.

From chapter 4, we learned that chitosan may damage the cell membranes of cyanobacteria, but that the effect seems to be strain specific and not so strong in a common bloom-forming species (*Microcystis aeruginosa*). Therefore, in chapter 5, the impact of chitosan on eight strains of *M. aeruginosa* was evaluated, which included determination of cyanotoxin release.

In chapter 6, a whole lake treatment was performed using the coagulant PAC together with the lanthanum modified bentonite, which was studied in chapter 2 and 3 . In chapter 6, the proposed treatment was first tested in sediment cores collected from the lake, where after it was performed on full-scale. The monitoring results were evaluated to shed light on the efficacy of the treatment in managing eutrophication and cyanobacterial blooms.

Lastly, in chapter 7 reflections on the findings of this thesis are given highlighting the importance of system analysis followed by tests on different scales to effectively manage eutrophication and cyanobacterial blooms.

2

Assessment of possible solid-phase sorbent to mitigate eutrophication: Influence of pH and anoxia

Maíra Mucci, Valentini Maliaka, Natalia Pessoa Noyma, Marcelo Manzi Marinho, and Miquel Lürling.
Science of The Total Environment 619-620 (2018) 1431-1440

Abstract

Managing eutrophication remains a challenge to water managers. Currently, the manipulation of biogeochemical processes (i.e., geo-engineering) by using phosphorus-adsorptive techniques has been recognized as an appropriate tool to manage the problem. The first step in finding potential mitigating materials is conducting a sequence of upscaling studies that commence with controlled laboratory experiments. Here, the abilities of 10 possible solid–phase-sorbents (SPS) to adsorb P were examined. Four materials adsorbed P, and two of these materials were modified, i.e., a lanthanum-modified-bentonite (LMB) and an aluminum-modified-zeolite (AMZ), and had the highest adsorption capacities of 11.4 and 8.9 mg P g^{-1}, respectively. Two natural materials, a red soil (RS) and a bauxite (BAU), were less efficient with adsorption capacities of 2.9 and 3.4 mg P g^{-1}, respectively. Elemental composition was not related to P adsorption. Since SPS might be affected by pH and redox status, we also tested these materials at pH values of 6, 7, 8 and 9 and under anoxic condition. All tested materials experienced decreased adsorption capacities under anoxic condition, with maximum adsorptions of 5.3 mg P g^{-1} for LMB, 5.9 mg P g^{-1} for AMZ, 0.2 mg P g^{-1} for RS and 0.2 mg P g^{-1} for BAU. All materials were able to adsorb P across the range of pH values that were tested. The maximum adsorption capacities of LMB and RS were highest at pH 6, AMZ was higher at a pH of 9 and BAU at a pH of 8. Thus, pH influenced P adsorption differently. Given the effects of pH and anoxia, other abiotic variables should also be considered. Considering the criteria that classify a useful SPS (i.e., effective, easy to produce, cheap and safe), only the two modified materials that were tested seem to be suitable for upscaling to enclosure studies with anoxic sediments.

Introduction

Phosphorus is a double edged sword, it is essential for all forms of life and crucial for the functionality of aquatic systems; however, if phosphorus is excessively present in waterbodies, it can constitute one of the main water quality issues worldwide: eutrophication (Moss, 1988; Smith and Schindler, 2009). Phosphorus (P) enters a water body naturally, through soil erosion caused by rainfall and degradation of organic matter. However, outflowing water from diverse anthropogenic activities, such as intensive agriculture, livestock and industrial/sewage discharge, are often the main sources of P that contribute to the intensification of eutrophication.

One of the key consequences of eutrophication is the blooming/overgrowth of phytoplankton, specifically cyanobacteria that may proliferate to very high densities and result in turbidity and malodor of the water, as well nocturnal oxygen deficiency that may lead to fish kills (Paerl and Huisman, 2009; Smith and Schindler, 2009). In addition, many cyanobacteria are able to produce toxins, which can cause serious and fatal liver, digestive and neurological damage in animals and humans; thus, eutrophication can pose a major threat to the use of lakes, reservoirs, ponds and rivers as sources of drinking water, for irrigation, fishing and recreation (Carmichael et al., 2001; Codd et al., 2005; Dittmann and Wiegand, 2006). Consequently, eutrophication has severe economic impacts, as water quality will be reduced, recreational activities will decrease, commercial fisheries and aquaculture will be impaired and property values will decrease (Dodds et al., 2009; Hamilton et al., 2013). For instance, in freshwater bodies alone, the USA has an estimated cost of approximately $2.2 billion annually as a result of eutrophication only in freshwater (Dodds et al., 2009). In Lake Tai (China), a single forced shutdown of an entire water supply system due to eutrophication/algal bloom had a cost of around $30 million. In the same lake - after nine incidents of fish kills - the cost was estimated to be $6.5 billion (Le et al., 2010). In England and Wales, the costs of damages caused by freshwater eutrophication are estimated to be between $ 105 - 160 million per year (Pretty et al., 2003).

The first step to managing eutrophication is to reduce the direct external source of P to the water body as much as possible (Cooke et al., 2005; Hilt et al., 2006). Such reductions may cause changes in some systems; however, delays in recovery may occur in most systems (Fastner et al., 2016) as a result of the release of P from a legacy pool stored in the sediments (Søndergaard et al., 2001). To speed up recovery and drastically reduce the time between nutrient control and the desired outcome in

a catchment, in-lake targeting of this legacy P has been proposed (Cooke, 2005; Spears et al., 2013c). In addition, in cases where catchment management is difficult (Huser et al., 2016b), or where relatively low diffuse loading has led to severe internal loading (Waajen et al., 2016a), the in-lake targeting of P through solid-phase P sorbents (SPS) has gained attention in the reduction of sediment P-release (Spears et al., 2013c). SPS can be divided into (1) natural occurring minerals or earth materials, such as carbonates, soils and sands; (2) natural or synthetically produced materials, such as allophane, hydrotalcites, iron- and aluminum-oxides and (oxy)hydroxides; (3) modified clay mineral or soil, such as expanded/thermally treated clay aggregates and rare earth modified clays, zeolites and soils; (4) mining, mineral processing and industrial by-products, such as red mud, slags and coal fly ash (Douglas et al., 2016).

To be considered as a potentially useful SPS, the compounds need to meet four criteria: (1) they should be effective, (2) they should be easy to manufacture and apply, (3) they should be relatively cheap and (4) they should be safe. These criteria are commonly determined in a series of upscaling experiments (Lürling et al., 2016). The first step to assess possible SPS in the sequence of upscaling experiments is through a series of laboratory studies that evaluate maximum adsorption capacities using isotherm models (such as the Langmuir model). Modified clays, such as Phoslock® and Aqual-P™, have a relatively high soluble reactive phosphorus (SRP) adsorption capacity compared to other natural materials (Gibbs et al., 2010; Noyma et al., 2016); however, the price of these products might be too high for large applications in developing countries, or for repeated interventions. Thus it remains important to search for cheaper alternatives. Of particular interest here are natural materials such as soils, bauxites and zeolites, because of their widespread occurrence, ease of retrieval and low transportation costs when obtained in close vicinity of the eutrophic water body.

Environmental factors may strongly influence the efficacy of SPS (e.g., Douglas et al., 2004; Gibbs et al., 2010; Lürling et al., 2014b; Noyma et al., 2016; Ross et al., 2008), and therefore, potential SPS should be subjected to controlled experiments that evaluate efficacy and potential side effects prior to application in the environment. Two important environmental factors are pH and redox state. The pH can vary considerably in waters due to photosynthesis, decomposition, buffering capacity and pollution, but in general, the pH in most lakes is between 6 and 9 (Wetzel, 2001). The pH has a considerable influence on the adsorption of P, as it changes both the charges of adsorbing surfaces as well as the speciation of the phosphate ion; in general, P adsorption tends to decline with higher pH levels

(Geelhoed et al., 1997). In addition to pH, the sensitivity of SPS to different redox potentials is of great importance, as most eutrophic lakes regularly experience anoxia, and thus, low redox potentials occur near the sediment. The bacterial breakdown of organic matter leads to the depletion of oxygen and, subsequently, leads to the reduction of iron, which results in the strong decrease in the P-binding capacity of sediments and the release of iron-bound P (Smolders et al., 2006). Since the main target of the SPS is this releasable P-pool in the sediment, SPS should not be redox sensitive.

In this study, eight natural materials (including zeolite, bentonite, bauxite and red soil) were studied and their phosphate (SRP) adsorption characteristics were evaluated and compared with two modified products: the lanthanum modified bentonite Phoslock® and the aluminum modified zeolite Aqual-P™. The main chemical composition of the tested materials was determined. We hypothesized that all compounds would adsorb SRP, albeit with different efficiencies, where the modified products were expected to perform best. Subsequently, the four best-performing materials were subjected to anoxia, and their maximum P adsorption capacities were studied at four different pH values. We expected a minor influence of anoxia on P adsorption due to the redox sensitivity of iron in the materials tested, and we expected a lower efficiency at elevated pH levels, because of competition with hydroxyl ions and charge differences.

Methods

Solid-phase P sorbents (SPS)

Bauxite from Brazil (BAU Br) was obtained from Mineração Rio do Norte S.A (Pará, Brazil). Bauxite 1 and 2 from Greece (BAU 1 Gr and BAU 2 Gr) were obtained from EUROPEAN BAUXITES S.A, (Amfissa, Greece). Bentonite (BEN NL) was obtained from Aldrich (The Netherlands); and red soil from Brazil (RS Br) was collected from the banks of the Funil Reservoir (Rio de Janeiro). Red soil from Greece (RS Gr) was collected from the surroundings of Lesser Prespa Lake (Prespa, Greece). Zeolites were obtained from the company Zeoprofit Hellas P.C. (Thessaloniki, Greece; ZEO Gr) and from Zeolites Products (Varsseveld, The Netherlands; ZEO NL). The La-modified bentonite Phoslock® (LMB) was obtained from Phoslock® Europe GmbH (Ottersberg, Germany). The Al-modified zeolite Aqual-P™ (AMZ) was obtained from Blue Pacific Minerals (Tokoroa, New Zealand) (Table 2.1).

Table 2.1: Overview of P-sorbents used

Solid phase P-sorbent	Abbreviation
Aluminium modified zeolite Aqual-P	AMZ
Lanthanum modified bentonite Phoslock®	LMB
Natural zeolite Netherlands	ZEO NL
Natural bentonite Netherlands	BEN NL
Natural red soil Brazil	RS Br
Natural bauxite Brazil	BAU Br
Natural red soil Greece	RS Gr
Natural zeolite Greece	ZEO Gr
Natural bauxite 1 Greece	BAU 1 Gr
Natural bauxite 2 Greece	BAU 2 Gr

Material composition

To analyze the chemical composition 200 mg of dried material of each SPS was brought in Teflon vessels, and 4 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30%) were added and the vessels were sealed. Subsequently, the materials were digested using a microwave oven (Ethos D microwave lab station; Milestone S.r.l., Sorisole, Italy), and unfiltered supernatant solution was collected and analyzed by ICP-AES (IRIS Intrepid II; Thermo Electron Corporation, Franklin, MA, USA), as described by Kleinebecker et al. (2013). The procedure was done using four replicates for each material. Phoslock® analysis for lanthanum and arsenic was performed in duplicate by ICP-MS in the Chemical-Biological Soil Laboratory of the Department of Soil Sciences (Wageningen University).

SRP adsorption experiments

Batch SRP (soluble reactive phosphorus) adsorption experiments were carried out to evaluate the SRP adsorption of the above-mentioned ten different materials (Table 2.1). Eight SRP solutions containing 0, 5, 10, 20, 40, 80, 120 and 160 mg P L^{-1} were prepared by dissolving K_2HPO_4 in nanopure water. A total volume of 200 mL SRP solution and 400 mg of each material was combined in 250 mL glass bottles; the bottles were closed with a screw cap. The experiment was conducted in triplicate. The suspensions were continuously mixed on a shaker at 180 rpm for 24 hours at 22°C; afterwards, samples were centrifuged for 10 minutes at 2500 rpm. The supernatant was filtered through unit filters (Aqua 30/0.45CA, Whatman, Germany). Filtrates were analyzed for their SRP concentration using a Skalar SAN+ segmented flow analyzer following the Dutch standard NEN-EN-ISO 15681-2:2005. This part of

ISO 15681 specifies the determination of orthophosphate contents by continuous flow analysis (CFA) (NNI, 1986). To calculate the maximum SRP adsorption the data were fitted using the isotherm Langmuir model:

$$\frac{Ce}{Qe} = \frac{1}{qmKL} + \frac{Ce}{qm}$$

in which *Ce* is the equilibrium concentration of the adsorbate (SRP, mg L^{-1}), *Qe* is the adsorption capacity at equilibrium (mg g^{-1}), *qm* is maximum adsorption capacity (mg g^{-1}), and *KL* is the Langmuir adsorption constant (L mg^{-1}). Plotting $\frac{Ce}{Qe}$ versus *Ce*, the slope will be $\frac{1}{qm}$ and the intercept will be $\frac{1}{qmKL}$ making it possible to calculate *qm* and *KL*. *Ce* and *Qe* will be obtained from the adsorption experiment:

$$Qe = \frac{(C_0 - Ce)V}{M}$$

where *Qe* is the amount of SRP adsorbed at equilibrium (mg g^{-1}), C_0 is the initial concentration of SRP in solution (mg L^{-1}), *Ce* is the concentration of SRP in solution at equilibrium (mg L^{-1}), *M* is the adsorbent mass used (g) and *V* is the volume of SRP solution (Langmuir, 1918). To assess the reaction speed of each material the Michaelis-Menten constant (K_m) was calculated, which is defined as the SRP concentration at half of the maximum adsorption capacity (*qm*) (Mosier and Ladisch, 2009):

$$V = \frac{qm\ Ce}{Km + Ce}$$

where *V* is the velocity at any time, *Ce*, *qm* and *Km* as defined previously. The maximum P adsorption capacities and *Km* were obtained by fitting the values in a non-linear regression of the Langmuir equation and in the Michaelis-Menten kinetics equation, respectively, using SigmaPlot version 13.0.

The influence of anoxia on maximum P adsorption

Batch experiments using the same methods described above were performed under anoxia. Hereto, all the glass bottles were bubbled with nitrogen gas for 5 minutes in order to create anoxic conditions. The materials used included AMZ, LMB, RS Br and BAU 1 Gr, which had shown SRP adsorption capacity in the first experiment.

After 24 hours samples were analyzed for phosphate as described before. Oxygen concentrations were measured with an oxygen electrode (WTW pH-320 meter) at the end of the experiment to confirm the anoxic condition of all the bottles. For LMB the same experiment was repeated three times, to determine variability among the experiments.

The effect of pH on P adsorption

Batch adsorption experiments using AMZ, LMB, RS Br and BAU 1 Gr were run to evaluate the effect of pH on SRP adsorption. Thus, the same approach was used as described before; however, this time, pH was changed by adding HCl (0.1 M) or NaOH (0.1 M) in order to obtain four different pH values (pH 6, 7, 8 and 9) for each material.

The effect of P-sorbents on the pH of ultrapure water

To evaluate if AMZ, LMB, RS Br and BAU 1 Gr affected the pH of the water, another batch experiment was performed, in which 0, 200, 400 and 800 mg of each compound was added to separate glass bottles containing 200 mL of ultrapure water, yielding 0, 1, 2 and 4 g L^{-1}. The ultrapure water had a pH of 6.8 ±SD 0.48. After 1 hour, pH was measured again using a WTW pH-320 meter. The experiment was run in triplicate. Final pH values were statistically compared running a two-way ANOVA, but since the test for normality (Shapiro-Wilk) failed ($p < 0.050$) and data transformation could not solve this issue, separate one-way ANOVAs were run for BAU 1 Gr, LMB and RS Br, and a non-parametric Kruskal-Wallis one way analysis of variance was used for AMZ as the data were not normally distributed. The latter was followed by a Tukey post hoc multiple comparison test, while the ANOVAs were followed by Holm-Sidak all pairwise multiple comparison procedures.

Results

Elemental composition of the ten tested solid-phase P-sorbents (SPS)

Aluminum (Al) was generally the second most abundant element measured in all materials (Table 2.2). In the AMZ, the Al mass fraction (24.07 g kg DW^{-1}) was lower than in the two unmodified zeolites (32.63 g kg DW^{-1} in ZEO Gr and 30.5 g kg DW^{-1} in ZEO NL; Table 2.2). The sum of Al and Fe was highest in the red soils (RS Br, RS Gr) and bauxites (BAU 1 Gr, BAU 2 Gr, BAU Br) comprising 85.2 to 223 g kg DW^{-1} of the major alkali-, earth and rare earth elements measured; in contrast, in the zeolites and bentonites, the sum of Al and Fe varied between 25.5 and 36.6 g kg DW

$^{-1}$ (Table 2.2). The mass fractions of Al+Fe ranged from 90.4 g kg DW $^{-1}$ in BAU 2 Gr and 223.6 g kg DW $^{-1}$ in BAU Br, while it was 19.6 g kg DW $^{-1}$ in LMB and as high as 36.6 g kg DW $^{-1}$ in ZEO Gr (Table 2.2). Potassium was highest in the zeolites, while Mg was highest in the bentonites; Ca had the highest mass fractions in BEN NL, LMB, ZEO Gr and ZEO NL; and Na, Mn, Si, S and P were present in lower abundances (Table 2.2). Other elements were measured and sometime showed differences between compounds; for instance, Cr had the highest mass fractions in the bauxites and red soils, and Ni was highest in the bauxites (Table 2.2). Lanthanum in LMB was on average 37.8 g kg DW $^{-1}$ (Table 2.2).

Table 2.2: Composition of the materials used. Major element (in the top) are in grams of elements per kg DW of each material. Minor elements (next page) are in milligram of elements per kg DW of each material. Mean values (n=4), except for La and As for LMB (*n*=2). Standard error (underlined) are reported.

Major elements (g Kg DW $^{-1}$)											
Materials	Al	Ca	Fe	K	Mg	Mn	Na	P	S	Si	La
AMZ	24.07	5.97	9.28	8.36	2.39	0.65	17.79	0.18	2.20	0.17	
	1.78	0.54	0.68	0.16	0.23	0.07	2.50	0.17	0.22	0.04	
LMB	12.65	10.19	7.01	0.86	4.39	0.21	5.36	0.51	0.45	0.98	37.8
	1.22	0.49	0.22	0.02	0.12	0.01	0.28	0.15	0.04	0.94	0.14
BEN NL	14.48	18.35	11.03	2.16	11.44	0.17	0.16	0.45	0.29	0.59	
	1.69	0.82	0.84	0.22	0.78	0.01	0.18	0.16	0.05	0.32	
ZEO NL	30.54	15.92	3.90	13.84	3.30	0.15	0.68	0.27	0.04	0.24	
	0.84	0.78	0.17	0.45	0.16	0.03	0.22	0.15	0.05	0.07	
ZEO Gr	32.63	16.15	4.01	17.88	2.35	0.11	1.13	0.19	0.08	0.37	
	1.77	0.60	0.48	1.05	0.36	0.01	0.26	0.13	0.03	0.11	
BAU 1 Gr	12.29	8.60	85.94	0.08	0.22	0.08	0.04	0.30	0.81	0.24	
	0.66	0.47	11.33	0.01	0.04	0.01	0.04	0.15	0.02	0.14	
BAU 2 Gr	17.07	3.93	73.37	0.30	0.39	1.66	0.13	0.18	0.04	0.24	
	0.66	0.05	14.48	0.01	0.07	0.20	0.16	0.15	0.03	0.13	
BAU Br	164.14	0.02	59.48	0.01	0.00	0.00	0.03	0.17	0.17	0.24	
	0.66	0.05	14.48	0.01	0.07	0.20	0.16	0.15	0.03	0.13	
RS Br	31.75	1.53	53.45	2.18	0.67	0.28	3.86	0.37	0.70	0.52	
	15.01	2.73	4.85	3.90	1.04	0.31	7.64	0.19	0.96	0.30	
RS Gr	27.27	1.78	52.19	1.98	1.39	0.73	0.08	0.72	0.15	1.27	
	3.21	0.03	2.72	0.29	0.07	0.07	0.09	0.17	0.04	1.46	

Minor elements (mg Kg DW $^{-1}$)

Materials	As	B	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	Sr	Zn
AMZ	19.22	25.29	0.68	2.10	11.86	3.74	0.02	651	0.13	1.51	30.81	39.16	45.02
	1.31	13.70	0.21	0.14	8.52	0.67	0.03	69.06	0.15	0.33	1.27	2.27	3.68
LMB	0.8	95.60	0.03	5.12	16.72	7.14	0.00	208	<0.01	4.50	31.44	>900	26.23
	0.9	27.01	0.04	0.24	8.32	0.83	0.00	14.29	0.01	2.83	2.32		2.05
BEN NL	5.66	38.99	0.10	6.15	29.03	13.93	0.01	171	0.00	17.95	28.14	55.88	60.26
	1.20	23.35	0.15	0.51	13.02	1.90	0.02	12.89	0.00	1.50	3.25	3.26	4.51
ZEO NL	2.10	19.98	0.01	0.72	6.17	2.55	0.21	150.86	0.17	1.59	22.18	292	16.71
	1.10	5.19	0.02	0.34	3.97	0.45	0.29	27.01	0.17	0.41	2.01	4.16	1.80
ZEO Gr	0.76	14.50	0.05	0.56	5.79	1.44	0.00	106	0.06	1.75	20.80	329	18.78
	0.81	2.95	0.11	0.65	2.98	1.67	0.00	9.00	0.09	0.38	5.75	13.73	5.69
BAU 1 Gr	44.90	208	0.46	28.55	425	3.33	0.00	80.52	3.57	334.13	61.69	9.42	27.86
	3.96	112	0.55	2.23	33.30	3.85	0.00	8.75	0.38	14.28	7.14	0.81	4.16
BAU 2 Gr	1.24	184	0.01	24.31	230	23.58	0.00	1664	0.03	95.31	65.91	9.20	96.64
	1.52	86.96	0.01	2.71	23.97	27.23	0.00	198	0.06	5.44	2.93	0.77	8.45
BAU Br	0.44	138	0.00	0.48	138	0.00	0.00	0.32	0.00	1.71	73.61	0.06	2.92
	1.52	86.96	0.01	2.71	23.97	27.23	0.00	198	0.06	5.44	2.93	0.77	8.45
RS Br	4.77	120	0.00	0.89	103	3.84	0.18	276	0.00	3.49	39.61	18.09	25.46
	7.97	69.73	0.00	0.72	39.28	4.69	0.26	306	0.00	1.59	19.15	17.95	21.82
RS Gr	22.48	121	0.18	29.67	115	6.01	0.00	734	0.15	24.89	45.04	19.02	39.15
	1.94	64.82	0.20	3.16	33.93	6.94	0.00	65.96	0.31	3.35	6.12	1.22	2.41

SRP adsorption

Four out of the ten SPS materials tested showed the capacity to adsorb SRP (Table 2.3; Fig. 2.1). The LMB and AMZ had the highest SRP adsorption capacities of 11.4 and 8.9 mg SRP g^{-1}, respectively. Both BAU 1 Gr and RS Br also showed the capacity to adsorb SRP, although their adsorption capacities were less than the two modified materials, with values of 3.4 and 2.9 mg SRP g^{-1}, respectively. The six other compounds (BAU Br, BAU 2 Gr, ZEO Gr, ZEO NL, RS Gr and BEN NL) demonstrated no SRP removal, and none of the compounds released any SRP (Table 2.3). LMB and BAU 1 Gr had the lowest *Km* with values of 3.4 and 8.2 mg SRP L^{-1}, respectively, demonstrating a faster SRP adsorption than AMZ and RS Br, which had *Km* values of 18.5 and 24.8 mg SRP L^{-1}, respectively (Table 2.3; Fig. 2.1).

Table 2.3: Maximum SRP sorption capacity of each SPS estimated using Langmuir isotherm (*qm* mean), including the including r^2 , *p* – and t –values. Also given is the possible SRP-release and Michaelis-Menten constant (*Km* mean). Values inside brackets are the standard error (SE) . < LOD = below level of detection (10 µg SRP L^{-1}).

Material	SRP Sorption capacity (mg SRP g^{-1})	r^2	*p*-value	t-value	K_m (mgSRPL^{-1})	SRP-release (µg SRPg^{1})
LMB	11.4 (1.5)	0.66	<0.001	7.6	3.4 (3.5)	< LOD
AMZ	8.9 (1.1)	0.82	<0.001	4.5	18.5 (8.9)	< LOD
RS Br	2.9 (1.1)	0.55	0.040	2.5	24.8 (32)	< LOD
BAU 1 Gr	3.4 (0.7)	0.61	0.003	4.7	8.15 (8.4)	< LOD
BAU Br	-	-	-	-	-	< LOD
ZEO Gr	-	-	-	-	-	< LOD
RS Gr	-	-	-	-	-	< LOD
BAU 2 GR	-	-	-	-	-	< LOD
BEN NL	-	-	-	-	-	< LOD
ZEO NL	-	-	-	-	-	< LOD

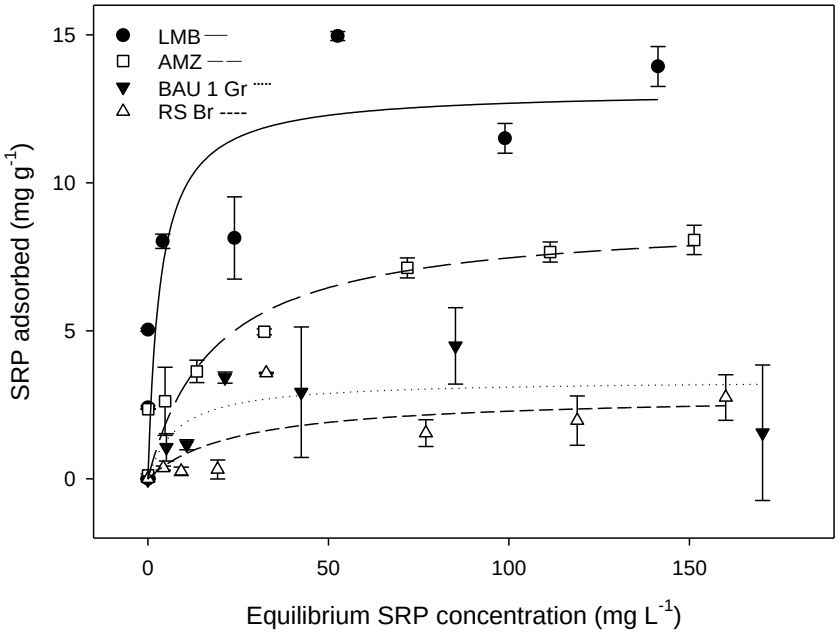

Figure 2.1: Langmuir adsorption isotherms under aerobe condition for the materials that showed SRP adsorption.

SRP adsorption capacity under anoxia

The four materials with the highest SRP uptake capacities (Table 2.3) all had a lower maximum SRP adsorption capacity under anoxia (Table 2.4). Whereas AMZ and LMB were still able to adsorb SRP under anoxic conditions, RS Br and BAU 1 Gr adsorbed very little SRP (Fig. 2.2; Table 2.4). None of the tested compounds released detectable amounts of SRP under anoxic condition (Table 2.4). A relatively small inter-assay coefficient of variation (11%) was found among the three LMB experiments. Based on the *Km* value, the SRP adsorption speed or binding affinity of LMB was approximately 120 times higher than AMZ (Table 2.4).

Table 2.4: Maximum SRP sorption capacities) of four P adsorbing materials under anoxic conditions determined with, including r^2 , *p* – and t – values of the Langmuir isotherms. Also given is the possible SRP-release from each material where < LOD = below limit of detection (10 µg SRP L^{-1}) and the Michaelis-Menten constant (K_m mean) reflecting the binding affinity where ND indicates that K_m could not be determined given the shape of the curve. Values inside the brackets are the SE.

Material	P Sorption capacity (mg P g^{-1})	r^2	*p-value*	*t-value*	K_m (mg SRP L^{-1})	P-release (µg P g^{-1})
LMB test 1	4.9 (0.9)	0.35	0.002	5.37	0.047 (0.06)	< LOD
LMB test 2	5.1 (1.1)	0.25	0.003	4.7	0.02 (0.02)	< LOD
LMB test 3	6.0 (0.5)	0.77	<0.0001	11.30	0.01 (0.001)	< LOD
AMZ	5. 9 (1.6)	0.38	0.010	3.6	3.17 (5.6)	< LOD
RS Br	0.2 (0.09)	0.22	0.062	2.4	ND	< LOD
BAU 1 Gr	0.2 (0.18)	0.22	0.072	2.2	ND	< LOD

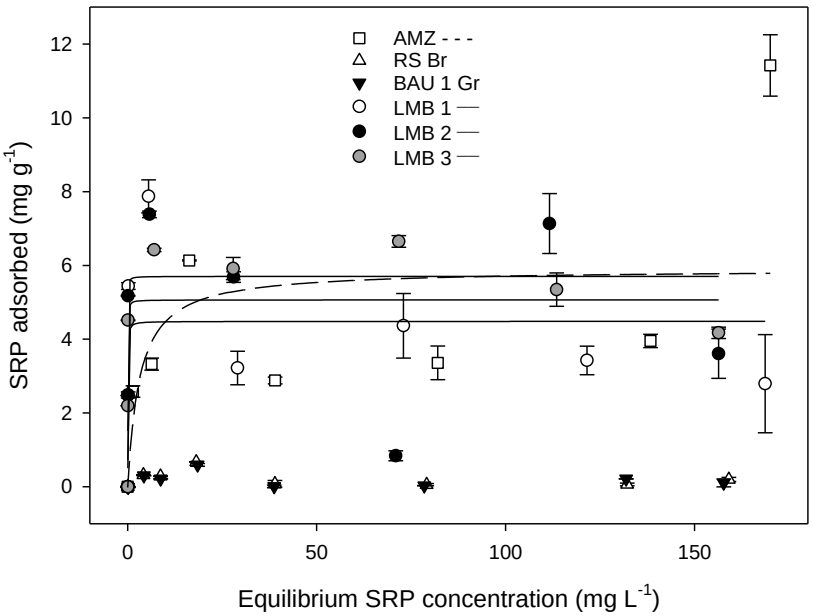

Figure 2.2: Langmuir adsorption isotherms under anoxic condition for RS Br, BAU 1 Gr, AMZ and the three experiments performed with LMB.

Effect of pH on SRP adsorption

In the batch experiments with AMZ, the SRP adsorption capacity was similar at pH values of 6, 7 and 8, but it was 35% higher at a pH of 9 (Table 2.5; Fig. 2.S1).

Nonetheless, the maximum SRP adsorption of 4.3-7.6 mg g^{-1} was lower than the 8.9 mg SRP g^{-1}, determined at pH of 8.8 ±SD 0.3 (Table 2.3). The *Km* values were similar at pH 7 and 8, higher at pH 9 and lowest at pH 6. The SRP adsorption capacities of LMB varied between 6.8 and 10.5 mg SRP g^{-1} (Table 2.5; Fig. 2.S2). At pH 9, the *Km* value was the highest, and the values at the other pH levels were similar. RS Br had the highest SRP adsorption capacity at pH 6 (4.5 mg g^{-1}) and gradually decreased in SRP adsorption capacity with an increasing in pH (Table 2.5; Fig. 2.S3). Considerable variation in SRP adsorption at different doses caused relatively poor *Langmuir* fits (r^2<0.2) (Table 2.5). At pH 6, the *Km* value was almost three times higher than at pH 8, while at pH 7 and 9, it was not possible to calculate *Km* because of poor model fits (Table 2.5). The adsorption capacity of BAU 1 Gr varied between 1.04 and 1.78 mg g^{-1}; *Km* values were high, and they increased with increasing pH to exceptionally high values (Table 2.5; Fig. 2.S4).

Effect of SPS on pH

The four positively tested compounds all influenced the pH of the resuspension; two materials (BAU 1 Gr and LMB) increased pH, while the other two materials (AMZ and RS Br) decreased pH (Fig. 2.3). The pH value in water treated with RS Br differed between treatments ($F_{3,8}$= 21.1; p < 0.001) and post hoc comparisons revealed that the pH of the control (7.05 ±SD 0.50) was significantly higher than the pH values of the three RS Br treatments, which all had similar pH values (5.41 ±SD 0.29). Likewise, AMZ influenced the pH (H_3= 10.4; p = 0.016), and Tukey's test revealed that pH was lower than the control only in the 4 g L^{-1} AMZ treatment. Adding LMB to the water increased the pH ($F_{3,8}$= 26.1; p < 0.001), and post hoc comparisons revealed that the pH in the control (7.02 ±SD 0.43) was significantly lower than the pH values of the three LMB treatments, which had similar pH values (8.41 ±SD 0.22). Also, BAU 1 Gr affected the pH ($F_{3,8}$= 36.1, p < 0.001), and post hoc comparisons revealed that the pH in all treatments were different from each other, except in the 2 and 4 g L^{-1} treatments, where the pH was highest (Fig. 2.3).

Table 2.5: Maximum SRP sorption capacity (*qm* mean) of four SPS at four different pH estimated using Langmuir isotherm, the possible SRP-release and the Menten constant (K_m mean± SE) reflecting the binding affinity where ND indicates K_m could not be determined given the shape of the curve. < LOD= bellow detected limit (10 µg SRP L^{-1}). Values inside the brackets are the SE.

Material	pH	P Sorption capacity (mg SRP g^{-1})	r^2	*p-value*	*t-value*	*Km* (mg SRP L^{-1})	P-release (µg SRP g^{-1})
LMB	6	10.5 (0.6)	0.90	<0.001	16.40	0.03 (0.01)	< LOD
	7	6.8 (1.1)	0.52	<0.001	6.10	0.01 (0.01)	< LOD
	8	7.9 (0.6)	0.86	<0.001	14.00	0.02 (0.01)	< LOD
	9	8.3 (0.6)	0.86	<0.001	14.20	0.11 (0.05)	< LOD
AMZ	6	4.8 (0.3)	0.86	<0.001	14.05	0.05(0.03)	< LOD
	7	4.5 (0.7)	0.51	<0.001	6.1	0.14 (0.02)	< LOD
	8	4.3 (0.3)	0.83	<0.001	13.59	0.13 (0.08)	< LOD
	9	7.6 (1.4)	0.64	0.002	5.01	7.14 (7)	< LOD
RS Br	6	4.5 (1)	0.26	0.006	4.19	34.49 (42)	< LOD
	7	2.1 (0.4)	0.34	0.003	4.77	ND	< LOD
	8	1.6 0.6)	0.13	0.830	2.81	12.98 (23)	< LOD
	9	1.5 (1.1)	0.37	0.221	1.40	ND	< LOD
BAU 1 Gr	6	1.44 (0.5)	0.12	0.036	2.67	368 (853)	< LOD
	7	1.04 (0.4)	0.14	0.031	2.80	937 (3177)	< LOD
	8	1.78 (0.8)	0.10	0.059	2.32	1260 (10791	< LOD
	9	1.59 (0.7)	0.09	0.062	2.29	$6.9e^{8}$ ($2e^{15}$)	< LOD

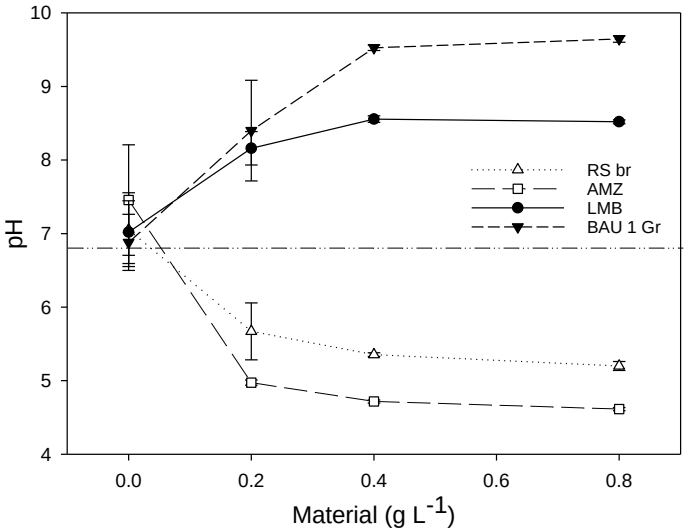

Figure 2.3: Influence on pH by adding 0, 1, 2 and 4 g of each clay per litre of ultrapure water. Dotted line represents initial pH (6.8, standard deviations =0.48; *n*=67). Error bar represents standard deviation (n=3).

Discussion

In this study, we evaluated the SRP adsorption capacity of ten possible solid phase P-sorbents (SPS) under controlled experiments. In contrast with our hypothesis, only four of the ten compounds showed SRP adsorption, and among those four compounds, two were modified compounds specifically designed to adsorb SRP. The low number of unmodified SRP-adsorbing compounds was not explained by their major elemental composition. For instance, both red soils (RS Br and RS Gr) had similar elemental profiles, with 31.7 and 27.3 g kg DW^{-1} Al, respectively and 53.4 and 52.2 g kg DW^{-1} Fe, respectively (Table 2.2); however, only RS Br had the capacity to adsorb SRP. Likewise, both Greek bauxites (BAU 1 Gr and BAU 2 Gr) had comparable compositions, yet only BAU 1 Gr had some capacity to adsorb SRP. Hence, elemental composition *per se* seems to not be the best predictor for SRP adsorption capacity *a priori*. Despite the fact that all compounds contained P, none of them released detectable amounts of SRP. Apparently, the form in which the elements are present, and most likely the mineralogy of the elements, determines the SRP adsorption capacity and the potential SRP release.

Trace elemental chemical analysis of the materials also revealed that some elements exceeded the maximum allowable concentration (MAC) in The Netherlands,

such as chromium (Cr), which reached 425 mg kg^{-1} dry weight in BAU 1 Gr; exceeding, the MAC value of 380 mg kg^{-1} dry weight sediment (https://rvs.rivm.nl/zoeksysteem/). Other elements also exceeded the MAC such as nickel (Ni) in BAU 1 Gr (334 mg kg^{-1}) and in BAU 2 Gr (95 mg kg^{-1}); both of these values that were above the MAC of 44 mg kg^{-1} DW for Ni. For LMB, arsenic and lanthanum were measured using ICP-MS because La creates a false positive at the As wavelength when measured using ICP-AES (Walton and White, 2015). In LMB, approximately 3.8% of La was present, which is lower than previously reported values 4.4% (Reitzel et al., 2013), 4.5% (Gibbs et al., 2011), 4.2% to 4.5% in five different LMB batches (own unpublished data), and 5% (Haghseresht et al., 2009). Hence, it appears that some differences between batches are possible.

The four compounds with SRP adsorption capabilities were subsequently subjected to anoxia, since anoxic conditions often develop near the bottom of eutrophic lakes and reservoirs due to high respiration rates. In this context, the redox sensitivity of Fe is of particular importance. The bacterial breakdown of organic matter results in oxygen consumption, nitrate reduction and the subsequent reduction of Fe and sulfate, which may cause a strong decrease in the P-binding capacity of the sediments and cause Fe-based amendments due to the formation of FeSx (Smolders et al., 2006). Hence, the redox sensitivity of Fe is viewed as a major drawback of using Fe in managing eutrophication in lakes (Douglas et al., 2004; Cooke et al., 2005). Indeed, under anoxia the two iron-rich compounds (RS Br and BAU 1 Gr - composed of 35% and 64% Fe, respectively) only had 7 and 6% of their SRP adsorption capacity, respectively, compared to oxic conditions. Similar results have been found for red soils (RS Br) by Noyma et al. (2016), who reported only 17% SRP adsorption under anoxic condition compared to oxic conditions.

The two modified compounds also showed lower SRP adsorption under anoxic condition compared to oxic conditions – AMZ: 38% and LMB: ~47% of the values under oxic conditions–implying that a considerable part of SRP adsorption is due to redox-sensitive binding (Fe and Mn). Meis et al., (2012) found that 21% of the P adsorbed by LMB was reductive-labile bound. The reduced P adsorption under anoxia in this study (53%) is closely aligned with the 51% found by Noyma et al (2016). The three Phoslock® adsorption experiments under anoxic condition showed similar values and showed a small variance (11%) among the experiments indicating a reliable experimental design. Although Gibbs and Özkundakci (2010) found that AMZ was equally able to adsorb and block the P release from the sediment under anaerobic and aerobic conditions, here, AMZ performance decreased by 38% of its capacity when subjected to anoxic condition. Using a relatively high dose to cap anoxic

sediments, however, may yield indistinguishable P releases from sediment under both aerobic and anaerobic conditions.

Several studies have determined the maximum SRP adsorption capacity of LMB (Table 2.6). While some studies observed a decrease in SRP adsorption with an increase in pH (Haghseresht et al., 2009; Ross et al., 2008), others did not (Gibbs et al., 2011). An analysis of all available literature (Table 2.6) yielded no relation between the pH and SRP adsorption capacity of LMB (linear regression on log-transformed adsorption data $F_{1,30}$ = 0.128; p = 0.723; r^2_{adj} = 0.00; Pearson product moment correlation coefficient = -0.0690; p = 0.712) and also in this study there was no clear relation between the SRP adsorption and pH variation.

Contrary to LMB, few studies have investigated the maximum SRP adsorption capacities of the other materials used in this work; additionally, very few have evaluated the influence of pH. For instance, one study evaluated the capacity of AMZ (also called Z2G1) to bind P and found a capacity of 20 mg P g^{-1} in synthetic water with a pH of approximately 6 (Gibbs and Özkundakci, 2010). This adsorption capacity is higher than obtained in our study for AMZ at any pH level and is 76% higher than the adsorption capacity of AMZ determined at the same pH. In our study, the SRP capacities of AMZ were approximately 40% lower at pH values below 9, with a small variance between pH 6, 7 and 8 (coefficient of variation of 0.05). In some cases, a lower pH has been suggested to affect the dissolution of the potential SRP adsorbents into its components, such as hydrotalcites (Douglas et al., 2004); however, we do not expect this for Al-based AMZ in the pH range of 6-8, where Al-hydroxides are dominant, and thus, lower SRP adsorption due to protonation, is not expected. Why AMZ had a lower SRP adsorption capacity at pH levels between 6-8 than at pH 9 remains unclear (Table 2.5). Nonetheless, the remaining capacity of 4.3-4.8 mg P g^{-1} is high enough to ensure effective SRP fixation at the sediment surface, where such pH values are common.

Table 2.6: Literature data of maximum SRP adsorption capacity of LMB (Phoslock®) in synthetic or natural water under different pH and oxic/anoxic conditions.

LMB SRP adsorption mg P kg^{-1}	Water type	pH	Redox	Reference
4.37	synthetic	5	oxic	Ross et al. 2008
4.36	synthetic	7	oxic	Ross et al. 2008
3.38	synthetic	8	oxic	Ross et al. 2008
3.19	synthetic	9	oxic	Ross et al. 2008
2.38	natural	8.45	oxic	Ross et al. 2008
9.54-10.54	synthetic	7	oxic	Haghseresht et al. 2009
~10.1	synthetic	5	oxic	Haghseresht et al. 2009
~9.8	synthetic	6	oxic	Haghseresht et al. 2009
~9.3	synthetic	7	oxic	Haghseresht et al. 2009
~8.75	synthetic	7.5	oxic	Haghseresht et al. 2009
~7.7	synthetic	8	oxic	Haghseresht et al. 2009
~7.2	synthetic	9	oxic	Haghseresht et al. 2009
5.22-5.47	synthetic	5.4	oxic	Haghseresht et al. 2009
4.63-4.99	synthetic	7	oxic	Haghseresht et al. 2009
23.75	synthetic	4.7-5.1	oxic	Meis et al. 2012
11.6	synthetic	6.1	oxic	Gibbs et al. 2011
11.3	synthetic	7.0	oxic	Gibbs et al. 2011
15.1	synthetic	8.9	oxic	Gibbs et al. 2011
11.6	synthetic	7	oxic	Zamparas et al. 2012
13.2	natural	7.5	oxic	Reitzel et al. 2013
12.6	natural	8.2	oxic	Reitzel et al. 2013
13.3	natural	7.9	oxic	Reitzel et al. 2013
11.3	natural	8.1	oxic	Reitzel et al. 2013
13.7	natural	8.2	oxic	Reitzel et al. 2013
12.3	synthetic	-	oxic	Noyma et al. 2016
6.0	synthetic	-	anoxic	Noyma et al. 2016

Divergent from AMZ and in agreement with our hypothesis, red soil from Brazil showed a higher SRP adsorption capacity at lower pH values and a proportional decrease at increasing pH. The same trend was observed by Dai and Pan (2014),

where at pH 5.5 the maximum capacity of a red soil from China almost doubled compared to the capacity at pH 8.5. Zhu (2007) also observed a slight increase in the adsorption efficiency of Fe-modified bentonite at lower pH levels. Likewise, natural bentonite, red mud, lanthanum-doped mesoporous SiO_2, iron-oxide tailings and another red soil from China all showed a tendency to reduce SRP adsorption at higher pH values (Ou et al., 2007; Zamparas et al., 2012; Zeng et al., 2004; Zhao et al., 2009; Zhu et al., 2007). These results are likely related to the higher concentration of hydroxyl ions in the solution, which promotes negative charges on the surface of the adsorbent, as well as, higher repulsion and increased competition between phosphate and hydroxyl for binding sites (Dai and Pan, 2014; Ferreira De Souza et al., 2006; McBride, 1994; Ou et al., 2007; Wasay et al., 1996). BAU 1 Gr does not seem to have any direct relation between pH and adsorption capacity, since all values were similar and independent of pH (coefficient of variation of 0.1). This deviates from the findings of Altundoğan and Tümen (2002), who found higher orthophosphate adsorption at pH 6 than at pH 9, yet the overall adsorption of 0.1-0.6 mg P g^{-1} was lower than the 1.0-1.8 mg P g^{-1} found in the present study. It is, however, important to note that both natural soils, RS Br and BAU 1 GR, showed a relatively poor *Langmuir* fit compared to the modified clays, AMZ and LMB.

The *Km* value provides insights about how quickly the adsorbent (SPS) reacts with the substrate (phosphate) while also considering the amount needed to be saturated (Mosier and Ladisch, 2009). Thus, lower *Km* values indicate that the SPS needs only small amounts of phosphate to achieve its maximum adsorption capacity, and higher *Km* values indicate the need for a higher concentration. It is important to consider that the SRP concentration of pore water in eutrophic systems is usually in the lower part of the tested adsorption range (0.25 to > 2.5 mg SRP L^{-1}) (Maassen et al., 2005), which means that the maximum capacity calculated for some compounds might never be achieved. Here, in all condition, LMB was much faster than the other compounds, suggesting better performance under realistic phosphate concentrations (Table 2.3).

Although all the compounds influenced the pH of ultrapure water, such an effect is probably lower in natural water due the higher alkalinity, and hence pH buffering. Thus, to check whether a buffer is necessary in a field application, tests should be performed with lake water. For LMB, these tests have been performed, specifically in algal growth medium and in pond water where no effect of even relatively large doses on pH was detected (van Oosterhout and Lürling, 2013).

The use and study of geoengineering materials has been increasing mainly due to environmental policies that require improvements in water quality and the need to manage eutrophication. Even though eutrophication is caused by an excess of both N and P, it has been stated that: "It is not important whether phosphate is currently the limiting factor or not, or even that it has ever been so; it is the only essential element that can be easily be made to limit algal growth" (Golterman, 1975). Likewise, strong in-lake P-reduction will render N reduction unnecessary. Consequently, the process of identifying materials that are able to target phosphate has attracted considerable interest. However, some methodological aspects should be taken into consideration.

First, it is necessary not only to test their efficiency to adsorb SRP but also to test adsorption capacities in realistic conditions. For instance, a high adsorption capacity of 144 mg P g^{-1} has been reported for goethite (FeO(OH)), at pH 5 (Peleka and Deliyanni, 2009), while 196 mg P g^{-1} was found for uncalcined hydrotalcite, at a temperature of 65˚C (Peleka and Deliyanni, 2009). Considering that eutrophic waterbodies have much higher pH values and most natural waters will never achieve such high temperatures, these materials might not be feasible for field applications.

Second, the cost-benefit analysis of the material being considered should be taken into account. The uncontrolled and unplanned population growth in developing countries intensifies eutrophication problems, but large upfront investment costs to tackle point sources such as sewage (van Loosdrecht and Brdjanovic, 2014), are unlikely to be realized in the short term. Thus, it is important to find affordable solutions for managing surface waters that are constantly subjected to nutrient pollution; these solutions should be able to be repeated regularly which implies that expensive materials or materials that are difficult to produce will not be feasible in this case. Synthetic materials that have high adsorption capacity, e.g. layered double hydroxides (LHDs) compounds modified with cations (Ca-Al, Ca-Fe or Mg_1Ca_3-Al) can adsorb between 47-70 mg P g^{-1} (Jiang and Ashekuzaman, 2015); however, these materials can be complex to produce. Consequently, even if a natural soil does not have the same efficiency as modified or synthesized materials, it might still be a viable alternative for economically developing countries. The decision of selecting the most suitable SPS should be based on system analyses, which includes the water and P fluxes as well as tests on efficacy and a cost-benefit assessment (Lürling et al., 2016).

Third, the SPS must be safe and should not cause any unwanted side-effects in organisms. The toxicity of SPS depends on their chemical composition and origin (Douglas et al., 2016a). RS Br does not have any compounds that exceed the MAC,

which may suggest it will not exert toxicity to exposed biota. However, adding solid-phase compounds such as RS Br to the water will cause turbidity and lead to elevated suspended solid concentrations in the water, which might have side effects on biota (Bilotta and Brazier, 2008). Unlike the common high-pH waste product– red mud – from bauxite refineries involved in aluminum production (Howe et al., 2011), raw bauxite might be less detrimental, yet applying bauxite will also increase the concentrations of suspended solid. The elevated Cr concentration in BAU 1 Gr is not expected to cause toxicity since after application the SPS will be diluted in the sediment. Nonetheless, further controlled ecotoxicological tests must be performed before whole-lake applications can be done. Concerning the modified clays, numerous studies have been conducted with LMB, and none of the studies indicated toxicity toward non-target biota (Afsar and Groves, 2009; Copetti et al., 2016; Lürling and Tolman, 2010; van Oosterhout and Lurling, 2011; Waajen et al., 2016a) . However, the active ingredient in LMB, La, accumulates in crayfish (van Oosterhout et al., 2014) and fish (Waajen et al., 2016b) demonstrating the importance of monitoring potential side effects after applications. AMZ did not show any effect on the survival or growth of crayfish, mussels or fish (Clearwater et al., 2014). Different methods and organisms have been used to assess SPS toxicity and have had variable results; thus, the most suitable approach would be running tests using water and biota collected from the target system to highlight possible side effects (Copetti et al., 2016). Such assays are a prerequisite in the sequence of tests prior to field application.

LMB has been applied and is able to successfully mitigate eutrophication in several environments (Copetti et al., 2016 (Table 2.1); Epe et al., 2017;Waajen et al., 2016a). However, the addition of LMB alone appeared to be less effective in an enclosure study (Lürling and Faassen, 2012) and in a whole-lake application (Lürling and van Oosterhout, 2013) than in controlled laboratory experiments (e.g., Ferreira and Marques, 2009; van Oosterhout and Lürling, 2013). The main reasons for the difference between the high P removal observed in laboratory experiments (e.g., Ferreira and Marques, 2009; van Oosterhout and Lürling, 2013) – and the poorer performance in lake water (e.g., Lürling and Faassen, 2012; Lürling and van Oosterhout, 2012; Ross et al., 2008) – lies in the composition/or predominant physicochemical conditions of the water. For example, humic substances (Lürling et al., 2014b), alkalinity (Reitzel et al., 2013), redox status and pH (Ross et al., 2008; Haghseresht et al. 2009; this study) each influence the performance of LMB to some extent. However, it should be noted that the extremely high affinity of lanthanum for phosphates might make that over time any of the initial hampering effects will be

overcome. This is for instance clearly evidenced from studies performed on humic acids (Dithmer et al., 2016a) and finds further support from analysis of how La and P are bound in sediments of treated lakes (Dithmer et al., 2016b).

Hence, prior to full-scale field applications, it is important to consider possible effects of such abiotic factors, as well as salinity and the potential interaction of factors on the efficacy of the chosen SPS. This requires more complex tests and an upscaling of experiments to achieve an in-depth knowledge of the SPS materials and any potential limitations for a possible successful field application. Considering the four criteria for a useful SPS (effective, easy to produce, cheap and safe), the two modified materials that were tested performed best under the experimental conditions employed. One of these, LMB, has already been applied widely in more than 200 surface waters (Copetti et al., 2016). The other one, AMZ, is not commercially available in large quantities, but the results of this study show that it has great potential, which makes it a good candidate for upscaling experiments and/or field application, which may eventually lead to commercial production and distribution.

Conclusion

Only four, lanthanum-modified bentonite (LMB), aluminum modified zeolite (AMZ), natural red soil from Brazil (RS Br) and bauxite 1 from Greece (BAU 1 Gr), out of the ten tested materials were able to adsorb phosphate. Modified clays showed greater adsorption capacities than natural materials. LMB expressed the fastest reaction and reached its maximum adsorption capacity at relatively low and realistic phosphate concentrations. The SPS elemental composition did not explain adsorption efficiency. All tested materials decreased their adsorption capacity under anoxic condition, indicating redox-sensitive bindings. All materials were able to adsorb SRP at pH 6, 7, 8 and 9. LMB and BAU 1 Gr were not affected by different pH values. RS Br showed a decline in SRP adsorption with increasing pH, while AMZ had the highest adsorption at the highest pH values. Thus, pH influenced each SPS material differently. Given the observed effects of pH and anoxia on SRP adsorption of SPS, other abiotic variables, such as salinity and alkalinity, should be evaluated in controlled experiments before field application. Considering the four criteria for a useful SPS (effective, easy to produce, cheap and safe), the two modified materials that were tested performed best under the experimental conditions employed. LMB is already widely used, AMZ shows great potential and both are the first candidates for upscaling

experiments. However, despite strongly reduced SRP adsorption under anoxic condition, the natural compounds RS Br and BAU 1 Gr still adsorbed some SRP, and thus, they could be considered for further testing.

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Supplementary information

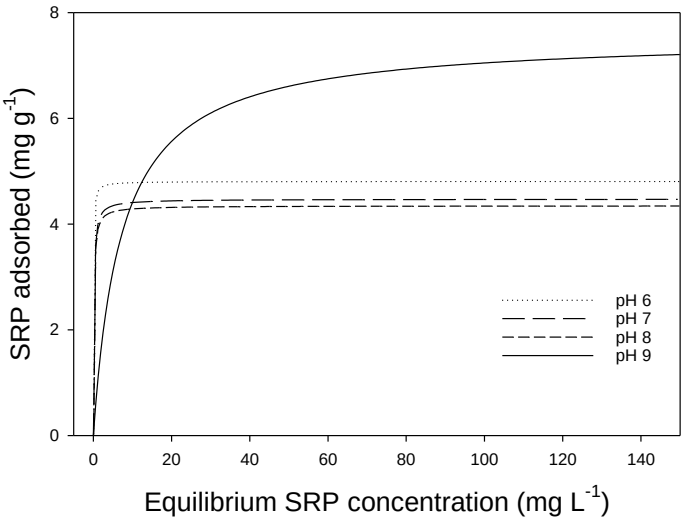

Figure 2.S1: Langmuir adsorption isotherm for AMZ (Aqual-P™) at four different pH.

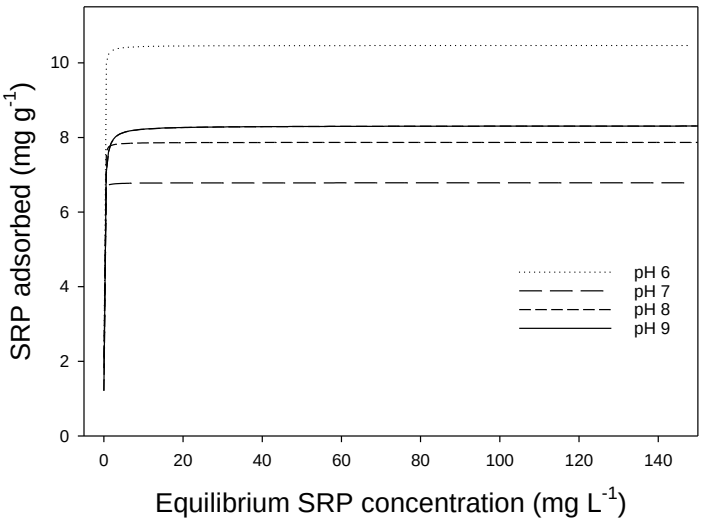

Figure 2.S2: Langmuir adsorption isotherm for LMB (Phoslock®) at four different pH.

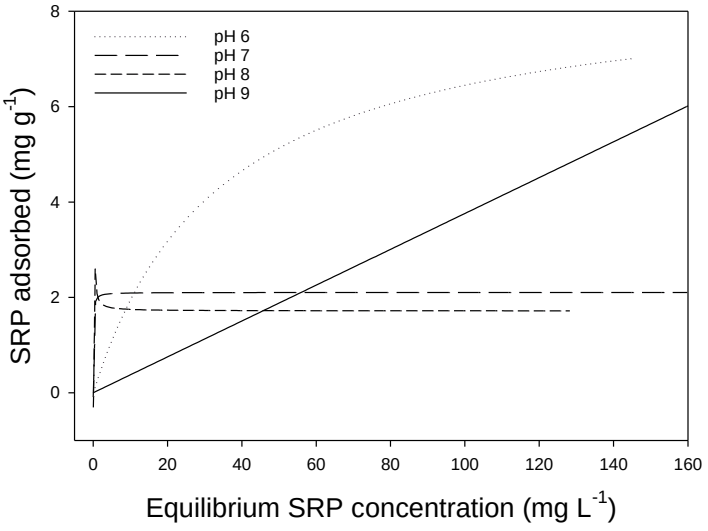

Figure 2.S3: Langmuir adsorption isotherm for red soil from Brazil at four different pH.

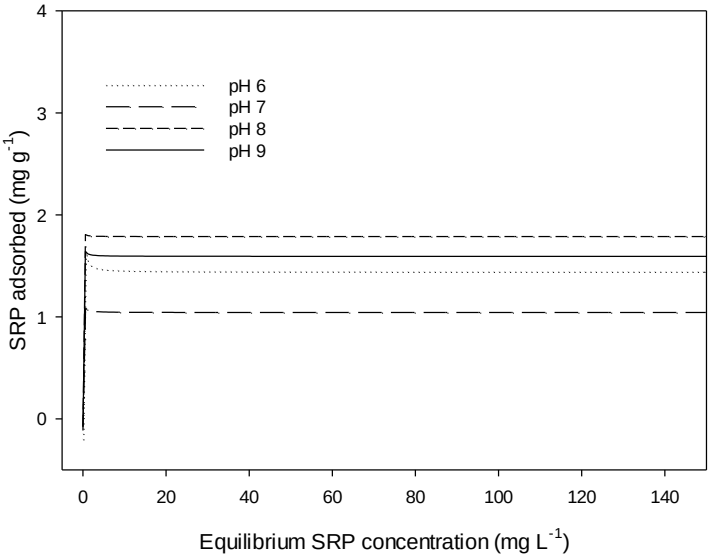

Figure 2.S4: Langmuir adsorption isotherm for BAU 1 Gr at four different pH.

3

Lanthanum modified bentonite (Phoslock®) behaviour and efficiency in adsorbing phosphate in saline waters

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Abstract

Lanthanum-modified bentonite (LMB) has been widely applied in freshwater systems to manage eutrophication. Little is known, however, about its behaviour and efficiency in binding filterable reactive phosphorus (FRP) in saline environments. We assessed if LMB would adsorb phosphate over a range of salinities (0-32 ppth) comparing the behaviour in seawater salts and equivalent concentrations of NaCl. Lanthanum release from the bentonite matrix was measured and the La species prevailing in saline environments were evaluated through chemical equilibrium modelling. We demonstrated that LMB was able to adsorb FRP in all the salinities tested. Filterable lanthanum (FLa) concentrations were similarly low (<5 μg L^{-1}) at all seawater salinities but considerably elevated, on occasion >2000 times greater in equivalent NaCl salinities. Mineralogical analysis indicates that La present in the clay interlayer was (partially) replaced by Na/Ca/Mg present in the seawater and possibly a secondary P-reactive phase was formed, such as kozoite ($LaCO_3OH$) or lanthanite ($La_2(CO_3)_3.8H2O$) that may be physically dissociated from the LMB. Geochemical modelling also indicates that most FLa dissociated from LMB would be precipitated as a carbonate complex. In light of the identification of reactive intermediate phases, further studies including ecotoxicologial assays are required to assess any deleterious effects from the application of LMB to saline waters.

Introduction

Over the past century in particular, human activities have resulted in the export of increasing quantities of nutrients to waterbodies resulting in the rise of eutrophication on a global scale (Downing, 2014; Smith and Schindler, 2009). A key consequence of eutrophication is the excessive growth of algae, mainly cyanobacteria in fresh and brackish waters (Paerl and Otten, 2013), eukaryote harmful algae in marine environments (Heisler et al., 2008; Smith et al., 1999), or co-occurrence of cyanobacteria and dinoflagellates in coastal waters (Paerl et al., 2008). These phytoplankton blooms may cause several negative effects in aquatic systems, such as increased turbidity, fish kills, and malodors (Paerl and Huisman, 2009; Smith and Schindler, 2009). In addition, some cyanobacteria can produce toxins that cause liver, digestive and neurological harm not only in the aquatic biota but also in humans, livestock and domestic animals (Carmichael et al., 2001; Dittmann and Wiegand, 2006; Lürling and Faassen, 2013). Similarly marine algal toxins may accumulate in shellfish that may cause severe intoxication when consumed (Gerssen et al., 2010). Consequently, eutrophication and harmful phytoplankton blooms impair the use of aquatic systems for fishing, aquaculture, irrigation, recreation and as a source of drinking water all of which may result in substantial negative socio-economic effects (Dodds et al., 2009; Hamilton et al., 2013). In coastal environments in Europe, the marine algal toxins impact tourism, recreation, and the shellfish industry with estimated losses of 720 M€ and 166 M€ respectively (Gerssen et al., 2010). From 1987 to 1992 the economic cost from red tides in US coastal waters were estimated to be US$ 500 M (Smith and Schindler, 2009).

Eutrophication of coastal environments is considered the second most important stressor on estuaries worldwide after habitat loss and alteration (Kennish, 2002) and an important factor in the creation of so-called “Dead Zones” (Diaz and Rosenberg, 2008). In addition to fluvial inputs from municipal, industrial and agricultural sources, coastal eutrophication may also be contributed to by commercial aquaculture which can create nutrient-rich effluents from excrement and unutilised food (e.g., Burford et al., 2003; Herbeck et al., 2013; Nóbrega et al., 2014; Soares et al., 2007).

With eutrophication is ultimately being an imbalance between nutrient inputs and outputs, the first step in managing is the reduction of external nutrient inputs (Cooke, 2005; Hilt et al., 2006; Paerl et al., 2014). Such actions generally require stringent catchment mitigation measures including reducing atmospheric nutrient

fluxes (Mackey et al., 2017), state-of the-art wastewater treatment, and where present, reductions in nutrient inputs to, and net nutrient exports from, aquaculture. In many cases, concurrent actions of such breadth and cumulative cost are often not feasible, or at least will not be fully implemented in the short term. Thus, cases of eutrophication have continued to increase (Bricker et al., 2008). Crucially, responses in aquatic systems to external load reductions may be delayed or have a seemingly small effects because recycling of in-situ nutrients as internal loading may continue to sustain eutrophication and nuisance of phytoplankton blooms (Lürling et al., 2016; Søndergaard et al., 2001). In these cases, primarily in freshwater systems, in-situ remedial actions via geo-engineering approaches have been proposed to accelerate system recovery (Lürling et al., 2016; Lürling and van Oosterhout, 2013; Spears et al., 2013a; 2014; Waajen et al., 2016). Geo-engineering practices typically use materials and techniques that seek to intervene in aquatic system biogeochemical cycles, particularly the interception and binding of P released from internal sediment inventories. One of the most widely applied and studied geo-engineering materials to adsorb P (usually determined as filterable reactive P, FRP) and mitigate P release from sediment (internal loading) is lanthanum modified bentonite (LMB) (Douglas, 2002; Douglas et al., 2016a). Lanthanum-modified bentonite, commercially called Phoslock®, was developed by the CSIRO in Australia (Douglas, 2002; Douglas et al., 2000, 1999), has been applied in around 200 freshwater environments worldwide as well as being studied extensively in laboratory and field experiments (Copetti et al., 2016).

The efficiency of LMB for FRP uptake has been tested in a broad range of environmental conditions, such as different pH, humic substance concentrations and redox conditions, (Gibbs et al., 2011; Lürling et al., 2014b; Mucci et al., 2018; Noyma et al., 2016; Reitzel et al., 2013; Ross et al., 2008; Spears et al., 2013c). Information on the performance of LMB in more saline conditions is, however, limited. Reitzel et al. (2013) found that FRP sequestration by LMB was equally efficient in freshwater and brackish waters of different salinities. Recent experiments in water and sediment from a brackish lagoon showed significant FRP uptake by LMB and interception of sediment P fluxes (Magalhães et al., 2018).

Information on behaviour of LMB in saline conditions is limited to FRP removal in NaCl solutions, which does not reflect saline waters (Zamparas et al., 2015), or to removal of the fish-killing haptophyte *Prymnesium parvum* (Seger et al., 2015). Little information exists on brackish waters (Reitzel et al., 2013). Magalhães et al. (2018) observed an effective decrease in FRP efflux from the sediment in cores treated with

LMB from a brackish tropical lagoon (~ 5 ppth) over 3 months. In the slightly brackish Sulphur Creek Reservoir (USA), LMB treatment reduced water column P and created a shift in sediment P towards more strongly bound forms (Bishop et al., 2014). Hence, LMB seems to possess a FRP removal capacity in brackish waters. In a cautionary note, however, Copetti et al. (2016) stated a caution that based on earlier experimentation (Douglas et al., 2000) that substantial amounts of La would be released in more saline waters and that therefore "*the application of the LMB in even moderately saline environments of >0.5 ppth is to be avoided* ".

In the absence of definitive studies of the performance of LMB in brackish to saline waters detailed information is required both on performance in terms of FRP uptake as well as the potential for the release of La, and hence potential ecotoxicity. In this study we evaluated LMB performance over a range of salinities prepared using either NaCl or sea salt, determined La speciation over a range of salinities using chemical equilibrium modelling, and characterized LMB using X-Ray Diffraction (XRD).

Methods

P-adsorption and La release experiments in different salinities

The effect of salinity on FRP adsorption by LMB was assessed over 14 days. Twenty-four graduated 1 L cylinders were filled with a stock solution of 1 mg FRP L^{-1} prepared using K_2HPO_4 (Sigma-Aldrich) and ultra-pure water (MilliQ). Six stock solutions of a synthetic seawater were prepared by dissolving commercially available sea salt (La Baleine, Sel de Mer) in ultra-pure water with 1 mg FRP L^{-1} at salinities of 0, 2, 4, 8, 16 and 32 (parts per thousand – ppth). To each cylinder 5 mL of 10 g LMB L^{-1} slurry was added by spraying it equally on the water surface, equivalent to a dose of 50 mg LMB L^{-1} that was sufficient to remove 50% of the FRP. The LMB was obtained from Phoslock Europe GmbH (Ottersberg, Germany). The experiment was run with four replicates per salinity. The cylinders were placed in a dark room at 21°C, and pH was measured after 1, 7 and 14 days of LMB addition using a WTW pH-320 pH meter. Samples were taken before the LMB application, and 1 hour, 1 day, 4 days, 7 days and 14 days after LMB addition. Twenty mL subsamples were filtered through unit filters (Aqua 30/0.45CA, Whatman, Germany), of which 10 mL was used to analyse FRP on a Skalar SAN+ segmented flow analyser following the Dutch standard NEN 6663 (NNI, 1986). The remaining 10 mL was acidified with 100 µL 7 M HNO_3 (Ultrex) and the lanthanum (La) concentration determined by ICP-MS in the Chemical Biological Soil Laboratory of the Department of Soil Sciences (Wageningen University). Ten mL

unfiltered subsamples were taken for analysis of total La concentration. An extra control was performed to test if the synthetic seawater would affect the FRP concentration. To this end, 15 glass tubes of 125 mL were filled with 100 mL FRP solution (1 mg L^{-1}) at 2, 4, 8, 16 and 32 ppth salinity. Subsamples for FRP analysis were taken before the salt addition and after 1 week; samples were filtered and analysed for FRP as described above. Solution pH, FRP and La concentrations were statistically evaluated via repeated measures ANOVAs ($\alpha = 0.05$) followed by Tukey post hoc tests. The extent of FRP removal was evaluated via a paired T-test of initial and final concentrations. Normality and homoscedasticity were tested with Shapiro-Wilk and Levene tests, respectively, using SPSS 22.0.

Lanthanum release experiments in NaCl and synthetic seawater solutions

Lanthanum release from the bentonite clay matrix was analysed in water with high salinity and lower FRP concentration to simulate a typical application scenario. Excess La (as LMB) was added over that required to bind the available FRP in the water column. This method was chosen as an LMB application is normally directed towards potential sediment P release, which constitutes a much higher dose than that required to bind P immediately present in the water column. Twenty-four Falcon bottles were filled with 50 mL stock solution of 0.1 mg FRP L^{-1} prepared using K_2HPO_4 (Sigma-Aldrich) and with a salinity of 32 ppth. For half of the Falcon bottles 32 ppth salinity was prepared by dissolving commercially available sea salt (La Baleine, Sel de Mer) as a synthetic seawater and the other half by dissolving NaCl (Sigma-Aldrich). The LMB was then added in different concentrations (0, 50 and 500 mg LMB L^{-1}). The experiment was undertaken using four replicates. The Falcon bottles were continuously mixed on a shaker at 180 rpm for seven days at 22°C in darkness. After one and seven days samples were taken and filtered through unit filters (Aqua 30/0.45CA, Whatman, Germany). Filtrates were analyzed for their La concentration as described above. The La concentrations between different LMB dose (0, 50 and 500 mg L^{-1}), salts (NaCl or sea salt) and time (1 and 7 days) were statistically tested using three way ANOVA.

Kinetic experiment

Batch experiments were carried out to evaluate the kinetics of FRP adsorption on LMB at the lowest (0 ppth) and highest salinity (32 ppth). The methodology details can be found at the supplementary information-Appendix A.

Chemical equilibrium modelling

To evaluate the lanthanum species prevailing at each salinity made with sea salt, chemical equilibrium modelling was performed using CHEAQS Next – version P2017.1 (Verweij, 2017). As input for the model we used the pH measured in the two weeks adsorption experiment, the amount of La and FRP added and the composition of standard seawater (Table 3.S1). In addition, extra model runs were performed at 32 ppth (pH 8 and in 1 mg FRP L^{-1}) to predict the amount of free La^{3+} released as function of La concentrations. To assess the difference in La species between NaCl and synthetic seawater solution, we used the amount of La and FRP used in the specific experiment (2.5 mg La L^{-1} and 0.1 mg FRP L^{-1}, respectively) and pH 8 as inputs in the model. In addition, model runs were performed at 32 ppth to predict La species as function of pH, using 2.5 mg La L^{-1} and 0.1 mg FRP L^{-1} as input.

LMB mineralogical analysis

Slurries of LMB (500 g L^{-1}) were prepared at 0, 4 and 32 ppth salinity and placed in 500 mL Schott bottles containing either 0 or 0.1 mg FRP L^{-1}. The bottles were continuously mixed on a shaker at 180 rpm for 24 hours at 22°C, after which they were centrifuged at 2500 rpm for 10 minutes and standing water removed. The pellet, which was mostly LMB, was washed with ultra pure water once and centrifuged again. The supernatant was discarded and the LMB dried in an oven for 24 hours at 60°C. X-ray diffraction (XRD; Bruker) analysis was undertaken using Cu radiation (1.548 Å) with a step size of 0.02° and step time of 1s between 5 and 65° 2θ.

Results

P-adsorption and La release experiment in synthetic seawater

The final FRP concentrations, after incubation with LMB, differed with synthetic seawater salinity (Figure 3.1, rmANOVA $F_{5, 18} = 29.669$, $p < 0.001$). The post hoc comparison between salinities showed no difference in FRP concentration 2, 8 and 16 ppth with that in 0 ppth ($p = 0.050$, $p = 0.976$, $p = 0.998$, respectively). The FRP concentration in 4 ppth was significantly lower than at 0 ppth ($p < 0.001$). At 32 ppth the FRP concentration was significantly higher than at 0 ppth ($p = 0.003$) reflecting a 8% lower FRP removal by LMB (Fig. 3.1). Statistical (Tukey's) testing revealed four homogeneous groups: 1) adsorption was highest at 4 ppth, 2) similar at 0, 2 and 8 ppth, 3) 0, 8 and 16 ppth were also similar and 4) lowest adsorption occurred at 32 ppth (Fig. 3.1). A paired t-test indicated that without LMB addition initial FRP

concentrations were similar to those measured after one week (p > 0.335). (Supplementary information Fig. 3.S1).

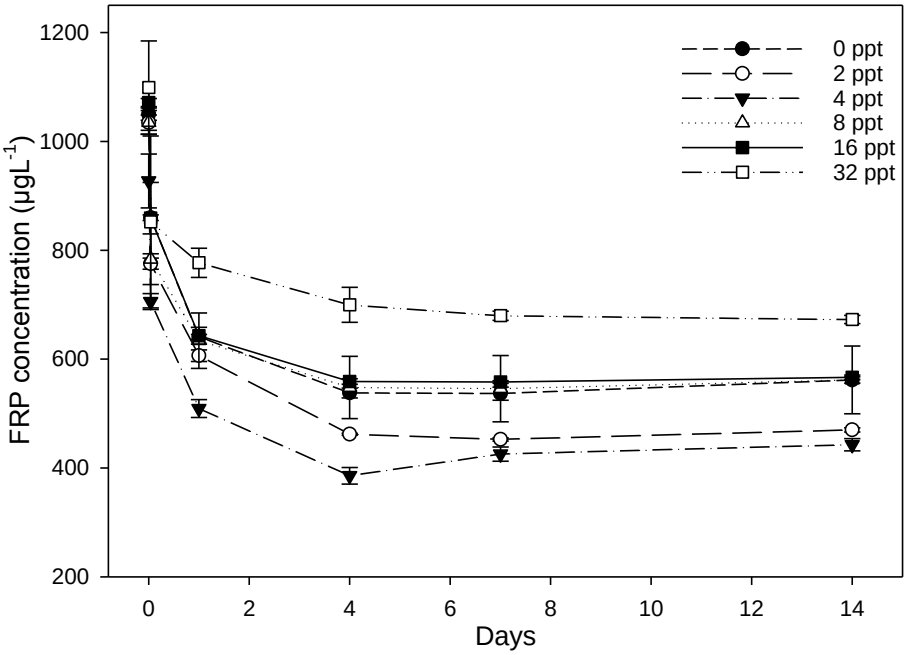

Figure 3.1: Filterable reactive phosphorus (FRP) concentration over time in different synthetic seawater salinities treated with 50 mg L^{-1} P. Errors bars indicate 1 SD (n=4).

Filterable lanthanum (FLa) concentrations were similar at all synthetic seawater salinities ($F_{5,18}$ = 2.619; p = 0.620), although marginally higher at the lowest salinities (Fig. 3.2). One hour after application FLa was highest at 2 ppth (12.4 µg FLa L^{-1}) and lowest at 32 ppth (2.1 µg FLa L^{-1}). After 1 day the FLa concentration declined to 5.1 µg FLa L^{-1} at 2 ppth and 0.5 at 32 ppth. After 14 days, the FLa concentration was the highest at 0 ppth (2.5 µg L^{-1}), while it was <1 µg FLa L^{-1} at all other salinities (2 – 32 ppth). After two weeks FLa concentrations were 0.11%, 0.04%, 0.02%, 0.008%, 0.009% and 0.007% of the initial concentration at 0, 2, 4, 8, 16 and 32 ppth salinities, respectively.

Total lanthanum (TLa) concentrations were similar among all synthetic seawater salinities ($F_{5, 18}$ = 0.750; p = 0.597), significantly different over time (p <0.001) but with a similar salinity versus time profile (p = 0.036) indicating that the decline in TLa concentrations over time was similar at all salinities (Fig. 3.S2). After 1 hour, TLa, was highest at 32 ppth (1073 µg TLa L^{-1}) and lowest at 0 pph (398 µg TLa L^{-1}). Only one day following LMB application the TLa concentration at 32 ppth had declined to 86 µg

L^{-1} whilst at 0 ppth it had declined to 113 µg L^{-1}. After 14 days the highest concentrations of 13 and 8 µg TLa L^{-1} were found at 0 ppth and in the 4 ppth treatments, respectively; in all the other salinities TLa was below 2 µg L^{-1} (Fig. 3.S2). The pH at the end of the experiment varied between pH 6.50 and pH 6.80 at all salinities (Table 3.S2).

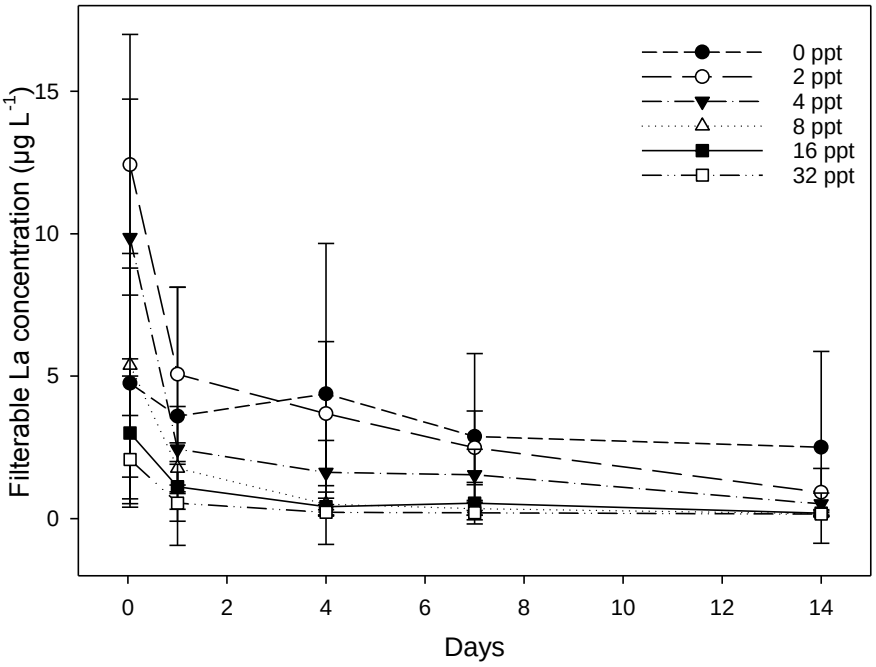

Figure 3.2: Filterable lanthanum over time in different synthetic seawater salinities in a solution with 1 mg FRP L^{-1} and 50 mg LMB L^{-1}. Errors bars indicate 1 SD (n=4)

Lanthanum release experiments in NaCl and synthetic seawater solutions

No filterable La (FLa) was found in the treatment where no LMB was added (data not shown). The three way ANOVA testing showed that FLa was significantly higher in NaCl solution than in synthetic seawater across a range of salinities ($F_{1,6}$ = 974; p <0.001). Lanthanum release was more than 2000 times higher in the 32 ppth NaCl solution than in the 32 ppth synthetic seawater solution (Fig. 3.3). Time was not influential as at day 1 and 7 FLa concentrations were similar ($F_{1,6}$ = 0.004; p = 0.950) for both type of salts used (p = 0.950) and also independent of LMB concentrations (p = 0.606). The main effects for LMB concentration were dependent on the type of salt used ($F_{1,6}$= 276.1; p <0.001) with NaCl solutions having a higher FLa concentration

when more LMB was added (500 mg L^{-1}), but in synthetic seawater the FLa concentration was similarly low and independent of the amount of LMB (Fig. 3.3).

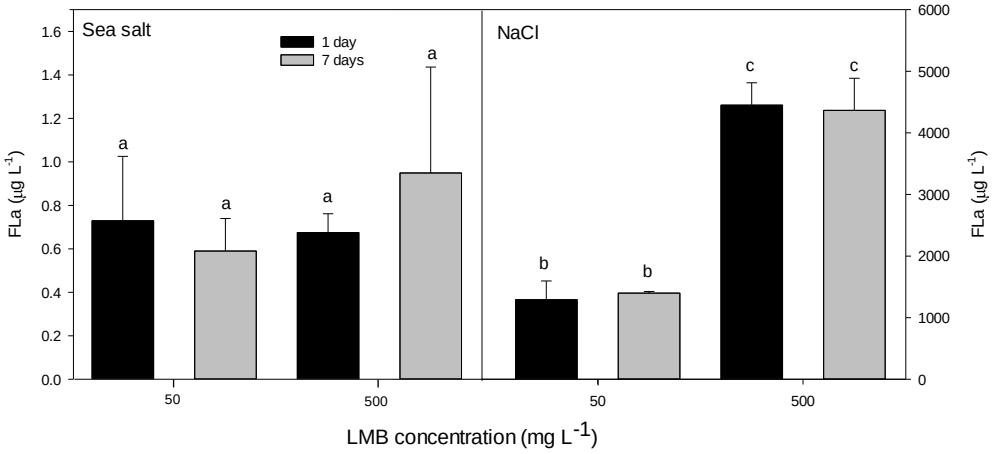

Figure 3.3: Filterable lanthanum concentrations (FLa) after one day (dark bars) and seven days (gray bars) in a FRP solution (0.1 mg L^{-1}) with 50 or 500 mg LMB L^{-1} in a water with 32 ppth of salinity made by dissolving commercially sea salt (left panel) or NaCl (right panel). Letters indicates significant differences.

Filterable Reactive Phosphorus Uptake Capacity

The maximum FRP adsorption capacity of LMB in seawater (32 ppth) and freshwater (0 ppth) were similar (Fig. 3.S3). Based on the *Langmuir* isotherm a maximum FRP adsorption capacity of 11.3 (±SD 3.2; p = 0.016) mg FRP per gram of LMB was found in synthetic seawater (32 ppth) and 11.4 (±SD 1.5; p <0.001) mg P g^{-1} in freshwater (Fig. 3.S3), data previously published (Mucci et al., 2018).

Chemical equilibrium modelling

Chemical equilibrium modelling indicated that for all seawater salinities, La would precipitate with phosphate as $LaPO_4$ (Table 3.1), with a very low concentration (≤1.57 x 10^{-6} mgL^{-1}) of free La species occurring in any of the salinities. In seawater (32 ppth), free La^{3+} ions would only start to appear when high concentrations of La were added (> 200 mg La L^{-1}; equating to 4 g of added LMB L^{-1}) (Fig. 3.4). At La concentrations below 200 mg La L^{-1}, all the La is precipitated with phosphate and carbonate. When the oxyanions have mostly been depleted, dissolved La starts to increase, of which a small fraction is predicted to consist free La^{3+} ions (Fig. 3.4).

Table 3.1: Chemical equilibrium modeling showing Free La^{3+} concentration (mgL^{-1}) and amount of La bound to $PO_{4\ (solids)}$ in each salinity in a solution with 3.06 mg FRP treated with 2.5 mg of lanthanum L^{-1} equating to 50 mg of LMB L^{-1}.

	0 ppt	2 ppt	4 ppt	8 ppt	16 ppt	32 ppt
free La^{3+}	2.39×10^{-10}	1.71×10^{-8}	2.70×10^{-8}	1.25×10^{-7}	5.10×10^{-7}	1.57×10^{-6}
$La(PO_4)_{(s)}$	2.50000	2.50000	2.50000	2.50000	2.499999	2.499994

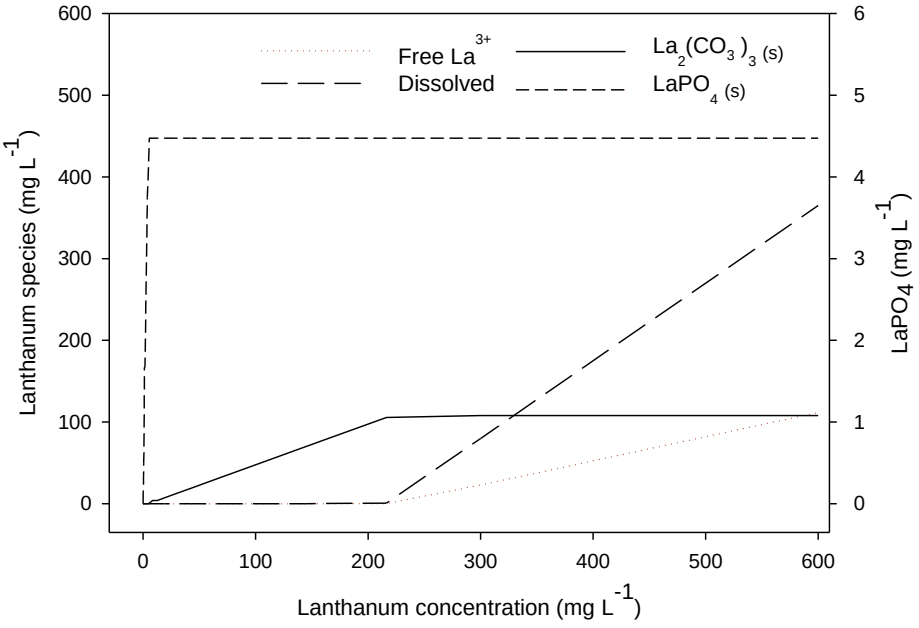

Figure 3.4: Different lanthanum species concentration in function of lanthanum added predict from chemical equilibrium modelling in a standard sea water (32 ppth) solution with 3.06 mg FRP L^{-1} at pH 8. Subscript s in the legend means solids.

In a scenario with 2.5 mg La L^{-1} and 0.1 mg FRP L^{-1} in seawater at pH 8, the majority of La is predicted to precipitate as $LaPO_4$ and as $La_2(CO_3)_3$. In contrast, in a solution of 32 ppth NaCl with 0.1 mg P L^{-1} at a pH 8, the chemical equilibrium modelling predicted that the majority of La would be present as free La^{3+} (Fig. 3.5).

At pH bellow 6.25, 26% of the La was present as free La^{3+} as limited carbonate was available for complexation (Fig 3.6). However, when pH increased above pH 6.25 free La^{3+} decreased via formation of $La_2(CO_3)_3$. At pH 6.5 free La^{3+} constituted only 7%

of all La species and at pH ≥ 7 it decreased to less than 1% (Fig. 3.6). At pH greater than 9, La precipitated as a hydroxide: $La(OH)_3$. In contrast, when in the presence of carbonate, La preferentially precipitated stoichiometrically with available PO_4 independently of the pH (Fig. 3.6).

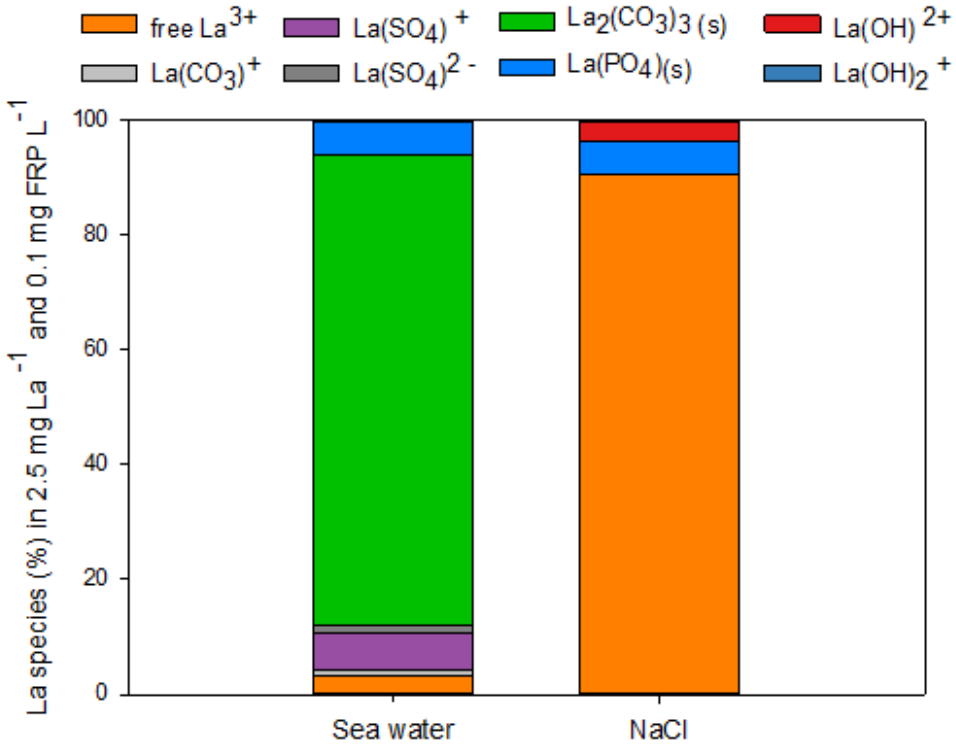

Figure 3.5: Different lanthanum species in % predict from chemical equilibrium modelling in seawater of 32 ppth (left bar) or NaCl solution of equivalent salinity right bar in 0.1 mg FRP L^{-1} solution at pH 8.

LMB mineralogical analysis

Mineralogical analysis of LMB prior to, and following the addition of P in waters of 0, 4 and 32 ppth salinity, prepared from synthetic seawater are shown in Figure 3.S4 with a differential XRD analysis (LMB minus LMB with P added) shown in Figure 3.S5. Analysis of the XRD spectra reveal a progressive decrease in the primary LMB interlayer distance from approximately 15.3 Å to 12.5 Å with increasing salinity. Primary peaks are also apparent for kozoite ($LaCO_3OH$) for the LMB at all salinities which disappears in the corresponding LMB reacted with P (Figure 3.S4). In contrast to kozoite, the product following reaction of the LMB/kozoite, rhabdophane is not immediately apparent in the XRD profiles in Figure 3.S4. Analysis of the differential

XRD spectra (LMB minus LMB-0.1P, Figure 3.S5) reveals both the disappearance of kozoite (positive peaks) and the formation of rhabdophane (negative peaks), particularly at ~20°, 29° and 31° 2θ where the most prominent peaks for neo-formed rhabdophane (Slade and Gates, 1999) generally occur (e.g. Diaz-Guillén et al., 2007; Roncal-Herrero et al., 2011)

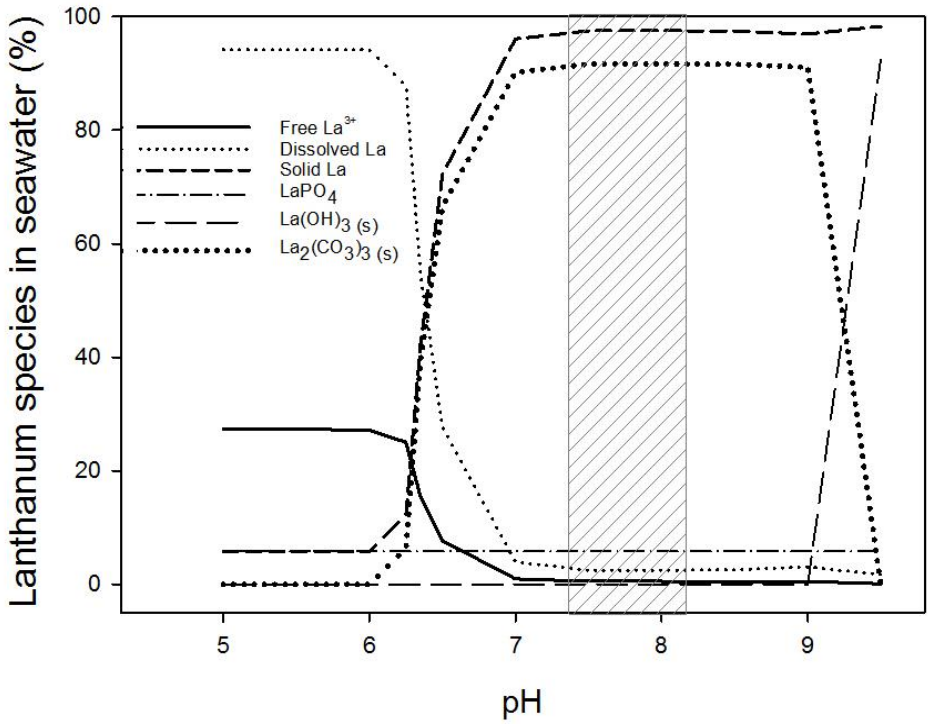

Figure 3.6: Different lanthanum species in % in function of pH predicted from chemical equilibrium modelling in a seawater (32 ppth) solution with 0.1 mg FRP L^{-1}. Subscript s in the legend means solids. Striped square is the range of oceanic pH (Chester and Jickells, 2012).

Discussion

Our study has revealed different behaviour of LMB in the presence of freshwater, synthetic seawater or NaCl of equivalent salinities. These aspects are discussed below in terms of both the composition of the solution, the mineralogy and structure of the LMB, and in the implications of these findings for the potential application of LMB in saline waters.

The LMB reduced FRP in all synthetic seawater salinities with a moderately enhanced removal at 4 ppth and a reduction at 32 ppth. Seawater, however, not only contains divalent cations, but also oxyanions, of which (bi)carbonate can precipitate with La (Firsching and Mohammadzadei, 1986) and potentially impede the rate and extent of FRP removal. This has been demonstrated in other studies where LMB removed less FRP in solutions with higher concentrations of bicarbonate (Liu et al., 2016). Similarly, FRP removal by expanded graphite loaded with lanthanum oxide was reduced most in presence of carbonate (Zhang et al., 2015). Fluoride removal by La was also substantially reduced in presence of bicarbonate/carbonate (Bansiwal et al., 2009; Kamble et al., 2009). Hence, the slightly lower FRP removal at higher salinities may be due to the formation of lanthanum carbonate or lanthanum carbonate/hydroxide precipitates such as kozoite ($LaCO_3OH$) as observed in this study, or lanthanite ($La_2(CO_3)_3.8H_2O$). While such carbonate compounds might impede phosphate removal in the short term as employed in our experiments, a potential exists for carbonate-bound lanthanum to eventually precipitate with phosphate via ligand exchange. This is supported by the chemical equilibrium modelling in this study in which lanthanum precipitates with phosphate. Humic substances are also known to impede the performance of FRP removal by LMB (Lürling et al., 2014b) in a short term, however, over time LMB also eventually binds with FRP (Dithmer et al., 2016a).

The maximum FRP adsorption capacities of 11.3 mg FRP g^{-1} LMB at 32 ppth and 11.4 mg FRP g^{-1} at 0 ppth are in close agreement with maximum FRP sorption capacities reported in the literature. In freshwater, a maximum sorption capacity of 12.3 mg FRP g^{-1} was found (Noyma et al., 2016). Douglas et al, (2002), Douglas (2010) showed a variation between 9.5 to 10.5 mg FRP g^{-1}. Reitzel et al. (2013) found only minor variances between the FRP adsorption capacity of LMB in waters with 1.5, 5 or 15 ppth salinity of 8.6, 8.6 and 8.7 mg FRP g^{-1}, respectively. Hence, there is little apparent difference in maximum sorption capacity of LMB in freshwater or seawater.

Filterable lanthanum concentrations remained low in all seawater salinities tested, indicating either (1) no substantial La release from the clay matrix, or (2) a release of La but rapid precipitation (Fig. 3.2) likely due the formation of kozoite. After one day of application in all simulated seawater salinities FLa and TLa concentrations were already below maximum allowed for Dutch water (10.1 and 150 µg La L^{-1}, respectively) (Sneller et al., 2000).

In the experiment, after 14 days between 0.11% (0 ppth) and 0.007% (32 ppth) of the LMB-La was present as FLa. These concentrations are comparable to the 0.02% found in leachate studies (Lürling and Tolman, 2010; NICNAS, 2001). Reitzel et al. (2013) found 0.0%, 0.0% and 0.8% of the La as FLa in water of 1.5 ppth, 5 ppth and 15 ppth, respectively. In addition, in our La release experiment in synthetic seawater at 32 ppth salinity where excess LMB was added relative to FRP, after seven days the FLa concentrations were between 0.59 µg L^{-1} (±SD0.15) and 0.95 µg La L^{-1} (±SD 0.49), ten times lower than the Dutch water quality criteria, equating to between 0.029% and 0.002% of the total La present as FLa (Fig. 3.3). In a previous seawater leaching study of a bentonite containing 4.3% La, only 0.7% or 16% of the total La was released (Spencer et al., 2007). Contrary to Spencer et al. (2011) who found substantially more La desorbed after day ten than day one, we did not observe a temporal effect in FLa concentration. However, Spencer et al. (2011) found the similar leaching behaviour in freshwater suggesting a potentially additional labile La component not present within the bentonite clay interlayers. Yuan et al. (2009) reported a La release from La modified clays of 0.006% at pH 6.1, and of 0.0007% at pH 7.85 and referred to it as the trivalent (free) La-ion. However, not all of the La in supernatant (or filtrate) should be viewed as La^{3+}, because of the potential for the formation of precipitates or the complexation with humic acids (Reitzel et al., 2017) or filterable colloids. To this end, Reitzel et al. (2017) demonstrated that a large portion of the La that was filtered through a 0.2 µm filter was colloidal and not free La^{3+}.

Our chemical equilibrium modelling indicated that in seawater only in excess of 200 mg La L^{-1} will free La^{3+} will be available (Fig. 3.4). Furthermore, no La free species could be expected in any salinities at an oceanic pH of between 7.5 and 8.4 (Chester and Jickells, 2012) since all La is likely to be precipitated as $LaPO_4(s)$ or $La_2(CO_3)_3(s)$ (Fig. 3.6). Spears et al., (2013b) showed that La^{3+} could reach up to 120 µg L^{-1} in low alkalinity waters, but that bicarbonate complexation would substantially diminish free La^{3+} concentrations thereafter. These results are in accordance with other studies that indicate the La will form either sparingly soluble rare-earth-metal phosphates (Firsching and Kell, 1993) or carbonates (Firsching and Mohammadzadei, 1986; Byrne and Kim, 1993).

Contrary to the limited FLa in the presence of synthetic seawater, primarily due to carbonate complexation, substantial La release occurred using NaCl at analogous salinities with FLa concentrations after one day of 1291 µg La L^{-1} (±SD 249) at 50 mg LMB L^{-1} and 4450 µg La L^{-1} (±SD 297) at 500 mg LMB L^{-1} added. This equated to a La release of 68% (±SD 13%) and 23% (±SD 2%) respectively or approximately 26 kg or 9 kg of La released from the LMB per tonne of LMB applied. After seven days, FLa concentrations were similar to day one concentrations, suggesting rapid release from the clay matrix consistent with a spontaneous cation-exchange process. Moldoveanu and Papangelakis (2013) showed that around 50% of the La present in natural clay was released after one hour contact with 0.5 M NaCl solution (~29 ppth) with most of the La liberated in the first 10 minutes. The much higher FLa present in NaCl solution relative to synthetic seawater at an equivalent salinity (32 ppth) reflects the absence of complexing oxyanions to precipitate the FLa. In analogous clay-REE (Rare Earth Elements) systems, other studies have shown that the REE may readily leach in contact with concentrated inorganic salts (e.g. NaCl and $(NH_4)_2SO_4$) via cation exchange (Moldoveanu and Papangelakis, 2013; Peelman et al., 2016). Thus, NaCl solution does not represent a seawater composition, albeit the Na^+ and Cl^- ions are major components of seawater (table 3.S1). Na, Ca and Mg cations collectively induce considerable cation exchange and La release from LMB, with, as discussed above, oxyanions such as (bi)carbonate, and where present phosphate, are crucial in regulating La solubility.

Chemical speciation modelling of La species in seawater demonstrates that most of the La in seawater was precipitated, depending on composition, as $La(PO_4)$ and $La_2(CO_3)_3$ (Fig. 3.5). In contrast, in NaCl solution leached La was only precipitated when PO_4^{3-} was present, in agreement with the elevated concentrations of FLa found in our experiments (Fig. 3.3).

Based on the solution analysis of the behaviour of LMB in synthetic seawater and NaCl of equivalent salinity, where it was demonstrated that the solute composition, as opposed to the salinity per se, is the major determinant of lanthanum speciation/precipitation, a complimentary mineralogical study using XRD has provided further insight into the behaviour of LMB during leaching. This study has revealed differences in both the mineralogy and structure of LMB, both as a function of salinity, and also following reaction with dissolved P. Reaction of LMB with synthetic seawater at 4 ppth and 32 ppth has revealed a substantial reduction in the primary LMB d-spacing from approximately 15.3 Å to 12.5 Å consistent with the cation exchange of La out of, and combination of Na, Ca and/or Mg from the seawater

entering into the bentonite interlayers. The propensity at which a significant proportion of La may exchange into solution in even moderately NaCl-based salinity as observed in solution-based studies is also evidenced by the change in the LMB primary d-spacing. The release of FLa in the presence of equivalent NaCl-only salinities, but not in synthetic seawater, and the absence of secondary crystalline phases identifiable by XRD, coupled with the uptake of P in parallel experiments, implies the formation of other cryptocrystalline/amorphous reactive phases when LMB reacts with seawater. Geochemical modelling (Fig. 3.5) indicates the likelihood that lanthanum carbonate phases such as lanthanite ($La_2(CO_3)_3.8H_2O$) or kozoite as observed in this study may form following interaction of the LMB with bicarbonate present in seawater. Thus, the presence of bicarbonate may serendipitously have the effect of ameliorating FLa, and with that a potential to modify La toxicity (as yet to be determined) in saline environments.

The presence of kozoite ($LaCO_3OH$) associated with the LMB at all salinities, and in particular at 0 ppth salinity suggests that this mineral is formed as a by-product of the production of LMB during La cation-exchange into the bentonite and may reflect the presence of residual bicarbonate present following Na-exchange of the Na-bentonite precursor used in LMB production. The loss of kozoite is also important in that this likely constitutes a reactive intermediate phase with respect to dissolved P with lanthanite potentially behaving in an analogous fashion.

Based on geochemical modelling, the association of lanthanite with the reacted LMB, for instance as a surface or edge precipitate is speculative, and more so given the absence of any secondary minerals identified in XRD analysis. Thus, a potential also exists for the formation of secondary P-reactive, possibly lanthanite phase, physically dissociated from the LMB. Subject to further studies on the association and fate of secondary lanthanite phases, this has major implications for the utility of LMB in saline waters as cryptocrystalline lanthanite will have substantially different hydrodynamic behaviour than the precursor LMB in addition to the potential, with its inherent smaller particle size relative to LMB for enhanced uptake/ingestions by aquatic biota (van Oosterhout et al., 2014; Waajen et al., 2017). The potential for cation exchange of a combination of Na, Ca and Mg cations into the LMB also has the potential to fundamentally alter the physical behaviour of the bentonite, in particular leading to the formation of cohesive, gel-like clay structures as observed in our laboratory-based studies, with the potential to adversely impact the critical sediment-water interface in saline aquatic systems. On the basis of this study, a combination of laboratory, and in-field mesocosm ecotoxicological studies,

are recommended before field applications in estuarine and marine environments, and most crucially in aquaculture given the potential for human health effects.

Acknowledgements

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Supplementary information

Appendix A

Kinetic experiment

Batch experiments were carried out to evaluate the kinetics of FRP adsorption on LMB at the lowest (0 ppth) and highest salinity (32 ppth). Seven FRP solutions containing 0, 5, 10, 20, 40, 80 and 120 mg FRP L^{-1} were prepared by dissolving K_2HPO_4 in ultra-pure water (0 ppth) or in ultra-pure water (MilliQ) water to which sea salt was added creating a salinity of 32 ppth. A total volume of 50 mL FRP solution and 100 mg of LMB was placed in 50 mL Falcon bottles. The experiment was conducted in triplicate for each FRP concentration. The Falcon bottles were continuously mixed on a shaker at 180 rpm for 24 hours at 22°C, after which samples were taken and centrifuged at 2500 rpm for 10 minutes. The supernatant was filtered through unit filters (Aqua 30/0.45CA, Whatman, Germany) and filtrates were analyzed for their FRP concentration as described before. To calculate the maximum FRP adsorption, we fitted the data in the isotherm Langmuir model:

$$\frac{Ce}{Qe} = \frac{1}{qmKL} + \frac{Ce}{qm}$$

in which *Ce* is the equilibrium concentration of the adsorbate (FRP, mg P L^{-1}), *Qe* is the adsorption capacity adsorbed at equilibrium (mg P kg^{-1}), *qm* is maximum adsorption capacity (mg P kg^{-1}) and KL is the Langmuir adsorption constant (L mg^{-1} P). Plotting

$\frac{Ce}{Qe}$ versus Ce, the slope will give $\frac{1}{qm}$ and the intercept $\frac{1}{qmKL}$ from which it is possible to calculate qm and KL. Ce and Qe will be obtained from the adsorption experiment:

$$Qe = \frac{(C_0 - Ce)V}{M}$$

Where Qe is the amount of FRP adsorbed at equilibrium (mg g^{-1}), C_0 is the initial concentration of FRP in solution (mg L^{-1}), Ce is the concentration of FRP in solution at equilibrium (mg L^{-1}), M is the adsorbent mass used (g) and V is the volume of FRP solution (Langmuir, 1918). The maximum P adsorption capacities was obtained by fitting the values in a non-linear regression of the Langmuir equation using SigmaPlot version 13.0.

Table 3.S1: Major ion composition of typical seawater. Source: http://www.lenntech.com/composition-seawater.htm

Ions	Major composition (mg L^{-1})
Chloride (Cl^-)	1898
Sodium (Na^+)	10556
Sulfate (SO_4^{2-})	2649
Magnesium (Mg^{2+})	1262
Calcium (Ca^{2+})	400
Potassium (K^+)	380
Bicarbonate(HCO_3^-)	140
Strontium (Sr^{2+})	13
Bromide (Br^-)	65
Borate (BO_3^{3-})	26
Fluoride (F^-)	1
Silicate (SiO_3^{2-})	1
Iodide (I^-)	1
Total dissolved solids (TDS)	34.483

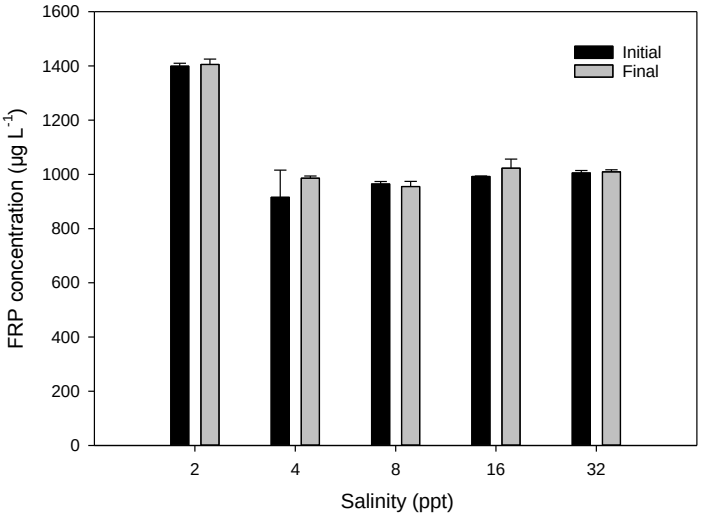

Figure 3.S1: FRP concentration at day 0 (Initial) and after one week (Final) with no addition of LMB in different salinities (*n*=3).

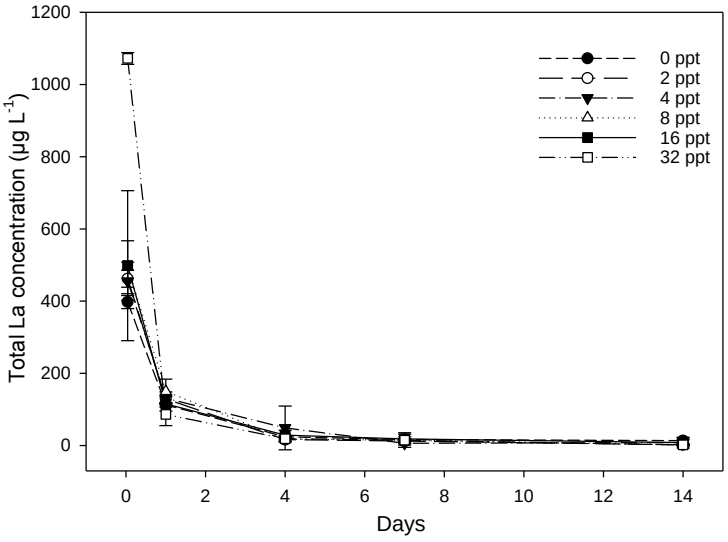

Figure 3.S2: Total lanthanum over time in different salinities in a solution with 1 mg FRP L^{-1} and 50 mg LMB L^{-1}. Errors bars indicate 1 SD (*n*=4)

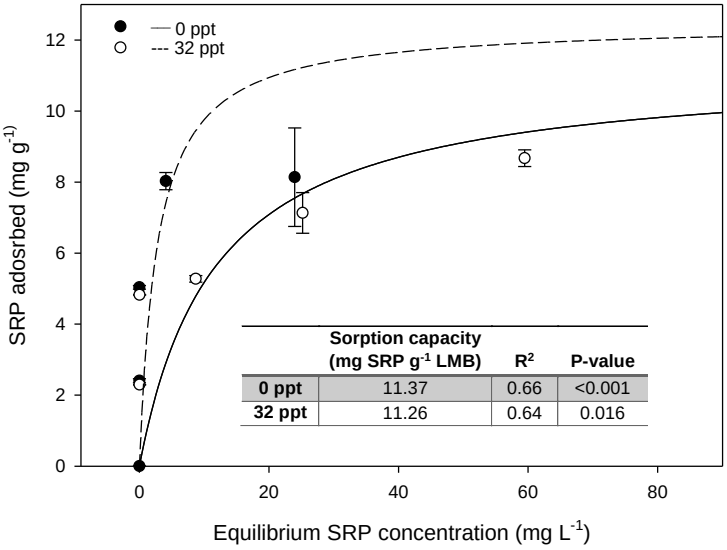

Figure 3.S3: Langmuir adsorption isotherm under solution of higher salinity (32ppth). Table inside shows maximum filterable reactive phosphorus (FRP) sorption capacity, the R square of the model and *p*-value.

Table 3.S2: Geometric average (standard deviation) over time between the different salinities in a solution with 1 mg FRP L^{-1} treated with 50 mg LMB L^{-1}. Similar letters represent homogeneous group.

Salinity	0 ppt	2 ppt	4 ppt	8 ppt	16 ppt	32 ppt
pH	6.80 (0.12)^{A}	6.64 (0.13)^{B}	6.54 (0.10)^{C}	6.53 (0.13)^{C}	6.49 (0.13)^{C}	6.50 (0.13)^{C}

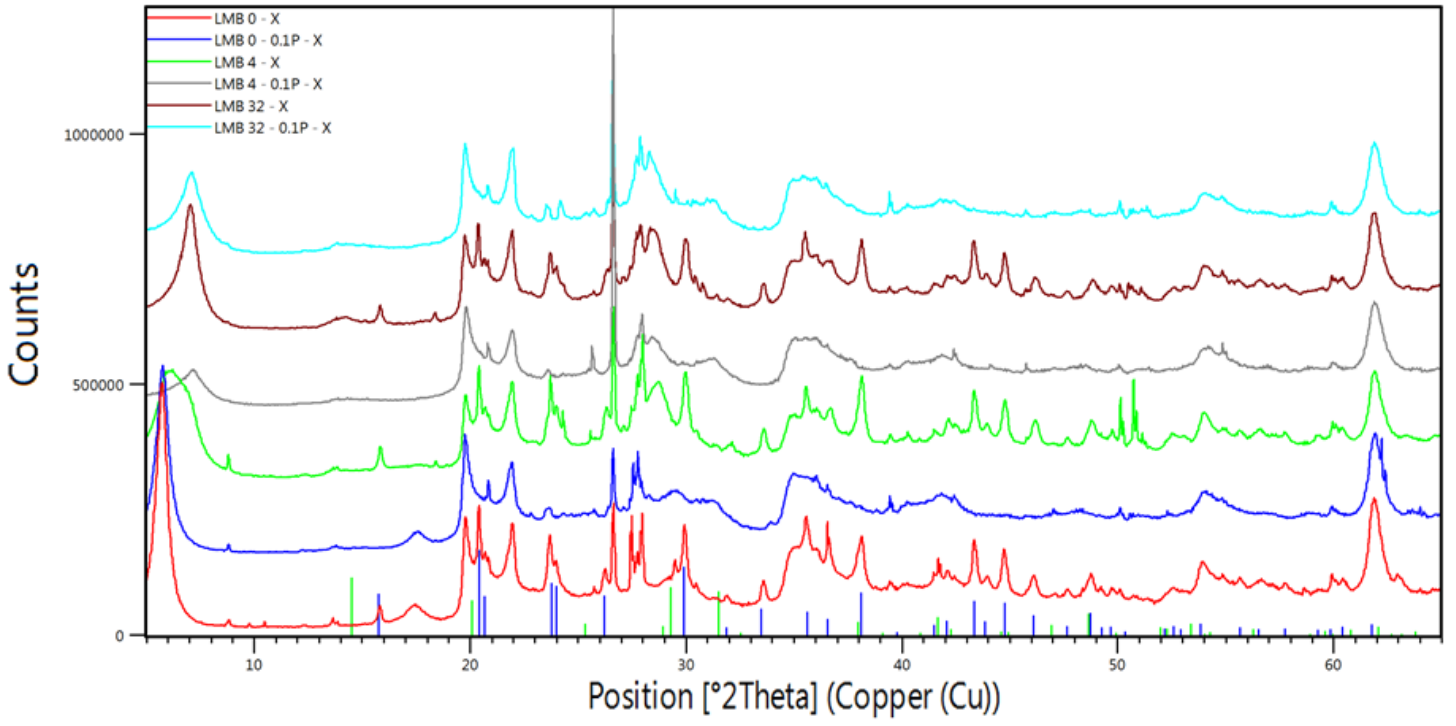

Figure 3.S4: XRD diffractogram. The numbers after LMB (0, 4 and 32) in the legend means salinity used (ppth). 0.1P means the treatment where FRP is present (0.1 mg L^{-1}) the absence means no FRP added.

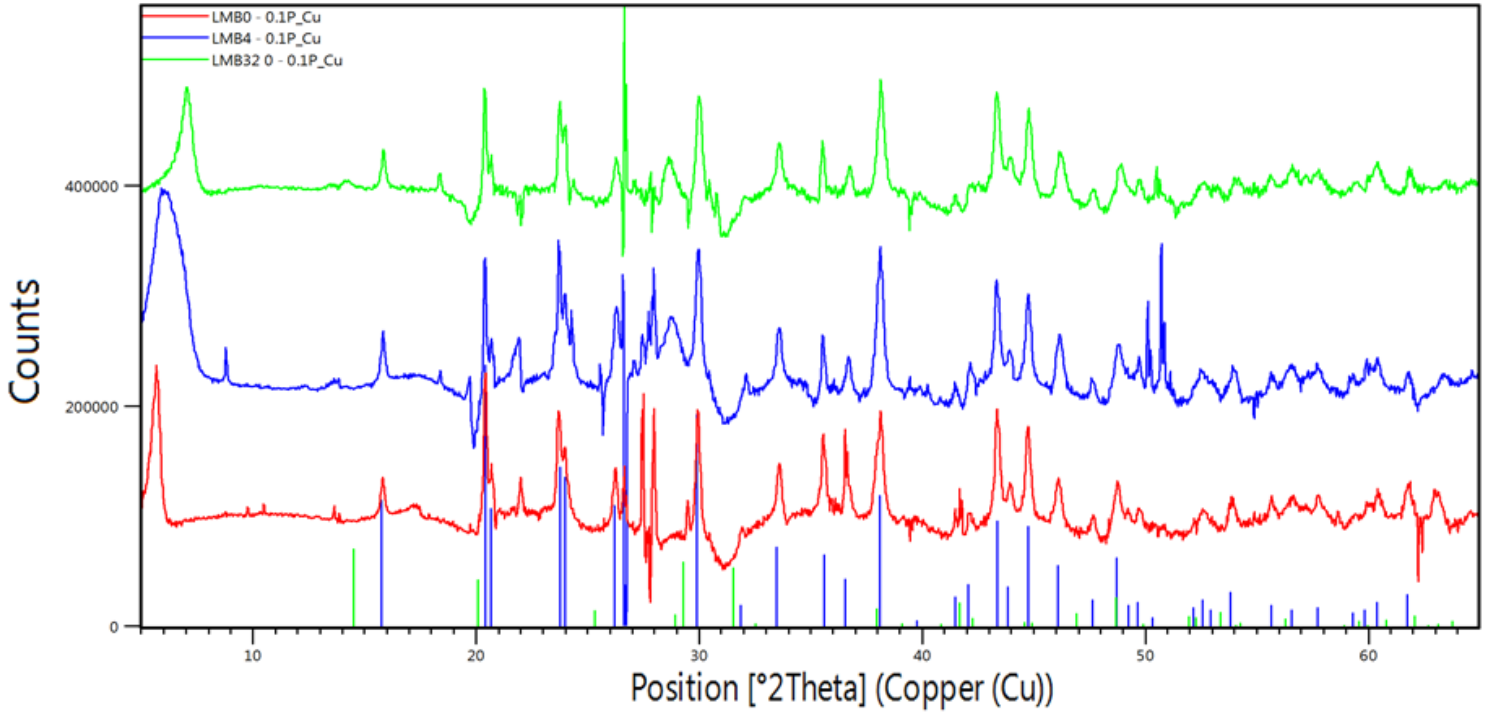

Figure 3.S5: Differential XRD diffractogram (LMB minus LMB-0.1P). The numbers after LMB (0, 4 and 32) in the legend means salinity used (ppth).

4

Chitosan as coagulant on cyanobacteria in lake restoration management may cause rapid cell lysis

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Abstract

Combining coagulant and ballast to remove cyanobacteria from the water column is a promising restoration technique to mitigate cyanobacterial nuisance in surface waters. The organic, biodegradable polymer chitosan has been promoted as a coagulant and is viewed as non-toxic. In this study, we show that chitosan may rapidly compromise membrane integrity and kill certain cyanobacteria leading to release of cell contents in the water. A strain of *Cylindrospermopsis raciborskii* and one strain of *Planktothrix agardhii* were most sensitive. A 1.3 hour exposure to a low dose of 0.5 mg l^{-1} chitosan already almost completely killed these cultures resulting in release of cell contents. After 24 hours, reductions in PSII efficiencies of all cyanobacteria tested were observed. EC_{50} values varied from around 0.5 mg l^{-1} chitosan for the two sensitive strains, via about 5 mg l^{-1} chitosan for an *Aphanizomenon flos-aquae* strain, a toxic *P. agardhii* strain and two *Dolichospermum cylindrica* (*Anabaena*) cultures, to more than 8 mg l^{-1} chitosan for a *Microcystis aeruginosa* strain and another *A. flos-aquae* strain. Differences in sensitivity to chitosan might be related to polymeric substances that surround cyanobacteria. Rapid lysis of toxic strains is likely and when chitosan flocking and sinking of cyanobacteria is considered in lake restoration, flocculation efficacy studies should be complemented with investigation on the effects of chitosan on the cyanobacteria assemblage being targeted.

Introduction

Cyanobacterial blooms are one of the most important water quality issues. Blooms arise when cyanobacteria proliferate to very high densities and/or accumulate at the water surface and lee-side shores in thick scums (Chorus et al., 2000). High densities of cyanobacteria may be a serious threat to the health of humans and wildlife, because many bloom-forming cyanobacteria may produce potent toxins (Carmichael et al., 2001; Dittmann and Wiegand, 2006). These toxins are mostly retained inside the cells (Sivonen and Jones, 1999) and ingestion of substantial amounts of cells may cause animal poisonings when the cells are being lysed in the gastrointestinal tract (e.g. Faassen et al., 2012; Lürling and Faassen, 2013). Likewise, treatment with algaecides to eradicate blooms from raw drinking water will cause cell lysis and liberate intracellular toxins (Jančula and Maršálek, 2011; Jones and Orr, 1994; Merel et al., 2013). Consequently, consumers of that drinking water may be exposed to dissolved cyanotoxins. For instance, treatment of a *Microcystis* bloom with copper sulphate led to liver damage in consumers in Armidale (Australia) (Falconer et al., 1983); an outbreak of hepatoenteritis at Palm Island (Australia) was caused by treating a *Cylindrospermopsis raciborskii* bloom with copper sulphate (Hawkins et al., 1985); boiling water with proliferating *Dolichospermum (Anabaena)* and *Microcystis* was the most likely cause of an outbreak of gastroenteritis in the area of the Itaparica Dam (Brazil) that led to some 2000 cases and 88 casualties (Teixeira et al., 1993), while in Caruaru (Brazil) patients from a haemodialysis clinic presented severe hepatotoxicosis after they had received water contaminated with cyanobacterial hepatotoxins (Azevedo et al., 2002). Effects from recreational exposures to cyanotoxins are less clear, although ingestion will likely cause similar problems as from contaminated drinking water; several, sometimes anecdotal, cases have reported a variety of effects varying from gastro-intestinal or dermatological complaints to presumed death (Falconer, 1999; Stewart et al., 2006). Cyanotoxins may also accumulate in the flesh of fish at higher concentrations than the recommended limit for human consumption (Freitas de Magalhães et al., 2001) and in crops via irrigation water (Saqrane et al., 2009). Thus, when blooms occur in a surface water they may hamper the use of the water for irrigation, fishing, aquaculture, recreation, industry process water and as source for drinking water, which makes mitigating cyanobacterial blooms a significant challenge to water quality managers.

Since cyanobacterial blooms usually result from eutrophication (Smith et al., 1999; Smith and Schindler, 2009), the first mitigation measure in eutrophic lakes is

reducing the external nutrient supply (Cooke, 2005; Paerl et al., 2014). But only a few lakes will respond rapidly to external load reduction, because continuing diffuse and internal loadings will delay recovery for decades to centuries (Carpenter, 2005; Cooke, 2005; Søndergaard et al., 1999). External load control is also not always possible (Huser et al., 2016b), or it is economically not feasible in developing countries because of large upfront investments (van Loosdrecht and Brdjanovic, 2014). Hence, in the majority of the cases an ongoing cyanobacterial nuisance is expected unless in-lake measures are implemented. In-lake mitigation measures can be applied to complement catchment measures in speeding-up recovery, while in-lake curative measures are the only possibility in controlling cyanobacterial nuisance in the short term when external load reduction has not been achieved.

In-lake interventions should be effective, cheap, easy to apply and safe, which means that unintended side-effects should be kept to the absolute minimum (Lürling et al., 2016). Consequently, strategies that liberate toxins from the cells are not preferred as management options (Merel et al., 2013). Thus, common curative interventions using algaecides should be reconsidered, and a promising alternative to algaecides is combining a coagulant and a ballast compound to flock and sink the cyanobacteria as aggregates out of the water column while remaining as intact cells (Lürling and Van Oosterhout, 2013; Pan et al., 2006b). In this approach different ballast and coagulant compounds have been used (e.g. Li and Pan, 2015; Lürling and Van Oosterhout, 2013; Noyma et al., 2016; Pan et al., 2006a, 2011a; Waajen et al., 2016a). Particularly, the organic coagulant chitosan has been promoted in the so-called "modified local soil induced ecological restoration" (MLS-IER) technology (Pan et al., 2011b) and as alternative to poly-aluminium chloride (PAC) (Li and Pan, 2013). Chitosan is commonly viewed as a non-toxic and eco-friendly coagulant (Li and Pan, 2013; Renault et al., 2009; Yang et al., 2016), however, it is also well-known for its antibacterial activity (e.g. Kong et al., 2010; No et al., 2002). Virtually all experiments on the effects of chitosan on cyanobacteria are restricted to trials with *Microcystis aeruginosa* (de Magalhães et al., 2016; Noyma et al., 2016; Pan et al., 2006b, 2006a; Pei et al., 2014). No detrimental effect in short term experiments (1-2 hours) on *M. aeruginosa* has been found (de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2016). In contrast, the first experiments performed with field samples dominated by *Cylindrospermopsis raciborskii* showed that chitosan caused rapid death of cyanobacteria promoting release of saxitoxins (Miranda et al., 2017). This effect is in line with the antibacterial properties of chitosan that vary with the type of bacteria (Kong et al., 2010; No et al., 2002). Inasmuch as chitosan is viewed as a benign

coagulant to control cyanobacterial nuisance (Li and Pan, 2015; Pan et al., 2011b), a thorough investigation on the short-term effect of chitosan on cyanobacteria is warranted to provide insight in possible rapid leakage of cyanobacterial cell constituents into the water. Therefore, in this study we tested the hypothesis that short-term exposure to realistic doses of chitosan, as commonly applied as cyanobacteria removal strategies in lake restoration trials, would cause rapid death of others species than *M. aeruginosa* being *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii, Planktothrix agardhii* and *Dolichospermum cylindrica* other than *M. aeruginosa*.

Materials and methods

Chitosan and cyanobacteria

Chitosan – made of shrimp shells – was obtained from Polymar Ciência e Nutrição S/A (Ceará, Brazil). We acidified the chitosan prior to using by first adding 100 μl of a 96% acetic acid solution (Merck analytical grade) to 100 mg chitosan in 20 ml milli-Q water, dissolved the chitosan and then diluted it in milli-Q water to 100 ml yielding a stock of 1 g l^{-1}. An additional stock of 100 μl of 96% acetic acid in 100 ml milli-Q water was made as control.

In this study, nine different cyanobacterial cultures were used, which were different strains except for *Dolichospermum*, where the same strain was obtained from two different culture collections (Table 4.1). Cyanobacteria were cultured on a modified WC (Woods Hole modified CHU10)-medium (Lurling and Beekman, 2006) in 250 ml Erlenmeyer flasks that were placed at 22 °C and in a 16:8 h light–dark cycle at ~45 μmol quanta m^{-2} s^{-1}. Considering the work and time needed, we conducted the tests in two separate experiments under identical conditions, but with different species, whereas an additional assay was run testing for cell viability.

Table 4.1: Cyanobacteria used in the experiments.

Cyanobacterium	Strain ID	Obtained from
Microcystis aeruginosa	MIRF-01	Laboratory of Ecophysiology and Toxicology of Cyanobacteria (LETC), Federal University of Rio de Janeiro (Brazil)
Aphanizomenon flos-aquae	CCAP 1446/1C	Culture Collection of Algae and Protozoa (Scotland)
Aphanizomenon flos-aquae	SAG 31.87	Sammlung von Algenkulturen der Universität Göttingen (Germany)
Cylindrospermopsis raciborskii	PMC 115.02	Paris Museum Collection (France)
Planktothrix agardhii	NIVA-CYA 116	Norwegian Institute for Water Research (Norway)
Planktothrix agardhii	NIVA-CYA 126	Norwegian Institute for Water Research (Norway)
Dolichospermum cylindrica	PCC 7122	Pasteur Culture Collection (France)
Dolichospermum cylindrica	CCAP 1403/2A	Culture Collection of Algae and Protozoa (Scotland) (=PCC 7122)

Experiment 1

In the first experiment, aliquots of *Aphanizomenon flos-aquae* (*A. flos-aquae*, CCAP 1446/1C and *A. flos-aquae* SAG 31.87), *Cylindrospermopsis raciborskii* (*C. raciborskii* PMC 115.02) and *Microcystis aeruginosa* (*M. aeruginosa* MIRF-01) were transferred into 30 ml reaction vessels (polystyrene cups with a polyethylene snap on lid) and diluted with fresh WC medium such that each vessel contained 25 ml of a cyanobacterial suspension. Cyanobacteria bloom concentrations of around 100 $\mu g\ l^{-1}$ chlorophyll-*a* were used that are commonly found in cyanobacteria dominated systems (Waajen et al., 2014) and correspond to a hypertrophic system (cf OECD, 1984). The effect of chitosan was tested in triplicate in the concentrations 0, 0.5, 1, 2, 4 and 8 $mg\ l^{-1}$. After chitosan was added to designated reaction vessels, the contents were mixed, and the vessels were placed at 22 °C on a laboratory table. For *A. flos-aquae* CCAP 1446/1C, *C. raciborskii* PMC 115.02 and *M. aeruginosa* MIRF-01 an additional series was included with acetic acid added in similar quantities as in the chitosan treatments that served as acetic acid control. After 1.3, 5 and 24 hours, samples were taken from each vessel and the chlorophyll-*a* concentrations and photosystem II (PSII) efficiencies as maximum quantum yield of PSII were measured using a PHYTO-PAM phytoplankton analyser (Heinz Walz GmbH, Effeltrich, Germany).

The maximum quantum yield of PSII, further referred to as PSII efficiency, was calculated according to Genty et al. (1989): $\Phi_{PSII,\,m} = (F_m - F_0) / F_m$

Where F_0 is the dark-adapted minimal fluorescence and F_m is the maximum fluorescence obtained when all photosynthetic reaction centres are closed with a saturating pulse of light. This photosynthetic yield can be used as an indicator of stress on the photosynthetic apparatus (Parkhill et al., 2001) and finds wide application in determining for instance the effect of hydrogen peroxide in killing cyanobacteria (Matthijs et al., 2012). After 24 hours also the pH in each vessel was measured using a WTW Inolab pH 7110 meter. In addition, for the *C. raciborskii* series 2 ml samples were filtered through 0.45 μm unit filters (Aqua 30/0.45CA, Whatman, Germany) and the filtrated was measured for chlorophyll-*a* concentrations and photosystem II efficiencies.

Experiment 2

In the second experiment, 25 ml suspensions of *Planktothrix agardhii* (*P. agardhii* NIVA-CYA116, *P. agardhii* NIVA-CYA126) and *Dolichospermum cylindrica* (*D. cylindrica* PCC7122 and D*. cylindrica* CCAP 1403/2A) were exposed to chitosan in concentrations of 0, 0.5, 1, 2, 4 and 8 mg l^{-1}. The experiment was performed similarly to the experiment 1. In addition, after 1.3 hour of incubation, for the *P. agardhii* NIVA-CYA116, series 2 ml samples were filtered through 0.45 μm unit filters (Aqua 30/0.45CA, Whatman, Germany) and measured on chlorophyll-*a* concentrations and photosystem II efficiencies.

Cell membrane permeability

To examine the effect of chitosan on the cell membrane integrity, an experiment was performed with *Cylindrospermopsis raciborskii* T3 cultures that were either exposed to chitosan (8 mg l^{-1}) or left untreated (control). The initial chlorophyll –*a* concentration was 100 μg l^{-1}. After 24 hours samples were taken to PSII efficiencies as described before. An aliquot of 15 ml was centrifuged at 5000 x g per 10 minutes, where after the pellets were incubated with Sytox ® Green (Thermo Fisher Scientific-Cat No. S7020) at a final concentration of 1nM for 30 minutes in the dark (Tashyreva et al., 2013). The dye binds to nucleic acids and is unable to penetrate the membrane of live cells. However, when the membranes are compromised, it easily penetrates the cell yielding a bright green fluorescence indicative of dead cells (Sato et al., 2004). The Sytox® Green treated samples were inspected with a fluorescence microscope

(ZEISS, Axioimager D2) using the filter long pass for Fluorescein (450 – 490 for excitation and 515 nm for emission).

Data analysis

Photosystem II efficiencies in the different cyanobacterial strains exposed for 1.3, 5 and 24 hours to different concentrations chitosan (0 – 8 mg l^{-1}) were analysed separately by one-way ANOVAs or Kruskal-Wallis One Way Analysis of Variance on Ranks when normality tests failed (Shapiro-Wilk) in the tool pack SigmaPlot 13.0. For each strain the concentration of chitosan that caused a 50% reduction in Photosystem II efficiency compared to PSII efficiency of the control (EC_{50}) were determined by iterative non-linear regression using a four-parameter logistic function in the tool pack SigmaPlot 13.0. Photosystem II efficiencies of cyanobacteria exposed to different concentrations of acetic acid were compared running a Parallel Lines Analysis in SigmaPlot 13.0.

Results

Experiment 1: *M. aeruginosa*, *A. flos-aquae* and *C. racibroskii*.

Chitosan caused a rapid increase in chlorophyll-*a* concentrations measured in *C. raciborskii* PMC 115.02 cultures after 1.3 hour of exposure, but not in both *A. flos-aquae* strains and in *M. aeruginosa* (Fig. 4.1A). After 5 hours, also in the *A. flos-aquae* CCAP 1446/1C cultures exposed to the highest chitosan chlorophyll-*a* concentrations increased (Fig. 4.1C), while this happened for all strains after 24 hours of exposure to higher chitosan doses (Fig. 4.1E). One-way ANOVA indicated chlorophyll-*a* concentrations in *M. aeruginosa* cultures were significantly different between treatments ($F_{5,12} = 14.1$; $p < 0.001$) after 24 hours exposure to chitosan, where a Holm-Sidak pairwise multiple comparison revealed that chlorophyll-*a* concentrations in the 4 and 8 mg chitosan l^{-1} treatments were significantly higher than those in the 0, 0.5, 1 and 2 mg chitosan l^{-1}. Likewise, in *A. flos-aquae* CCAP 1446/1C ($F_{5,12} = 4.67$; $p = 0.013$) chlorophyll-*a* concentrations between 2 and 4 mg chitosan l^{-1} treatments differed, while in SAG 31.87 ($F_{5,12} = 45.6$; $p < 0.001$) only in the 8 mg chitosan l^{-1} treatment significantly higher chlorophyll-*a* concentrations than those in the other treatments were found. *C. raciborskii* still remained higher chlorophyll-*a* concentrations in treatments than in controls (Fig. 4.1E) and analysis of 0.45 μm filtrate revealed that a large portion was filterable (Fig. 4.2) indicating cell lysis.

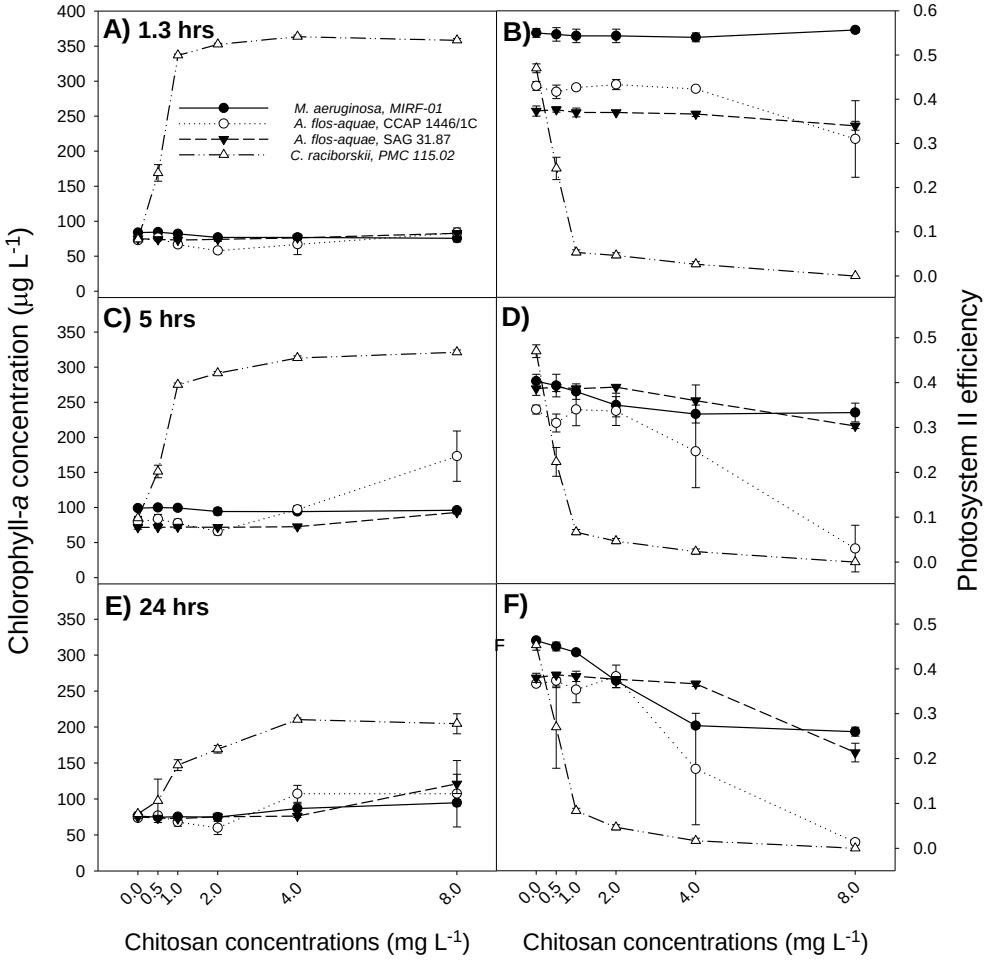

Figure 4.1: Total chlorophyll-a concentration (µg l^{-1}) and Photosystem II efficiencies for *Aphanizomenon flos-aquae* CCAP 1446/1C, Aphanizomenon flos-aquae SAG 31.87, *Cylindrospermopsis raciborskii* PCM 115.02 and *Microcystis aeruginosa* MIRF-01 after 1.3 hours (A, B), 5 hours (C, D) and 24 hours (E, F) exposure to different chitosan concentrations (0 – 8 mg l^{-1}). Error bars indicate one standard deviation (*n*=3).

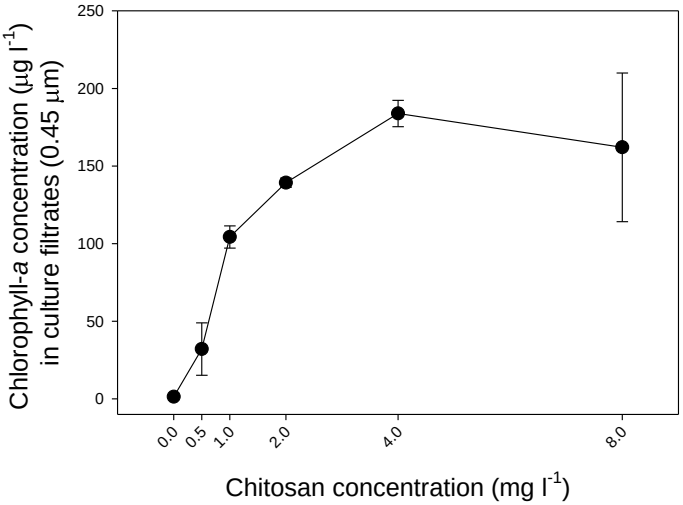

Figure 4.2: Dissolved Chlorophyll-a concentration (µg l^{-1}) in 0.45 µm filtrates from culture of *Cylindrospermopsis raciborskii* (PMC 115.02) exposed for 24 hours to different chitosan concentration (0 – 8 mg l^{-1}). Error bars indicate one standard deviation (*n*=3).

Additional proof for cell damage was obtained from the PSII efficiency measurements that already after 1.3 hours exposure revealed a strong decrease in *C. raciborskii* cultures with increasing chitosan dose (Fig. 4.1B). The PSII efficiencies were significantly different (Table 2) and four homogenous groups were detected: 1) higher PSII-efficiency in the control; 2) significantly lower in the 0.5 mg chitosan l^{-1} treatment; 3) lower in the 1, 2 and 4 mg chitosan l^{-1} treatments; and 4) lowest in the 8 mg chitosan l^{-1} treatment. This remained after 5 hours and then also appeared in *A. flos-aquae* CCAP 1446/1C, where the PSII efficiency in the 8 mg chitosan l^{-1} treatment was significantly lower than in the rest and in *M. aeruginosa* were the PSII-efficiencies in the 4 and 8 mg chitosan l^{-1} treatments were lower (Table 4.2; Fig. 4.1D). After 24 hours also significantly lower PSII efficiencies (Table 4.2) at higher chitosan doses in *A. flos-aquae* SAG 31.87 and in *M. aeruginosa* cultures were found (Fig. 4.1F).

Only for *C. raciborskii* at each exposure duration and for *A. flos-aquae* CCAP 1446/1C after 5 and 24 hours EC_{50} values could be calculated; for *M. aeruginosa* and *A. flos-aquae* SAG 31.87 EC_{50} values were exceeding the highest dose tested and thus were > 8 mg chitosan l^{-1} (Table 4.3). One-way ANOVA indicated that EC_{50} values in *C. raciborskii* PMC 115.02 were similar ($F_{2,6}$ = 0.79; p = 0.498) at each exposure duration and on average 0.50 (± 0.07) mg chitosan l^{-1}. Similarly, a t-test revealed that EC_{50} values for *A. flos-aquae* CCAP 1446/1C were similar (t_4 = 1.05; p = 0.354) and on average 5.2 (±SD 1.6) mg chitosan l^{-1} (Table 4.3).

Table 4.2: *F*- and *p*-values of one-way ANOVAs and *H*- and *p*-values of Kruskal-Wallis One Way Analysis of Variance on Ranks when normality tests failed (Shapiro-Wilk) for Photosystem II efficiencies in four different cyanobacterial strain exposed for 1.3, 5 and 24 hours to different concentrations chitosan (0 – 8 mg l^{-1}). Significant differences in bold.

Cyanobacterial Strain	Exposure duration (exp. 1)		
	1.3 hours	5 hours	24 hours
MIRF-01	$F_{5,12}$ = 0.69; p = 0.643	$F_{5,12}$ = 6.60; p = **0.004**	$F_{5,12}$ = 275.3; p < **0.001**
CCAP 1446/1C	H_5 = 9.51; p = 0.090	$F_{5,12}$ = 22.1; p < **0.001**	H_5 = 12.7; p = **0.027**
SAG 31.87	H_5 = 9.96; p = 0.076	H_5 = 11.0; p = 0.052	$F_{5,12}$ = 108.1; p < **0.001**
PMC 115.02	$F_{5,12}$ = 726.8; p < **0.001**	H_5 = 16.7; p = **0.005**	H_5 = 16.7; p = **0.005**
	Exposure duration (exp. 2)		
	1.3 hours	5 hours	24 hours
CYA 116	$F_{5,12}$ = 306.4; p < **0.001**	$F_{5,12}$ = 3607; p < **0.001**	H_5 = 15.8; p = **0.007**
CYA 126	$F_{5,12}$ = 0.61; p = 0.692	H_5 = 2.37; p = 0.795	$F_{5,12}$ = 940.2; p < **0.001**
PCC 7122	H_5 = 10.7; p = 0.059	H_5 = 9.64; p = 0.086	$F_{5,12}$ = 34.2; p < **0.001**
CCAP 1403/2A	$F_{5,12}$ = 11.2; p < **0.001**	$F_{5,12}$ = 139.5; p < **0.001**	$F_{5,12}$ = 80.8; p < **0.001**

A Parallel Lines Analysis revealed that the slopes of the PSII efficiencies against the acetic acid concentrations were not different ($F_{8,36}$ = 1.66; P = 0.144). The overall regression yielded PSII = 0.419 + (0.00137 × acetic acid) with a r^2 = 0.005, which indicates the slope is not different from zero (Supplementary information; Fig. 4.S1). The pH values in the various chitosan treatments after 24 hours varied between on average pH 7.21 and pH 7.84, while in the acetic acid control it varied between pH 7.40 and pH 7.94 (Supplementary information, Table 4.S1).

Table 4.3: Mean EC_{50} values (mg l^{-1}; values inside brackets represent SD; n = 3) of chitosan for the Photosystem II efficiency in different cyanobacterial strains. Similar symbols per column (A,B) indicate homogeneous groups (Holm-Sidak pairwise comparisons; $p < 0.05$).

Cyanobacterial strain	EC_{50} - 1.3 hour (mg l^{-1})	EC_{50} - 5 hours (mg l^{-1})	EC_{50}- 24 hours (mg l^{-1})
MIRF-01	> 8	> 8	> 8
CCAP 1446/1C	> 8	5.93 (1.86)^{A}	4.59 (1.22)^{A}
SAG 31.87	> 8	> 8	> 8
PMC 115.02	0.50 (0.02)	0.46 (0.05)^{B}	0.54 (0.13)^{B}
CYA 116	0.41 (0.07)	0.27 (0.04)^{B}	0.44 (0.05)^{B}
CYA 126	> 8	> 8	5.58 (1.07)^{A}
PCC 7122	> 8	8.19 (0.82)^{A}	5.65 (1.47)^{A}
CCAP 1403/2A	> 8	7.62 (0.27)^{A}	5.53 (1.11)^{A}

Experiment 2: *Dolichospermum* and *Planktothrix*

After 1.3 hour exposure to chitosan, fluorescence–based chlorophyll-*a* concentrations were significantly influenced (Fig. 4.3A). A sharp increase was observed in *P. agardhii* CYA 116 with increasing chitosan doses, except 4 and 8 mg l^{-1} being significantly different from each other ($F_{5,12}$ = 363.3; $p < 0.001$). This increase was caused by leakage of pigments in the surrounding medium as 0.45 μm filtered culture medium had significantly higher ($F_{5,12}$ = 148.3; $p < 0.001$) chlorophyll-*a* concentrations (Fig. 4.4), where all treatments differed significantly from each other, but all with zero PSII efficiency (data not shown). Also in the other *P. agardhii* strain (CYA 126) significant differences in chlorophyll-*a* concentrations were detected ($F_{5,12}$ = 26.3; $p < 0.001$). Despite smaller than in CYA 116 the increase from on average 43 μg l^{-1} in controls to 52 and 54 μg l^{-1} in 4 and 8 mg chitosan l^{-1} treatments, respectively (Fig. 4.3A), was significant due to relative small within group variability. In both *Dolichospermum* strains, significant differences between different concentrations of chitosan were also detected (PCC 7122: H_5 = 12.2; p = 0.032 and CCAP 1403/2A: $F_{5,12}$ = 32.9; $p < 0.001$). The *post hoc* comparisons revealed that only chlorophyll-*a* concentrations in the 8 mg chitosan l^{-1} treatment was significantly higher than in the other treatments.

The above observed pattern was similar after 5 hours exposure to chitosan, where fluorescence–based chlorophyll-*a* concentrations were still significantly influenced (Fig. 4.3C). In *P. agardhii* CYA 116 the controls, 0.5 and 1 mg l^{-1} treatments were significantly different ($F_{5,12}$ = 182.6; p < 0.001) from each other and from the 2, 4 and 8 mg l^{-1} treatments that formed one homogenous group. In *P. agardhii* CYA 126, three homogenous groups were found: 1) the controls, 0.5, 1 and 2 mg l^{-1} treatments, 2) the 2 and 4 mg l^{-1} treatments, and 3) the 4 and 8 mg l^{-1} treatments ($F_{5,12}$ = 37.4; p < 0.001). In *D. cylindrica* PCC 7122 only the highest chitosan dose was identified as causing significantly increased chlorophyll-*a* concentrations (H_5 = 11.1; p = 0.049). Omitting this highest dose yielded normally distributed data (Normality Test (Shapiro-Wilk): p = 0.619) with equal variances (Equal Variances Test Brown-Forsythe: p = 0.453) and an one-way ANOVA that indicated all chlorophyll-*a* concentrations in the chitosan doses 0 – 4 mg l^{-1} were not different ($F_{4,10}$ = 2.24; p = 0.137). Since the power of the *F* –test (0.26) was less than the desired power (0.80) some caution is needed, but the actual magnitude of the measured chlorophyll-*a* concentrations strongly suggests that this result would not have been different running a multitude of replicates (Fig. 4.3C). Likewise, in the other *Dolichospermum* series only the highest chitosan dose caused significantly increased chlorophyll-*a* concentrations ($F_{5,12}$ = 47.1; p < 0.001).

After 24 hours exposure to chitosan, the observed patterns of measured chlorophyll-*a* concentrations (Fig. 4.3E) were not that different from those observed after 5 hours (Fig. 4.3C). In *P. agardhii* CYA 116, still significant differences were found ($F_{5,12}$ = 106.5; p < 0.001) with three homogenous groups: 1) the control and 0.5 treatment; 2) the 1 mg l^{-1} treatment; and 3) the 2, 4 and 8 mg l^{-1} treatments. In *P. agardhii* CYA 126, the control and the 8 mg l^{-1} treatment were significantly different from each other (H_5 = 14.1; p = 0.015). In *D. cylindrica* PCC 7122 the highest chitosan dose caused significantly increased chlorophyll-*a* concentrations compared to the other treatments ($F_{5,12}$ = 15.2; p < 0.001; Fig. 4.3C), whereas in the other *D. cylindrica* series (CCAP 1403/2A) no differences were detected anymore ($F_{5,12}$ = 1.04; p = 0.438).

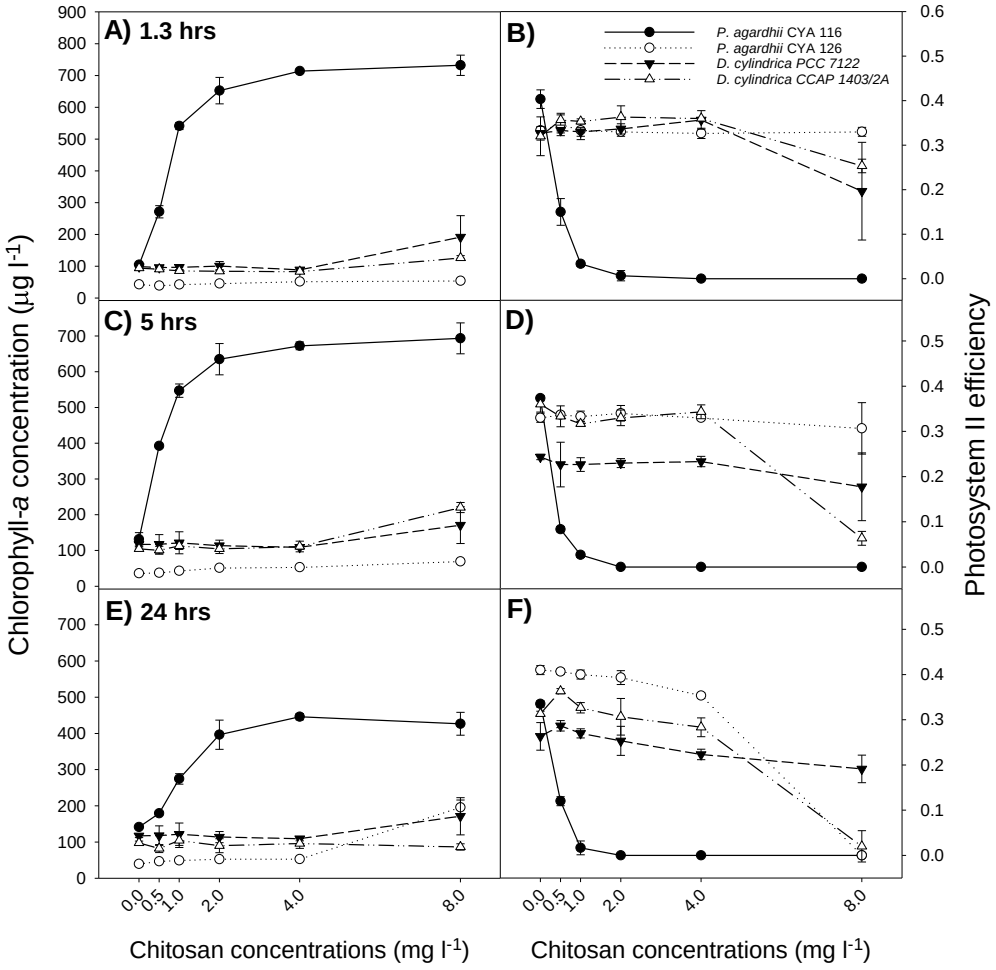

Figure 4.3: Chlorophyll-a concentrations (µg l^{-1}) and Photosystem II efficiencies for *Planktothrix agardhii* NIVA-CYA 116 and NIVA-CYA 126, and for *Dolichospermum cylindrica* PCC 7122 and CCAP 1403/2A after 1.3 h (A, B), 5 h (C, D) and 24 h (E, F) exposure to different chitosan concentrations (0 – 8 mg l^{-1}). Error bars indicate one standard deviation (*n* = 3).

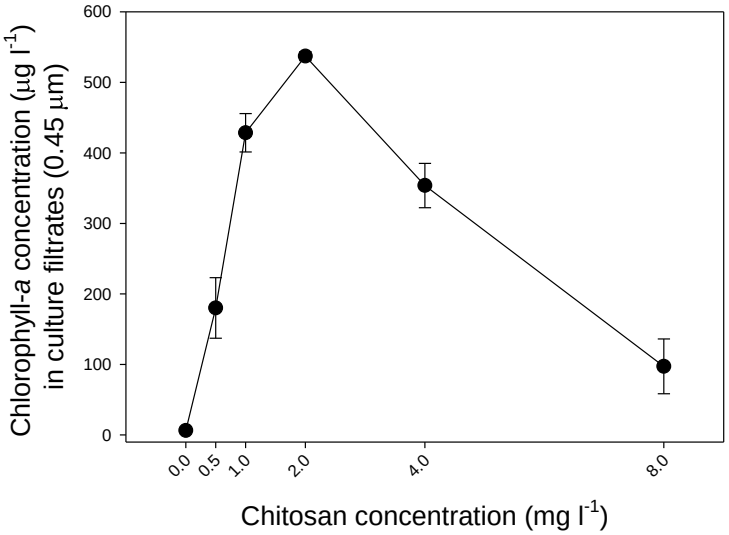

Figure 4.4: Chlorophyll-a concentrations (µg l^{-1}) in 0.45 mm filtrates from culture of *Planktothrix agardhii* NIVA-CYA 116 exposed for 1.3 h to different concentrations chitosan (0 – 8 mg l^{-1}). Error bars indicate one standard deviation (*n*= 3).

Photosystem II efficiencies were strongly reduced in *P. agardhii* CYA 116. After 1.3 hour, PSII efficiencies dropped significantly in the 0.5 mg l^{-1} treatment and virtually became zero in all other treatments (Fig. 4.3B; Table 4.2). After 5 hours, PSII efficiency of *P. agardhii* CYA 116 in the control was significantly higher than in the 0.5 mg l^{-1} treatment, which was higher than in the 1 mg l^{-1} treatment, while PSII efficiencies were zero in the 2, 4 and 8 mg l^{-1} treatments (Fig. 4.3D). This pattern did not change after 24 hours (Fig. 4.3F). In *P. agardhii* CYA 126, PSII efficiencies were not affected by chitosan after 1.3 and 5 hours exposure (Fig. 4.3B, D; Table 4.2), but after 24 hours, where PSII efficiencies remained similar in the control, 0.5, 1 and 2 mg l^{-1} treatments, it was significantly lowered in the 4 mg l^{-1} treatment and became zero in the highest chitosan dose (Fig. 4.3F; Table 4.2).

Although in *D. cylindrica* PCC 7122 PSII efficiency in the highest chitosan dose showed a tendency to be lower than in the other treatments, it was only significant after 24 hours incubation (Table 4.2). In the *D. cylindrica* CCAP 1403/2A series PSII efficiencies in the highest chitosan dose were significantly lower at all three exposure durations (Fig. 4.3B,D,F; Table 4.2)

One-way ANOVA indicated that EC_{50} values for chitosan exerting a negative effect on PSII efficiencies in *P. agardhii* CYA 116 differed ($F_{2,6}$ = 8.36; p = 0.018), with EC_{50} after 5 hours being lower than after 1.3 and 24 hrs (Table 4.3). After 5 hours or

24 hours exposure to chitosan EC_{50} values for *D. cylindrica* PCC7122 were similar (t_4 = 2.61; p = 0.059), while they differed in CCAP 1403/2A (t_4 = 3.19; p = 0.033). Comparing the EC_{50} values for the five strains in which it could be determined after 5 hours of exposure yielded significant differences ($F_{4,10}$ = 269.2; p < 0.001) and two homogeneous groups: 1) *C. raciborskii* and *P. agardhii* CYA 116; and 2) *A. flos-aquae* CCAP 1446/1C, *D. cylindrica* PCC 7122 and CCAP 1403/2A. This outcome was similar for EC_{50} values determined after 24 hours exposure ($F_{5,12}$ = 19.2; p < 0.001), where the now included *P. agardhii* CYA 126 ended up in the second group with *A. flos-aquae* CCAP 1446/1C, *Dolichospermum* PCC 7122 and CCAP 1403/2A (Table 4.3).

PSII efficiencies were not influenced by the acetic acid used to dissolve the chitosan, as values remained as high as in the controls in acetic acid concentrations between 0.5 µl l^{-1} and 8 µl l^{-1}, which is identical to the acetic acid concentrations in the chitosan treatments (Supplementary information; Fig. 4.S1). The slopes of the PSII efficiencies against the acetic acid concentrations were not different (Parallel Lines Analysis; $F_{10,44}$ = 1.84; P = 0.081). The overall regression yielded PSII = 0.336 + (0.00142 × Acetic acid) with a r^2 = 0.005, which indicates the slope is not different from zero (Supplementary information; Fig. 4.S1).

The pH values in the various chitosan treatments after 24 hours were varied slightly between pH 7.34 and pH 8.17, while in the acetic acid control it varied between pH 7.57 and pH 8.83 (Supplementary information, Table 4.S1).

Cell membrane permeability

The cell membrane integrity test showed clear differences between chitosan exposed *C. raciborskii* T3 cells and non-exposed cells (Fig. 4.5). After 24 hours incubation with chitosan a very bright green fluorescence was observed (Fig 4.5A).

This green fluorescence indicates penetration of the dye in the *C. raciborskii* T3 cells, confirming membrane damage, which was also confirmed by a low PSII value (0.06) (Fig 4.5A). In the control, after 24 hours only the natural red fluorescence of the cells was detected (Fig. 4.5B), indicating no membrane damage which is in agreement with a high PSII value (0.53) measured.

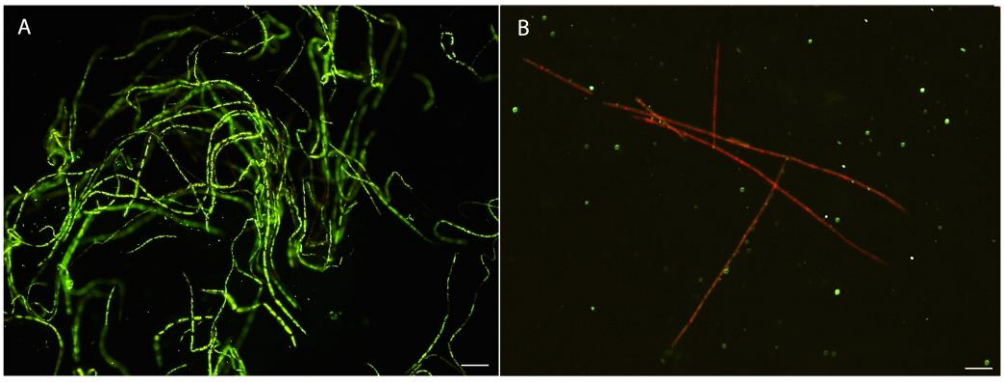

Figure 4.5: Fluorescence images of *C. raciborskii* T3 cells. The panel A shows intracellular accumulation of Sytox Green fluorescence (green) at chitosan 8 mg treatment after 24h. The panel B represents the control (no addition of chitosan) and shows autofluorescence (red) and no intracellular Sytox accumulation. Scale bar = 20 µm.

Discussion

The results of this study are only partly in agreement with our hypothesis that cyanobacteria other than *M. aeruginosa* would be killed rapidly by exposure to realistic doses of chitosan as proposed for cyanobacterial nuisance control in lakes. Two of the eight strains tested, namely *C. raciborskii* PMC 115.02 and *P. agardhii* CYA 116, were affected by low doses of chitosan within 1.3 hours of exposure. EC_{50} values were around 0.5 mg chitosan l^{-1}, which is below the normal application doses of 1-2 mg chitosan l^{-1} used at comparable cyanobacteria blooming concentrations (Li and Pan, 2015, 2013; Pan et al., 2006b). This detrimental effect of chitosan maintained throughout the exposure duration. After 5 hours exposure in three other strains (*A. flos-aquae* CCAP 1446/1C, *D. cylindrica* PCC 7122 and *D. cylindrica* CCAP 1403/2A) also EC_{50}s could be determined, while a sixth strain (*P. agardhii* CYA 126) could be added after 24 hours. There were two groups distinguishable: the two very susceptible strains with EC_{50}s around 0.5 mg chitosan l^{-1} and four others with EC_{50}s around 5 mg chitosan l^{-1}. It should, however, be noted that after 24 hours in the highest chitosan dose also in the two remaining strains (*M. aeruginosa* MIRF-01 and *A. flos-aquae* SAG 31.87) in which no EC_{50} could be determined- PSII efficiencies had dropped to 56% of the values in the controls. Hence, all strains were affected by chitosan, albeit for some strains only after one day at a dose of 8 mg l^{-1}. In agreement with our results, Noyma et al. (2016) and de Magalhães et al. (2016) did not observe any chitosan effect on PSII efficiencies after 1 hour exposure of lake water with a *M. aeruginosa* bloom.

Since, in the current experiment an effect became visible after 24 hours, it is advisable to explore the chitosan effect under prolonged exposure. The rapid decrease in PSII efficiencies in strains *P. agardhii* CYA 116 and *C. raciborskii* PMC 115.02 and the concomitant increase in dissolved fluorescent pigments reflect cell lysis. It is well known that water soluble extracellular phycocyanin can contribute considerably to the detected fluorescence signal, which does not reflect an increase of biomass (Bastien et al., 2011). Hence, the strong increase in the filterable chlorophyll-*a* that occurred without any PSII efficiency can be used as an indicator of cell leakage. In general, the release of intracellular components is an indication of membrane damage (Liu et al., 2004). Loss of membrane integrity was confirmed by the positive staining with Sytox® green of chitosan exposed *C. raciborskii* T3, but not in controls. Sytox® green has been shown to be the most suitable dye to distinguish between live and dead cells in cyanobacteria (Tashyreva et al., 2013). The observed effect of compromised membrane integrity by chitosan has high similarity to what has been observed in other bacteria (Liu et al., 2004).

The acetic acid used to dissolve and protonate the chitosan had no measurable influence on the cyanobacteria; both chlorophyll-*a* concentrations and PSII efficiencies remained unaffected. The pH was only slightly lowered at the highest chitosan doses and therewith the highest acetic acid concentrations, but stayed well within the optimal growth range with values between pH 7.21 and pH 8.83. Thus, pH effects can be excluded leaving a direct effect of chitosan as the most probable cause of the rapid leakage and cell death as observed in *P. agardhii* CYA 116 and *C. raciborskii* PMC 115.02.

When protonated in acidic medium, chitosan behaves as a typical cationic polyelectrolyte, because the protonated free amino groups of chitosan allow electrostatic interactions with the negatively charged cell wall constituents of cyanobacteria (Renault et al., 2009). Flocculation is caused when the long chain polymers attach to cyanobacteria thereby forming bridges that subsequently can entrap particles when settling or uprising (Chen et al., 2014; Renault et al., 2009; Yang et al., 2016). Besides being a good flocculant under certain environmental conditions – flocks were observed in all strains exposed to chitosan in our experiment – chitosan is also known for its antibacterial activity (Kong et al., 2010; Younes et al., 2014). The mechanism behind chitosan's antibacterial activity is complex and has not been fully elucidated, as it depends on the characteristics of chitosan, such as the amount of amino groups, protonation, molecular weight; environmental factors, such as ionic strength and pH and, bacterial properties like cell surface characteristics (Bellich et al., 2016; Kong et al., 2010). Inasmuch as we have used only one type of chitosan and

environmental conditions were kept as similar as possible, most probably the variability in susceptibility to chitosan among the cyanobacteria tested is caused by differences between the cyanobacterial strains.

There is no clear pattern distinguishable in susceptibility to chitosan based on taxonomy. The least sensitive strains were a member of the order of the Chroococcales (*Microcystis*) and one of the Nostocales (*Aphanizomenon*), while the most sensitive strains belonged to the Nostocales (*Cylindrospermopsis*) and to the Oscillatoriales (*Planktothrix*). Interestingly, a second strain of the same species *P. agardhii* appeared much less sensitive. Both *Dolichospermum* strains were equally sensitive, which makes sense as, although obtained from two different culture collections, they originated from the same isolate. Variability in sensitivity to chitosan has also been observed in other bacteria (No et al., 2002). Although it is unclear what is the underlying mechanism causing the obvious differences in susceptibility to chitosan among the cyanobacterial strains tested, the type and degree of polymeric substances that surround most cyanobacteria as a protective barrier between the cell and the environment (De Philippis et al., 2001; Kehr et al., 2015) may play a role. Extracellular polymeric substances in *M. aeruginosa* have been proposed as providing cells with a protective envelope (e.g., Gao et al., 2015) that weakens upon chitosan flock storage causing membrane damage and cell leakage after some days (Pei et al., 2014). The polysaccharide composition of the envelope as well as the amount of polysaccharide produced may differ greatly even in closely related taxa (Forni et al., 1997). In addition, or related to the polymeric envelope, in other Gram-negative bacteria the negative charge density on the cell surface appeared a major factor in determining the susceptibility of the bacteria to chitosan (Chung et al., 2004). The Gram-negative cell envelope is composed of a cytoplasmic membrane and an outer membrane, where the outer membrane is comprised of lipopolysaccharides and proteins (Durai et al., 2015). These compounds are stabilised by divalent cations and either poly-protonated chitosan (at pH < pKa) or chelating chitosan (at pH > pKa) is proposed to destabilise the electrostatic interactions that keep together the outer membrane (Kong et al., 2010). When outer membrane integrity is compromised, the cytoplasmatic membrane may be exposed to chitosan leading to destabilization, leaking and cell death (Kong et al., 2010).

The leakage of intracellular substances is of particular relevance when this occurs rapidly during the process of flocking and settling, because then intracellularly stored toxins and odoriferous compounds may be liberated into the water column. Cell lysis might release geosmin and 2-methylisoborneol (MIB) that are one of the main causes of taste- and odour complaints to water suppliers (Journey et al., 2013;

Suffet et al., 1996). Consequently, under such circumstances chitosan might not be the preferred coagulant in an environmentally safe management strategy (Merel et al., 2013), especially when the bloom occurs in a water storage reservoir (Jones and Orr, 1994).

In contrast, a delayed effect in settled flocks, as has been observed by Pei et al. (2014) for chitosan flocked *M. aeruginosa*, can be viewed as beneficial. It is well-known that cyanobacteria may survive for prolonged periods on the sediment and that sediment in lakes and reservoirs may, for example, contain large biomass of viable *Microcystis* colonies (Brunberg and Boström, 1992; Latour and Giraudet, 2004; Reynolds et al., 1981). The settled cyanobacteria, while remaining alive, may potentially serve as an inoculum to the over-standing water, or be resuspended by wave- or bioturbation actions. Hence, a rather rapid lysis of flocked and settled cyanobacteria (within a few days) will strongly reduce the possibility of recolonization of the water column, whereas the near the sediment liberated toxins can be degraded by a rich community of decomposing bacteria (e.g., Grützmacher et al., 2010; Holst et al., 2003; Li and Pan, 2015). Modified chitosan soil has also been successful tested together with a soil/sand to cap the flocks settled near to the sediment reducing algal resuspension as well as nutrients flux and microcystins release (Li and Pan, 2015; Pan et al., 2012).

The sensitive *P. agardhii* strain CYA 116 does not produce microcystin (MCs), while the other far less sensitive strain CYA 126 produces MCs: under our culturing conditions this strain in its stock culture produces dmRR (83.0%), RR (0.3%), YR (1.2%), dmLR (15.5%) and LR (0.1%), which was determined by LC-MS/MS following the protocol as described in Lürling and Faassen (2013). Also the other highly susceptible strain, *C. raciborskii* PMC 115.02 is not a known toxin producer (Berger et al., 2006). Hence, rapid lysis of these strains will not likely evoke an undesired condition from release of cyanotoxins. A generalisation, however, towards higher susceptibility of non-toxic strains is not warranted as Miranda et al. (2017) observed higher dissolved saxitoxin concentrations when field samples dominated by *C. raciborskii* were exposed to chitosan and chitosan mixed with different ballast compounds.

Moreover, amount and composition of extracellular polymeric substances in *Microcystis* was not related to the ability to produce MCs (Forni et al., 1997). Therefore, more strains, for instance various *C. raciborskii* strains and various *M. aeruginosa* strains, should be tested on their susceptibility to relatively low concentrations of chitosan to get insight in the among strain variability and also in the potential of chitosan to rapidly liberate intracellular toxins. Based on the results of this study, when chitosan-modified flocking and sinking of cyanobacteria is

considered in lake restoration, flocculation efficacy studies should be complemented with investigation of the effects of the chitosan on the cyanobacteria assemblage being targeted.

Conclusions

- The organic coagulant chitosan is used as a flocculant in lake restoration, yet in a realistic low dose of 0.5 mg l^{-1} it caused within 1.3 hours rapid cell lysis in two of eight cyanobacteria tested (*C. raciborskii* PMC 115.02 and *P. agardhii* CYA 116).
- Cell lysis was indicated by strong increase of filterable chlorophyll-*a* as detected by Phyto-PAM, but without any photosynthetic activity and confirmed by strong increase in green fluorescence of the Sytox® Green dye in chitosan exposed *C. raciborskii* T3, but not in controls.
- After one day, six of the eight tested cyanobacteria showed clear signs of being affected by chitosan; neither the diluted solvent acetic acid, nor slight pH differences caused these effects.
- Differences in sensitivity to chitosan are related to cyanobacteria specific characteristics and probably to polymeric substances that surround cyanobacteria.
- Rapid cell lysis in the water column is not wanted, but cell lysis in settled cells on the sediment is beneficial.
- In lake restoration, prior to each application, studies on the effects of chitosan on the cyanobacteria assemblage being targeted should be included.

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Supplementary information

Table 4.S1: pH values measured in cyanobacterial suspensions after 24 hours exposure to different concentrations of chitosan (means; values in the brackets represent SD; *n* = 3) or the corresponding acetic acid concentration.

	pH values in cyanobacterial suspensions in experiment 1			
Chitosan (mg l^{-1})	*M. aeruginosa* MIRF-01	*Aph. flos-aquae* CCAP 1446/1C	*Aph. flos-aquae* SAG 31.87	*C. raciborskii* PMC 115.02
0	7.69 (0.02)	7.63 (0.03)	7.72 (0.03)	7.84 (0.03)
0.5	7.70 (0.02)	7.63 (0.03)	7.70 (0.01)	7.68 (0.02)
1	7.69 (0.04)	7.61 (0.06)	7.68 (0.01)	7.59 (0.02)
2	7.61 (0.02)	7.63 (0.03)	7.65 (0.01)	7.55 (0.01)
4	7.57 (0.01)	7.49 (0.08)	7.58 (0.02)	7.45 (0.02)
8	7.44 (0.02)	7.21 (0.06)	7.45 (0.06)	7.34 (0.03)
	pH values in cyanobacterial suspensions in experiment 2			
Chitosan (mg l^{-1})	*P. agardhii* NIVA-CYA 116	*P. agardhii* NIVA-CYA 126	*D. cylindrica* PCC 7122	*D. cylindrica* CCAP 1403/2A
0	8.17 (0.04)	7.74 (0.02)	7.89 (0.04)	7.88 (0.04)
0.5	7.77 (0.03)	7.78 (0.03)	7.82 (0.02)	7.91 (0.04)
1	7.63 (0.04)	7.76 (0.01)	7.86 (0.02)	7.88 (0.06)
2	7.49 (0.02)	7.72 (0.01)	7.81 (0.01)	7.83 (0.03)
4	7.44 (0.00)	7.65 (0.01)	7.69 (0.01)	7.74 (0.05)
8	7.34 (0.01)	7.43 (0.01)	7.38 (0.06)	7.41 (0.01)

	pH values in acetic acid controls						
Acetic acid as in chitosan (µl l^{-1})	MIRF-01	CCAP 1446/1C	PMC 115.02	NIVA-CYA 116	NIVA-CYA 126	PCC 7122	CCAP 1403/2A
0	7.87	7.81	7.94	8.12	7.80	8.04	7.96
0.5	7.7	7.81	7.92	8.83	7.78	7.90	8.06
1	7.4	7.77	7.87	8.12	7.76	8.09	8.00
2	7.78	7.72	7.8	8.21	7.78	8.00	7.83
4	7.66	7.61	7.69	8.07	7.71	7.76	7.79
8	7.48	7.4	7.44	7.93	7.57	7.63	7.69

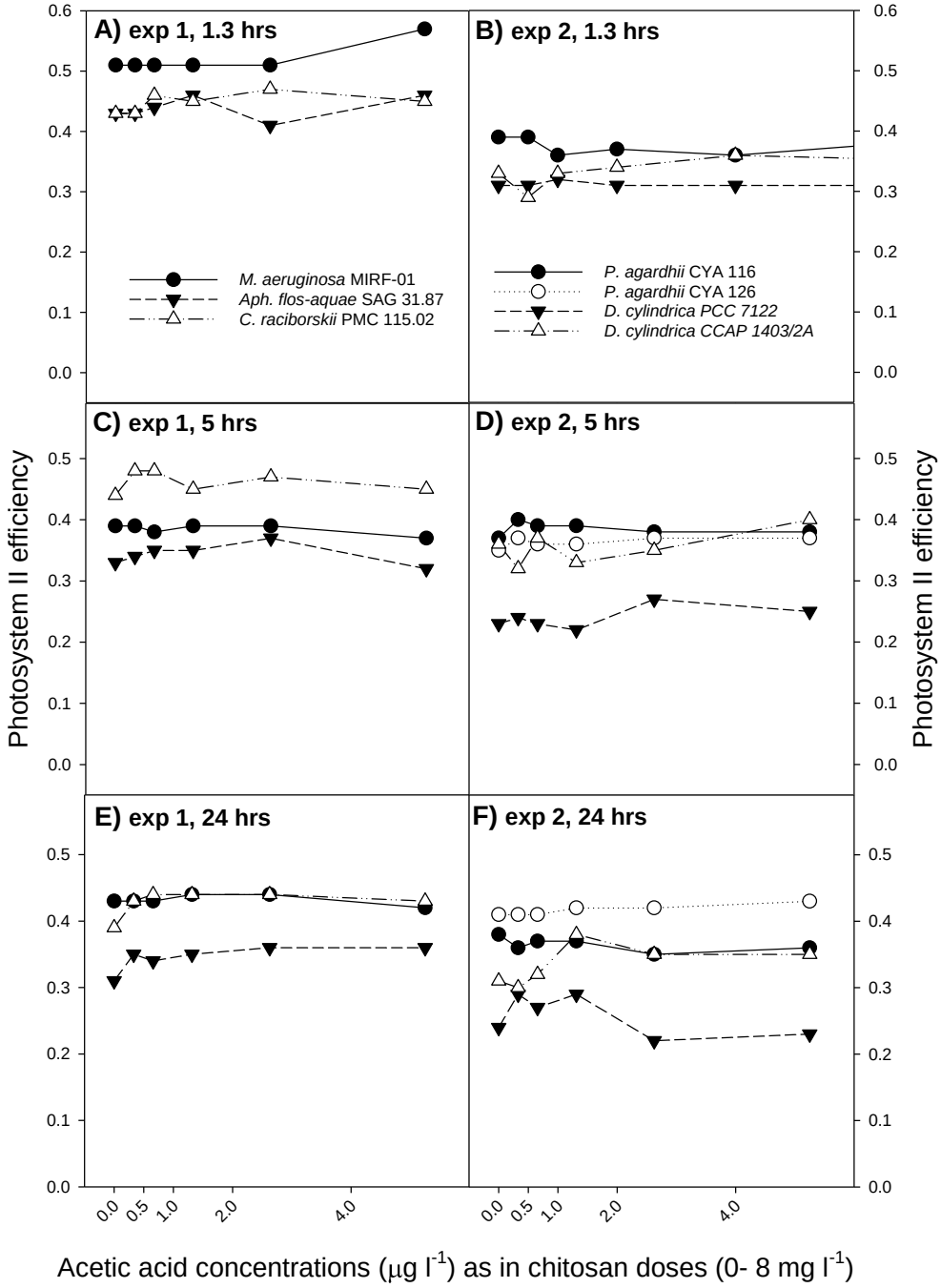

Figure 4.S1: Effect of acetic acid (mg l^{-1}), used as solvent to dissolve chitosan and applied in similar doses as in chitosan treatments, on Photosystem-II efficiency of different cyanobacterial strains exposed for 1.3, 5 and 24 hours.

5

Chitosan used to remove cyanobacteria can cause toxin release

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Submitted to *Water Research*

Abstract

Chitosan has recently been tested as a coagulant to remove cyanobacteria nuisance. While its coagulation efficiency is well studied, little is known about its effect on the viability of the cyanobacterial cells. The aim of this study was to test eight strains of the most frequent bloom forming cyanobacterium, *Microcystis aeruginosa,* exposed to a realistic concentration range of chitosan used in lake restoration management (0 to 8 mg chitosan l^{-1}). The effect of chitosan on photosystem II efficiency, chlorophyll-a release, cell membrane integrity and extracellular microcystin concentration was evaluated. We found that after 1 hour of contact with chitosan, in seven of the eight strains tested photosystem II efficiency was decreased, and after 24 hours all the strains tested were affected. EC_{50} values varied from 0.47 to > 8 mg chitosan l^{-1} between the strains, which was not related to the ability of producing microcystins, but might be related to the amount of extracellular polymeric substances. The acid nucleic staining (Sytox Green®) illustrated the loss of membrane integrity in all the strains tested, and subsequently leakage of pigments was observed as well as release of intracellular microcystin. Consequently, chitosan applications in drinking water reservoirs should be considered carefully. Our results indicate that among strain variability hampers generalization about species response to chitosan exposure. Hence, when used as a coagulant to manage cyanobacterial nuisance, chitosan should be first tested on the natural site specific biota and not only on cyanobacteria removal efficiency, but also on cell integrity aspects.

Introduction

Cyanobacteria were key photosynthetic organisms that gradually increased the oxygen concentration in the Earth's atmosphere, changing it from a reducing to an oxidizing one, therewith allowing the development of other life forms (Schopf, 2012). Also nowadays, cyanobacteria play an important role in the oxygen production, being responsible for half of the primary production in the oceans (Gadd and Raven, 2010). However, some cyanobacterial species are able to form intense blooms under certain conditions, which have severe impacts on water bodies, such as increased water turbidity, nocturnal depletion of oxygen, fish kills and malodour (Paerl and Huisman, 2009; Smith and Schindler, 2009). Furthermore, some cyanobacteria are able to produce toxins which can be harmful to aquatic and terrestrial organisms including humans and dogs (Azevedo et al., 2002; Carmichael et al., 2001; Lürling and Faassen, 2013). These blooms therefore impede the use of water bodies for recreational use, drinking water production, fishing and agricultural use and consequently cause severe economic losses (Dodds et al., 2009; Hamilton et al., 2013).

The main cause behind cyanobacterial blooms is the excess of nutrient supply to waterbodies - eutrophication (Smith et al., 1999). Eutrophication is a major water quality issue worldwide and phosphorus (P) enrichment of lakes is one of the most important keys to trigger and maintain cyanobacteria blooms (O'Neil et al., 2012; Rangel et al., 2012; Smith and Schindler, 2009). Thus, to manage the problem, nutrients must be limited. The classical and most straight forward approach is to reduce the external nutrient input (Cooke, 2005; Hilt et al., 2006), however, adequate catchment control is not always feasible due to economic reasons (Huser et al., 2016b). In addition, in cases where the internal loading is the main nutrient source due to long term diffuse load (e.g., Lürling and Van Oosterhout, 2013; Waajen et al., 2016a), reduction of external nutrient sources will be inefficient, virtually impossible or will take decades to centuries to have an effect (Carpenter, 2005; Fastner et al., 2016). Hence, to speed-up the system recovery and minimize the nuisance, in-lake measures have been recognized as a feasible solution (Huser et al., 2016b). Mainly in cases where the single source of drinking water is suffering from cyanobacterial blooms, a fast and safe solution can be most suitable, even if its effect is not long lasting. A proper system analysis (SA) consisting of a nutrient balance evaluation, biological make-up, and cost-benefit analysis will guide to the most promising set of measures to mitigate blooms (Lürling et al., 2016).

In this context, geo-engineering materials, for example the use of low doses of flocculants (e.g., polyaluminium chloride – PAC or iron chloride) followed by the addition of natural soils or modified clays (Noyma et al., 2016; 2017), have gained attention as useful tools to mitigate the effects of eutrophication for a shorter time-frame. This 'Flock & Sink' technique is able to remove cyanobacteria from the water column whilst blocking P efflux from the sediment. This approach has been implemented effectively a few times using PAC or iron chloride as coagulant (Lürling and Van Oosterhout, 2013; Waajen et al., 2016a). Recently, an organic coagulant, chitosan, has gained attention as a possible alternative for inorganic metal based coagulants (Li and Pan, 2013).

Chitosan is an organic polymer synthesized by alkaline deacetylation of chitin, which is a biopolymer extracted from shellfish and crustaceans sources (Yang et al., 2016). Chitosan acts as a cationic polyelectrolyte when protonated in acidic medium, thus its free amino groups interact with the negative charged cyanobacterial cell wall (Chen et al., 2014; Renault et al., 2009; Yang et al., 2016). In addition, due its long polymer chain, chitosan can also attach to the cells, thereby forming bridges that entrap the cells (Yang et al., 2016). Chitosan is frequently viewed as an eco-friendly and non-toxic coagulant (Li and Pan, 2013; Pan et al., 2011a, 2006b; Renault et al., 2009), however beside the coagulation property, chitosan is also known for its antimicrobial activities (Allan and Hadwigei, 1979; Kendra et al., 1984; Kong et al., 2010; No et al., 2002; Sudarshan et al., 1992) and it has been even used to preserve food, such as fish (Jeon et al., 2002), oyster (Cao et al., 2009) and strawberries (Campaniello et al., 2008).

Several studies have used chitosan to remove cyanobacteria or dinoflagellates from the water column, some using chitosan-modified local soils/sand (MLS, e.g., Li and Pan, 2015, 2013, Pan et al., 2012, 2011a, 2006ab; Wang et al., 2016; Zou et al., 2006) and others adding first only chitosan, followed by soils/clays (e.g., de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2017, 2016). However, in only few of these studies the possible chitosan effects on the viability of the algal cells was investigated. From these studies, some did not find any negative effect on the cyanobacterium *Microcystis aeruginosa* (e.g., de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2017, 2016), whereas others showed a detrimental effect on the cyanobacterium *Cylindrospermopsis raciborskii* and growth inhibition in the dinoflagellate *Amphidinium carterae* (Miranda et al., 2017; Pan et al., 2011a). A more recent study from our group indicated rapid cell lysis of some cyanobacterial species when incubated with chitosan, but a less severe impact on *M. aeruginosa*

(Mucci et al., 2017), but in this study, cyanotoxin release was not analysed. In fact, only few studies so far have addressed cyanotoxin release caused by chitosan, in some of these studies toxins were released and others they were not (Li and Pan, 2015; Miranda et al., 2017; Pan et al., 2011b; Pei et al., 2014; Wang et al., 2015).

Our present study aims to extend the knowledge on possible side effects caused by chitosan. Possible materials to manage blooms must be efficient, easy to apply, cheap and safe (Lürling et al., 2016). Therefore, an environmentally safe management strategy should be selected and methods that cause cell damage and toxin release must be applied carefully or avoided (Merel et al., 2013).

We tested the response of the most frequently encountered bloom forming cyanobacterium, *Microcystis aeruginosa* (Harke et al., 2016; O'Neil et al., 2012; Srivastava et al., 2013) to a realistic concentration range of chitosan as used in lake restoration management (Pan et al., 2011b). Since intraspecific variation was observed in other species (Mucci et al., 2017), we tested eight different strains of *M. aeruginosa*. The effect of chitosan was evaluated by analysing the photosystem II efficiency and filterable chlorophyll-a concentration. In addition, we analysed cell membrane integrity and the extracellular microcystin concentration. We hypothesized (1) that chitosan would negatively affect all the *M. aeruginosa* strains tested only at high dose, (2) that sensibility to chitosan will not differ between strains, and (3) that cell lysis followed by toxin release will be observed only at high chitosan dose.

Methods

M. aeruginosa cultures

The eight different strains used in the experiments were obtained from different culture collections (Table 5.1) and were cultivated on modified WC medium (Lurling and Beekman, 2006) under controlled conditions at 22°C with 16:8 h light-dark cycle and 45 µmol quanta m^{-2} s^{-1} light intensity. Prior to the experiment the cultures were refreshed twice (around two weeks interval), always in the exponential phase.

Chitosan

Chitosan was obtained from Polymar Ciência e Nutrição S/A (Ceará, Brazil) and the deacetylation degree was 86.3% (Batch-010913). The chitosan (made of

shrimp shells) was acidified with 96% acetic acid solution (Merck, analytical grade) yielding a final concentration of 0.1% acetic acid.

Table 5.1: *Microcystis aeruginosa* strains used in the experiments.

Strain ID	Acquired from	Toxins produced
MIRF-01	Laboratory of Ecophysiology and Toxicology of Cyanobacteria (Brazil)	dm-MC-LR, MC-LR, MC-LY, MC-LW, MC-LF (Marinho et al., 2013)
PCC7806 ΔmcyB	Pasteur Culture Collection (France)	Non-toxic (Dittmann et al., 1997; this study)
PCC 7806	Pasteur Culture Collection (France)	dm-MC-LR, MC-LR (Ger et al., 2016)
PCC 7005	Pasteur Culture Collection (France)	Non-toxic (this study)
PCC 7820	Pasteur Culture Collection (France)	dm-MC-LR, MC-LR, MC-LY, MC-LW, MC-LF (Lürling et al., 2014a)
SAG 14.85	Sammlung von Algenkulturen der Universität Göttingen (Germany)	dm-MC-LR, MC-LR (unpublished data)
SAG 17.85	Sammlung von Algenkulturen der Universität Göttingen (Germany)	dm-MC-LR, MC-LR, MC-YR (Ger et al., 2016)
CYA 140	Norwegian Institute for Water Research (Norway)	dm-MC-LR, MC-LR (Ger et al., 2016)

Experimental design

Aliquots of *M. aeruginosa* were transferred to 100 ml Erlenmeyer containing 50 ml of modified WC medium yielding a final concentration of 100 µg chlorophyll-a l^{-1}. Six concentrations of Chitosan were used (0, 0.5, 1, 2, 4 and 8 mg l^{-1}) based on the frequently used concentrations to flocculate cyanobacteria in lake restoration (de Magalhães et al., 2016; Li and Pan, 2015; Noyma et al., 2016; Pan et al., 2006ab; Zou et al., 2006). The experiment was done in triplicate. To check if the acetic acid in which chitosan was dissolved had any influence on *M. aeruginosa* cells, an extra control was added in which only acetic acid was added in the same dose as in the chitosan treatment. After the addition of chitosan or acid acetic, the flasks were mixed and placed in the laboratory at 22°C in 16:8 h light-dark cycle at 45 µmol quanta m^{-2} s^{-1}. After 1, 4 and 24 hours subsamples were taken to measure the total chlorophyll-a

concentration and Photosystem efficiency II (PSII) through PHYTOPAM phytoplankton analyser (Heinz WalzGmbH, Effeltrich, Germany). Additionally, at the end of the experiment 3 ml samples from each flask were filtered through a filter unit (Aqua 30/0.45CA, Whatman®, Germany) and measured again in the PHYTOPAM to quantify chlorophyll-a released from the cells. After 24 hours, pH was measured in each flask and 8 ml samples were filtered through glass fibre filters (GF/C, Whatman®, Germany) and placed in glass tubes for dissolved microcystin (MC) analysis. The samples were dried in a Speedvac concentrator (Savant™ SPD121P, Thermo Fisher Scientific) and were reconstituted in 900 µl methanol solution (J.T. Baker®, 97%). After that, the reconstituted samples were transferred to a 1.5 ml tube with a cellulose-acetate filter and centrifuged for 5 min at 16.000 x *g*. The filtrates were transferred to amber glass vials and analysed for eight MC variants (MC - dmRR, RR, YR, dmLR, LR, LY, LW and LF) using LC-MS/MS according to Lürling and Faassen, 2013. The MC analysis was done for the strains MIRF-01, PCC 7806, PCC 7820 and CYA 140 (table 5.1).

Cell membrane permeability

To evaluate the effect of chitosan on membrane integrity, immediately after 24 hours of exposure, an aliquot from each replica was taken, joined and centrifuged at 5000 x g for 10 min. The pellet was stained with Sytox® Green (Thermo Fisher Scientific) at a final concentration of 1 nM for 30 min in the dark. The samples were observed under a fluorescence microscope (ZEISS, Axioimager D2) using the filter long pass for Fluorescein (450–490 for excitation and 515 nm for emission). Sytox® Green binds to nucleic acid, but it cannot penetrate the cell membrane. However, a damaged membrane allows the stain to infiltrate resulting in a green fluorescence colour when analysed in a fluorescence microscope.

Matrix effect on MC analysis

The possible effect of chitosan on the toxins analysis was evaluated by incubating pure microcystin mix standards (all 8 variants) for 24 hours in a solution with 8 mg Chitosan l^{-1} dissolved in WC medium. The control series contained only WC medium and pure microcystin mix standards. The test was performed using three replicas and MC analysis was executed as mentioned before.

Data analysis

The PSII for each strain at each time point was compared between different chitosan concentrations using one-way ANOVA or *Kruskal-Wallis* One Way Analysis of

Variance on Ranks when normality test (*Shapiro-Wilk*) or Equal Variance test (*Brown-Forsythe*) failed.

For each strain, the chitosan concentrations that caused a 50% reduction in their PSII efficiency compared to the control (EC_{50}) were determined by non-linear regression using four parameter logistic curve in the software Sigma Plot 13.0. EC_{50} values were statistically compared between strains using one-way ANOVA. Extracellular MC and filterable chlorophyll-a concentration were compared between different chitosan concentrations using one-way ANOVA or *Kruskal-Wallis* One Way Analysis of Variance on Ranks when normality test (*Shapiro-Wilk*) failed. Chitosan effect on MC standards was tested through Student's T-test between the treatment with and without chitosan.

Results

The eight *M. aeruginosa* strains tested were affected differently by chitosan. Considering the effect of chitosan on the PSII efficiency, we could divide the *M. aeruginosa* strains based on their response in two groups: (1) the strains that have a delayed response to chitosan (MIRF-1, PCC 7806 ΔmcyB, PCC 7806 and PCC 7820) and (2) the more sensitive strains with earlier response (SAG 14.85, CYA 140, PCC 7005 and SAG 17.85) (Fig. 5.1). After 1 hour of contact with chitosan in the first group hardly an effect of chitosan on PSII efficiency could be observed (Fig. 5.1A). Nonetheless, ANOVAs indicated significant differences between the chitosan concentrations in the strains PCC 7806 ΔmcyB, PCC 7806 and PCC 7820, but not for MIRF-01 (Table 5.2). After 4 hours, effects on PSII efficiency became visible at the highest concentration in PCC 7806 ΔmcyB and from 1 mg l^{-1} in PCC 7820 (Fig. 5.1B), while after 24 hours these effects became more pronounced (Fig. 5.1C). Now also the PSII efficiencies in strains PCC 7806 and MIRF-1 were reduced at 8 mg chitosan l^{-1} (Fig. 5.1C). ANOVAs revealed that in each strain PSII efficiencies between chitosan concentrations were statistically different (Table 5.2).

The response of strains from the second group was clearly different, where SAG 14.85, CYA 140, PCC 7005 and SAG 17.85 all showed already after 1 hour a clear typical sigmoidal decrease in PSII efficiency with higher chitosan concentrations (Fig. 5.1D). This pattern persisted after 4 and 24 hours of chitosan incubation (Fig. 5.1E and 5.1F). The ANOVA output showed, after 1, 4 and 24 hour of incubation, a significant difference in PSII efficiency between the chitosan concentrations in all four strains tested (Table 5.2).

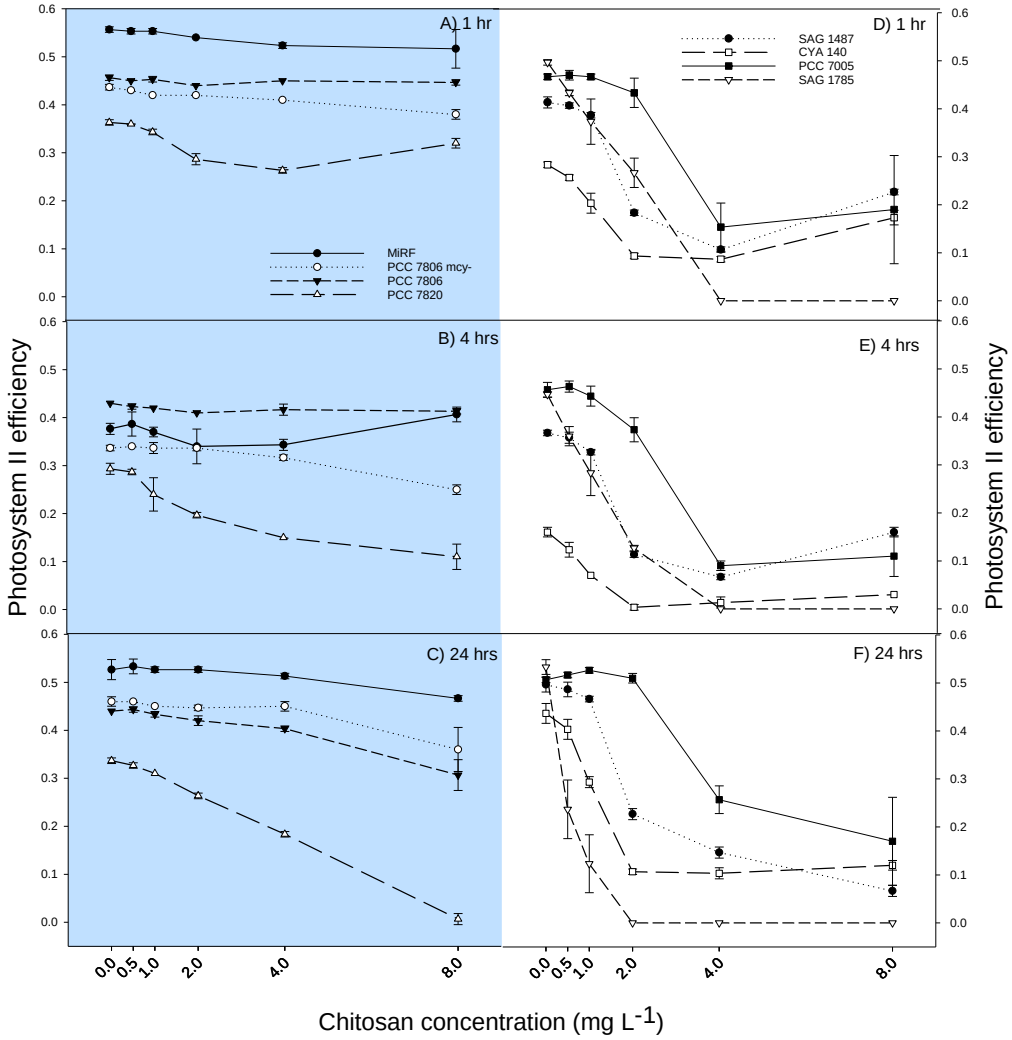

Figure 5.1: Photosystem II efficiency (PSII) for all 8 strains tested after 1 hour (A,D), 4 hours (B,E) and 24 hours (C,F) exposure to different concentrations of chitosan (0 to 8 mg l^{-1}). Error bars indicate standard deviation (*n*=3). Blue graphs on the left show strains with late response (MIRF, PCC 7806 ΔmcyB-, PCC7806 and PCC 7820) and graphs on the right show more sensitive strains with early response (SAG 1487, CYA 140, PCC 7005 and SAG 1785).

The strains PCC 7820 and SAG 17.85 showed a clear increase in chlorophyll-a concentrations as a function of the chitosan concentration used (Fig. 5.S1 Supplementary information). For both strains, at the end of the experiment when exposed to 8 mg chitosan l^{-1}, the chlorophyll-a concentration was around 3 times higher than in the control. The others six strains used (MIRF-1, PCC 7806 ΔmcyB, PCC

7806, SAG 14.85, PCC 7005 and CYA 140) only showed an increase in chlorophyll-a compared to control after 24 hours and at the highest chitosan concentration used. In all strains, variation in pH between treatments remained below 0.5 units (Fig. 5.S2). Addition of acetic acid had no effect on PS II efficiency (Fig. 5.S3).

Table 5.2: *F*- and *p*-values of one-way ANOVAs and *H*- and *p*-values of *Kruskal-Wallis* One Way Analysis of Variance on Ranks when normality tests failed (*Shapiro-Wilk*) for Photosystem II efficiencies in eight different *M. aeruginosa* strain exposed for 1, 4 and 24 hours to six different concentrations chitosan (0 to 8 mg l^{-1}). Statistical significant differences are in bold.

M. aeruginosa	Exposure duration		
Strain	1 hour	4 hours	24 hours
MIRF-01	H_5= 9.93; p=0.077	$F_{5,12}$ = 4.65; **p=0.014**	$F_{5,12}$ = 13.87; **p<0.001**
PCC 7806 ΔmcyB	H_5= 16.70; **p=0.005**	$F_{5,12}$ = 66.34; **p<0.001**	H_5= 12.70; **p=0.026**
PCC 7806	H_5=12.24; **p=0.032**	H_5= 11.28; **p=0.046**	H_5= 15.81; **p=0.007**
PCC 7820	$F_{5,12}$ = 89.62; **p<0.001**	$F_{5,12}$ = 47.06; **p<0.001**	$F_{5,12}$= 1079.0 **p<0.001**
SAG 14.87	H_5= 16.317; **p=0.006**	$F_{5,12}$ = 876.16; **p<0.001**	H_5= 16.1; **p=0.007**
CYA 140	$F_{5,12}$ = 149.94; **p<0.001**	$F_{5,12}$ = 144.96; **p<0.001**	$F_{5,12}$ = 338.1; **p<0.001**
PCC 7005	H_5= 14.13; **p=0.015**	$F_{5,12}$ = 187.49; **p<0.001**	H_5= 15.16; **p=0.01**
SAG 17.85	H_5=16.74; **p=0.005**	H_5= 16.74; **p=0.005**	H_5= 16.56; **p=0.005**

In the first group of strains (MIRF-1, PCC 7806 ΔmcyB, PCC 7806 and PCC 7820), extracellular chlorophyll-a concentrations were clearly elevated at the highest chitosan dose (Fig. 5.2A). However, in the second group also at much lower chitosan dose elevated extracellular chlorophyll-a concentrations were observed (Fig. 5.2B). Extracellular chlorophyll-a concentrations differed considerably among strains with the highest concentration found in strain PCC 7820 (257 µg extracellular chlorophyll-a l^{-1}) and the lowest in strain CYA 140 (16 µg extracellular chlorophyll-a l^{-1}), both at the 8 mg chitosan l^{-1} treatment (Fig. 5.2).

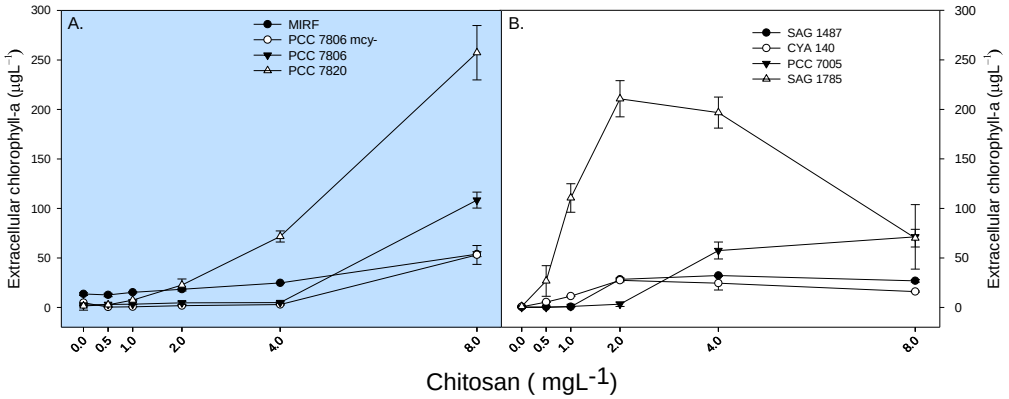

Figure 5.2: Extracellular chlorophyll-a concentration for MIRF, PCC 7806 ΔmcyB , PCC 7806 and PCC 7820 (A) and for SAG 1487, CYA 140, PCC 7005 and SAG 1785 (B) after 24 hours exposure to different concentrations of chitosan (0 to 8 mg l^{-1}). Error bars indicate standard deviation (*n*=3).

EC_{50} values for MIRF-1, PCC 7806 ΔmcyB and PCC 7806 could not be calculated, because the values were exceeding the highest dose used (8 mg l^{-1}) (Table 5.3). SAG 17.85 was the most sensitive strain with the lowest EC_{50} value (0.47 mg chitosan l^{-1}) followed by CYA 140, SAG 14.85, PCC 7005 and PCC 7820 with EC_{50}'s of 1.06, 1.71, 3.44 and 4.51 mg chitosan l^{-1}, respectively (Table 5.3). One-way ANOVA showed difference between the strains ($F_{4,10=}$ 47.74; p<0.001) and the Tukey post-hoc test divided the strains in three different groups: 1) SAG 17.85, CYA 140 and SAG 14.85 were the most sensitive, 2) followed by PCC 7005 and 3) PCC 7820 (table 3). MIRF-1, PCC 7806 ΔmcyB and PCC 7806 were least sensitive.

Extracellular MC concentrations were below the level of detection in filtrates from incubations of MIRF-1 exposed to 0 to 4 mg chitosan L^{-1}, while the variant MC-LR was detected at 8 mg chitosan l^{-1}, but below the level of quantification. Likewise, in strain CYA 140 no extracellular MCs were detected in incubations exposed to 0 to 1 mg chitosan l^{-1}, whereas MC-LR was detected, yet not quantifiable, at 2, 4 and 8 mg chitosan l^{-1}. On the other hand, in incubations with PCC 7820 and PCC 7806 extracellular MCs were quantified. Filtrates from PCC 7806 showed a significant increase at the highest chitosan concentration used (*p*=0.006*; H_5*=16.251) (Fig. 5.3). In strain PCC 7820 extracellular MCs clearly increased with chitosan concentration *(p*<0.001*; $F_{5,11}$*=4516.5*)* and already at 1 mg chitosan l^{-1} the extracellular MC concentration was significantly higher than in the control (Fig. 5.3). Tukey's test disclosed five significantly different groups in strain PCC 7820 from lower to higher chitosan concentrations: 1) 0 and 0.5 mg l^{-1}, 2) 0.5 mg l^{-1} and 1 mg l^{-1}, 3) 2 mg l^{-1} 4) 4

mg l^{-1} and 5) 8 mg l^{-1}. A significant positive linear relation between MC concentration and filterable chlorophyll-a was observed (r^2= 0.98 p<0.0001 for PCC7820 and r^2= 0.99 p<0.0001 for PCC 7806) (Fig. 5.3). Student`s T-test showed no difference in treatments with MC standards incubated with 8 mg of chitosan l^{-1} or without chitosan (p=0.552) (Fig. 5.S4).

Table 5.3: Mean EC_{50} values (mg l^{-1}; values inside brackets represent SD , *n* = 3) of chitosan for the Photosystem II efficiency in different *M. aeruginosa* strains. Letters (A, B and C) represent homogenous group (Tukey pairwise comparisons).

M. aeruginosa Strain	EC_{50} - 24 hours (mg L^{-1})
MIRF-01	>8
PCC 7806 ΔmcyB	>8
PCC 7806	>8
PCC 7820	4.51 (0.37) r^2=0.98; **p<0.0001^A**
PCC 7005	3.44 (0.42) r^2=0.94; **p<0.0001^B**
SAG 14.85	1.71 (0.08) r^2=0.98; **p<0.0001^C**
CYA 140	1.06 (0.04) r^2=0.98; **p<0.0001^C**
SAG 17.85	0.47 (0.05) r^2=0.96; **p<0.0001^C**

For the five most sensitive strains (SAG 14.85, CYA 140, PCC 7005, PCC 7820, SAG 17.85), the cell membrane permeability test showed clear differences between cells exposed to chitosan at each of the concentrations used and the non-exposed (control) cells. For instance, in strain PCC 7820 the non-exposed cells only showed the natural red fluorescence (Fig. 5.4; panel control B) and no intracellular accumulation of Sytox green (Fig. 5.4; panel control C), however, at 2 mg chitosan L^{-1} intracellular accumulation of Sytox was observed (Fig. 5.4; panel 2 mg l^{-1} C), while the accumulation was even stronger at the highest chitosan dose (8 mg l^{-1}) indicating membrane damage (Fig. 5.4; panel 8 mg l^{-1} C). In the less sensitive strains MIRF-1, PCC 7806 ΔmcyB and PCC7806 only at the highest concentration such intracellular accumulation of Sytox was observed.

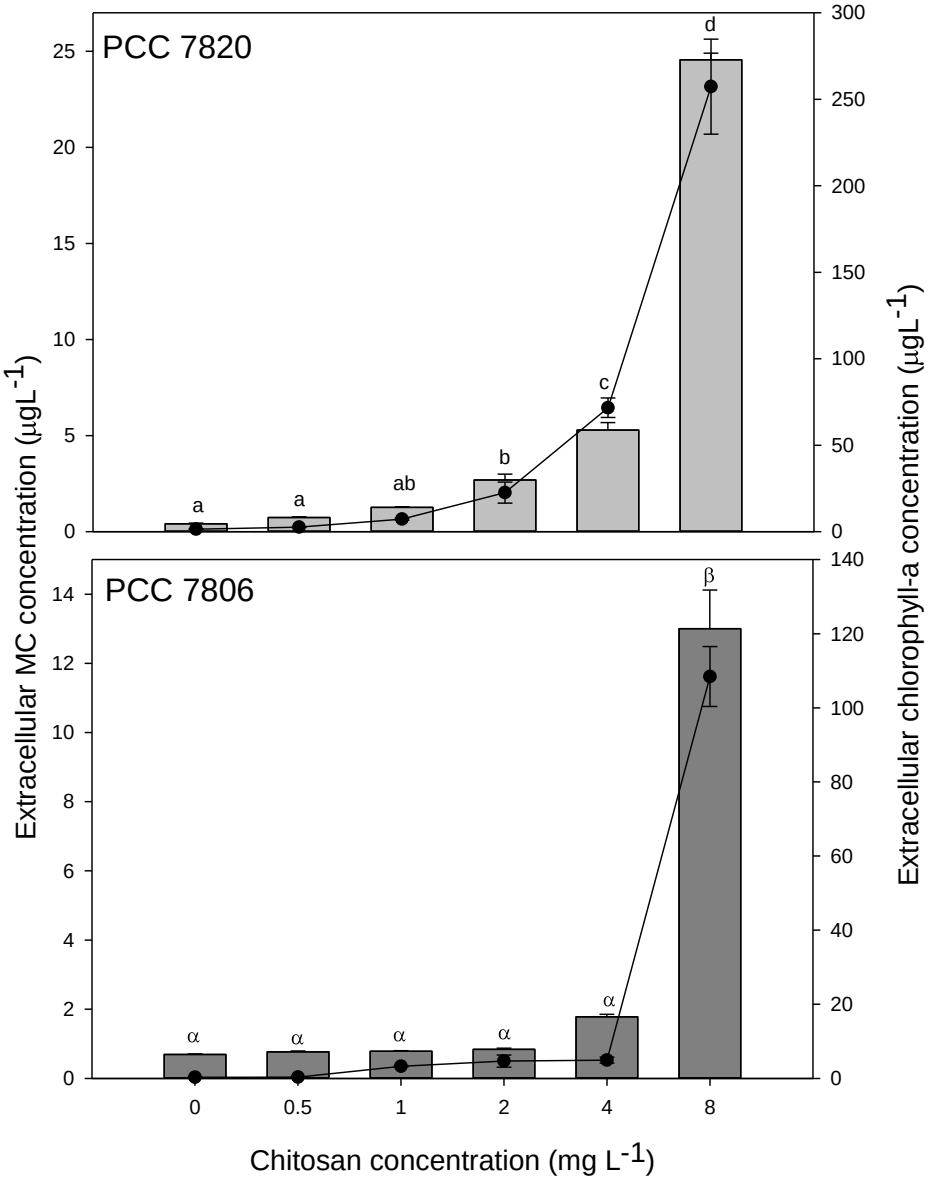

Figure 5.3: Extracellular MC (bars) and extracellular chlorophyll-a (line) in all different chitosan concentration after 24 hours for PCC 7820 (upper graph) and PCC 7806 (graph below). Errors bars indicate standard deviation (*n*=3). Letters and asterisk represent statistical difference.

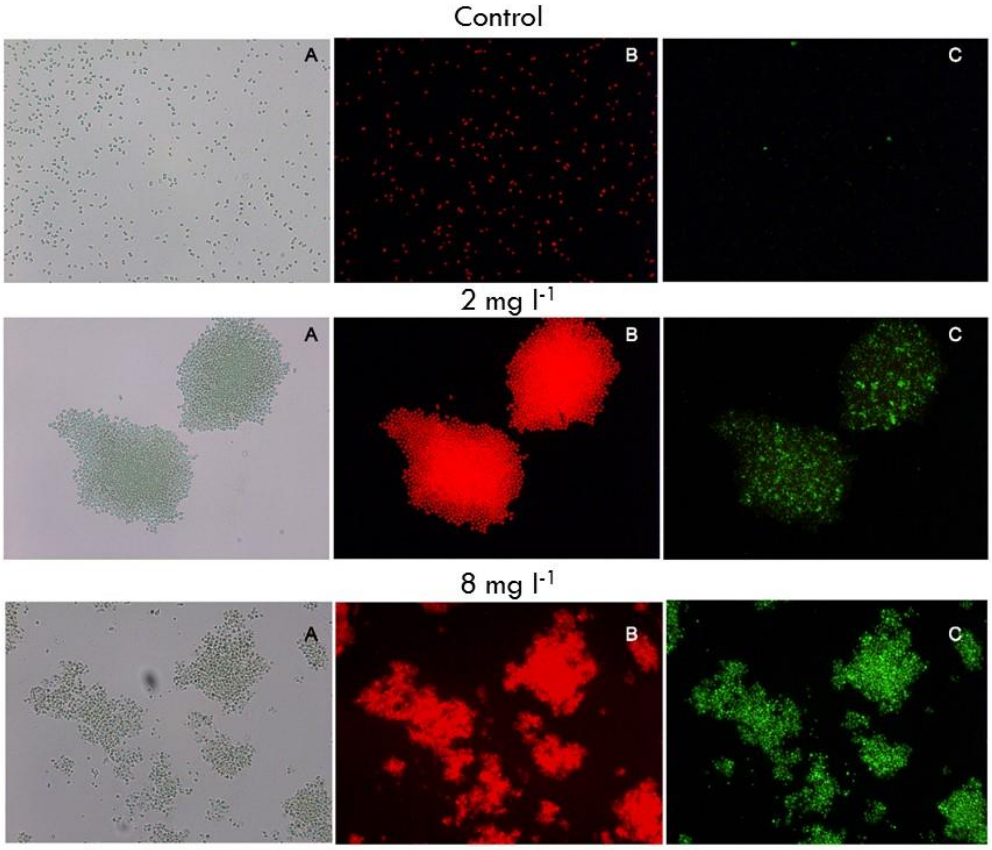

Figure 5.4: Fluorescence images of PCC 7820 cells in the control (upper pictures), 2 mg (middle pictures) and 8 mg chitosan L^{-1} (lower pictures). The panels A show bright field images, B the cyanobacteria auto fluorescence (red) and C the intracellular accumulation (or not) of Sytox® Green (green).

Discussion

Chitosan has recently received increased attention as a coagulant to remove cyanobacteria from aquatic bodies. It has good flocking properties depending on water chemistry (Lürling et al., 2017) and combined with ballast in freshwaters, cyanobacteria may be removed effectively from the water column (Pan et al., 2011b; Li and Pan, 2013; Noyma et al., 2016). Effects on the viability of flocked cyanobacteria has received less attention, but is of great importance because chitosan may cause cell membrane damage in bacteria (Liu et al., 2004) and therefore may cause toxin release (Miranda et al., 2017). Only very few studies included cyanotoxin analysis in

trials with only chitosan addition (Pei et al., 2014; Miranda et al., 2017), few others tested it for chitosan combined with a ballast (Li and Pan, 2015; Miranda et al., 2017; Pan et al., 2011b; Wang et al., 2015). In the latter studies, Miranda et al. (2017) tested the response of two distinct cyanobacterial assemblages to chitosan. Only one other study explored intraspecific variability between two strains of few cyanobacteria species (Mucci et al., 2017), but not among *Microcystis* strains. Our study filled in this research gap by testing the response of eight different *Microcystis* strains to chitosan, whilst also measuring extracellular MCs.

The results of our study are not in agreement with the first hypothesis that chitosan would affect all the *M. aeruginosa* strains tested only at a high dose. In five strains a rapid negative impact could be detected yielding an EC_{50} between 0.47 and 4.51 mg L^{-1}, while in three less sensitive strains (MIRF-1, PCC 7806 and PCC 7806 ΔmcyB) after 24 hours a significant reduction in PSII efficiency was observed at the highest dose tested of 8 mg chitosan l^{-1}. PSII is one of the reaction centres responsible for the transport of energized electrons to accomplish photosynthesis (Witt, 1996); thus, a decrease in PSII reflects damage to the thylakoid membrane and gives insight in the physiological status of the cells. A significant reduction in the PSII efficiency of MIRF-1 cells was also observed in our previous work (Mucci et al., 2017). In contrast, some studies found no decrease in PSII efficiency at similar chitosan concentrations (de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2017, 2016). These studies had incubated lake water infested with cyanobacteria only for one hour, which might be too short to evoke a measurable effect. The strains SAG 14.85, CYA 140, PCC 7005 and SAG 17.85 were strongly affected after 1 hour of incubation, but in MIRF-1, PCC 7806, PCC 7820 and PCC 7806 ΔmcyB negative effects became apparent after 24 hours incubation. Hence, exposure time might be an important factor. All our strains were uni-and bicellular which implies that the among strain variability seems to be caused by strain specific characteristics rather than a colonial or unicellular appearance The reduction in PSII efficiency most probably reflects increased membrane permeability and cell lysis that is a results of cationic NH_3^+ groups of chitosan interacting with negatively charged cyanobacterial cell membranes (Liu et al., 2004; Li et al., 2015). The among strain variability might then be caused by differences in the composition of the outer layer and in the composition and amount of extracellular polysaccharides (EPS) (Forni et al., 1997). EPS are mainly composed of polysaccharides and proteins (Xu et al., 2013). Due to the large number of negatively charged functional groups, their efficiency in removing heavy metals and organic contaminants provides protection to the cells (Bai et al., 2016; De Philippis et al., 2011 and references therein; Gao et al., 2015; Ozturk and Aslim, 2008; Zhang et al., 2006).

EPS also protects *M. aeruginosa* against strong oxidizers like hydrogen peroxide (Gao et al., 2015) and consequently follow ups could explore the role of EPS in among strain variability and among species variability in sensitivity to chitosan. Clearly, presence or absence of MCs is not related to chitosan sensitivity, as both the MC producing wild-type PCC 7806 and its MC lacking mutant PCC 7806 ΔmcyB were equally sensitive.

Another factor that might play a role in the sensibility to chitosan is charge density of the membrane in each species/strain. Positively charged chitosan will have electrostatic interactions with the negatively charged cell wall of the cyanobacteria, where a higher negative charge density will lead to a stronger interaction with chitosan (Chung et al., 2004). Stronger interactions can cause membrane destabilization and disruption of the membrane leading to leakage of intracellular substances (Kong et al., 2010), as observed in our study.

Besides PSII efficiency, there are other parameters that can reflect the physiological status of the cell and also indicate cell lysis. For instance some studies measured the release of intracellular substances (e.g., K^+, M^{2+}, pigments and toxins), the membrane integrity through acid nucleic staining, as done here, or growth inhibition (table 5.S1). About half of the works found in the literature did not analyse any cell health aspects (table 5.S1). From the works that included a cell viability indicator after contact with chitosan, one third revealed a negative effect of chitosan on the cells. Considering chitosan as a tool to be applied in water bodies to remove cyanobacteria, it is important not only to look at removal/coagulation efficiency, but also on possible side effects on cyanobacterial cells.

The results of our study refute the second hypothesis that the sensibility to chitosan would be similar in all strains; the EC_{50} varied from 0.47 to >8 mg chitosan l^{-1}. Based on the absence of an effect on *M. aeruginosa* in short term (1 hour) exposures (de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2017, 2016) and a weak effect of about 45% reduction in PSII-efficiency after 24 hours in one *M.* aeruginosa strain (Mucci et al., 2017), we hypothesized that all *M. aeruginosa* would be more or less equally resistant. However, our results evidently demonstrated that among strain variability in senstitivity to chitosan is as large as has been found for other cyanobacteria. For instance, Mucci et al. (2017) found an intraspecific variability (EC_{50} of 0.41 and >8 mg chitosan l^{-1}) between two strains of *Planktothrix agardhii* equal to what we observed here for *M. aeruginosa*.

In analogy with our expectation that *M. aeruginosa* would only be affected at the highest dose, we hypothesized that only in these exposures microcystins would be released from cells. Our results, however, are not in line with this third hypothesis. Extracellular microcystins (MCs) could be detected in filtrates from all the strains

tested, albeit not always at levels allowing quantification. Nonetheless, in strain PCC 7820 already at 2 mg chitosan l^{-1} MC release was significantly higher than in the controls. In strain PCC 7806 extracellular MC concentration was elevated at 4 mg l^{-1} and increased at 8 mg chitosan l^{-1}. Since the MC concentrations in these treatments were high, such chitosan doses should be used with care when used to treat toxic blooms in drinking water supplies. The capacity of chitosan to remove extracellular MCs has been reported and can be substantial (e.g., Pei et al., 2014; Ma et al., 2016a). Also the study of Miranda et al. (2017) using field samples dominated by *M. aeruginosa* showed that exposure for two hours to chitosan significantly lowered extracellular MC concentration. Probably the positively charged chitosan molecules interact with negatively charged microcystins that have a -1 charge over a large pH range (Lee and Walker, 2011). In our study, however, a clearly different effect of chitosan was observed, namely release of intracellular MCs. It is likely that extracellular MCs were first reduced, but evidently the chitosan induced cell leakage led to significantly enhanced extracellular MC concentrations compared to non-exposed cells. Likewise, when Miranda et al. (2017) used water dominated by the sensitive cyanobacterium *Cylindrospermopsis raciborskii*, they not only found strongly reduced PSII efficiency, but also enhanced extracellular saxitoxins. Hence, differences in sensitivity of *Microcystis* used, exposure duration too short to evoke cell lysis (e.g., Ma et al., 2016b; Miranda et al., 2017) or matrix effects on the MC detection could underlie the apparent differences. Pei et al (2014) used an ELISA kit to measure MCs, but possible matrix effects on the antibodies were not determined. Our study showed that chitosan did not interfere with the LC-MS/MS method for MC analysis used in this study.

Studies that combined chitosan and a ballast compound all revealed a reduction in extracellular MCs. A mesocosm experiment done by Pan et al. (2011b) showed that a bay one and two months after treatment with chitosan-modified soil (MLS), had lower dissolved MC compared to outside the bay. Similarly, Li and Pan (2015) found a reduction in MC when MLS was applied, however when only chitosan was added an increase in MC was observed indicating that the decreased MC concentrations might be related to the soil MC adsorption capacity instead of chitosan. Miranda et al. (2017) found that extracellular MC concentrations were significantly reduced in treatments where chitosan was combined with a ballast than in chitosan only treatments. In contrast, they found higher extracellular saxitoxin concentrations when *C. raciborskii* was exposed to either chitosan alone, or chitosan combined with soils and clay (Miranda et al., 2017). Where electrostatic interactions of chitosan with MCs can be expected, this is less likely for positively charged

saxitoxins (Shimizu et al., 1981). The study of Miranda et al. (2017) underpins that when chitosan is to be applied in drinking water reservoirs, depending on the cyanobacteria prevailing, corresponding cyanotoxin analysis is strongly advised.

Any material used to mitigate cyanobacterial nuisance that causes release of toxins is a double-edged sword. For one side, if the nuisance is reduced this will be viewed as positive, but if cells are killed rapidly and toxins released, the water body might not be suitable for drinking, irrigation or recreational purpose (Chorus and Bartram, 1999). However, when such cell death happens later close to the sediment, released toxins can be degraded (e.g., Li and Pan, 2015) with far less impact on ecosystem functionality. Thus, the use of ballast together with chitosan (a "flock and sink" approach or the MLS technique) seems a better strategy than using only chitosan, not only because it might prevent higher concentrations of toxins in the water column, but also because a ballast prevents cell accumulation at the water surface (e.g., Noyma et al., 2017).

The increase in chlorophyll-a (Fig. 5.S2) does not reflect an increase in biomass, but is a result of pigments leaking out of the cells, which was confirmed by the increase of extracellular chlorophyll-a (Fig. 5.2). A rapid increase in extracellular chlorophyll-a is a strong indicator of cell lysis, as is a rapid increase in extracellular MC concentration. Cell lysis implies membrane damage, which was confirmed by the membrane viability assay, where in all the strains tested a green fluorescence was observed at the highest chitosan concentration used (for example Fig. 5.4). Sytox green has a high affinity with nucleic acids, however, is not able to penetrate living cells, yet, when the membrane is compromised the stain is able to colour the genetic material with a bright green colour (Tashyreva et al., 2013), as observed here in the chitosan treatments. The absence of green colour in the control, but the presence of natural red fluorescence of cyanobacteria indicates no membrane damage in the controls (Fig. 5.4). In the strains in which it was possible to quantify MCs a significant positive linear relation between MC concentration and filterable chlorophyll-a was observed, hence, when dissolved toxins analysis is not possible filterable chlorophyll-a might be used as a surrogate to give insight in possible toxin release.

Geo-engineering materials used to manage eutrophication and to control cyanobacterial blooms must be efficient, easy to apply, cheap and safe, which means it is important to be aware of all the consequences that any material can cause in the ecosystem (Lürling et al., 2016). Chitosan, here, was able to damage *M. aeruginosa* cells causing cell lysis and consequently microcystin and pigment release. These effects were, however, strain dependent. Evidently, these trials need a follow up with natural seston dominated by *M. aeruginosa*, which in the field usually is found in its

typical colonial form contrasting the unicellular morphology in laboratory cultures (Geng et al., 2013). Considering the high diversity of cyanobacteria when chitosan is considered to be used in lake restoration, the best approach to understand its effects is to test it directly on the natural biota being targeted. Such tests should not only include coagulation efficiency, but also cell viability. We highlighted the importance of controlled experiments to understand the implications and efficiency of materials used to mitigate cyanobacterial nuisance, such tests are the first step, and to predict real effects a tiered approach from laboratorial to field tests is needed.

Conclusions

- Chitosan, as a coagulant, affected after one hour seven of the eight *M. aeruginosa* strains tested and after 24 hours all the strains had their photosystem II efficiency reduced.
- EC_{50} values varied from 0.47 to > 8 mg chitosan l^{-1} between the strains tested, such intraspecific variability might be related to the different amount of extracellular polymeric substances or membrane density charge of each strain.
- Chitosan caused microcystin (MC) release in all the toxic strains tested. Thus chitosan application in drinking water reservoirs should be considered with care.
- Fluorescence microscopy, through nucleic acid staining, proved cell lysis in all eight strains exposed to chitosan. Some strains were more sensible to lower doses of chitosan than others.
- Chitosan caused release of intracellular pigments in all strains tested. Extracellular chlorophyll-a concentrations was positively correlated to extracellular MC concentrations.
- The variability in strain sensibility requires that when chitosan is used as a coagulant to manage cyanobacterial nuisance, it should be tested first on the natural biota being targeted checking not only removal efficiency, but also cell viability.

Acknowledgements

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Supplementary information

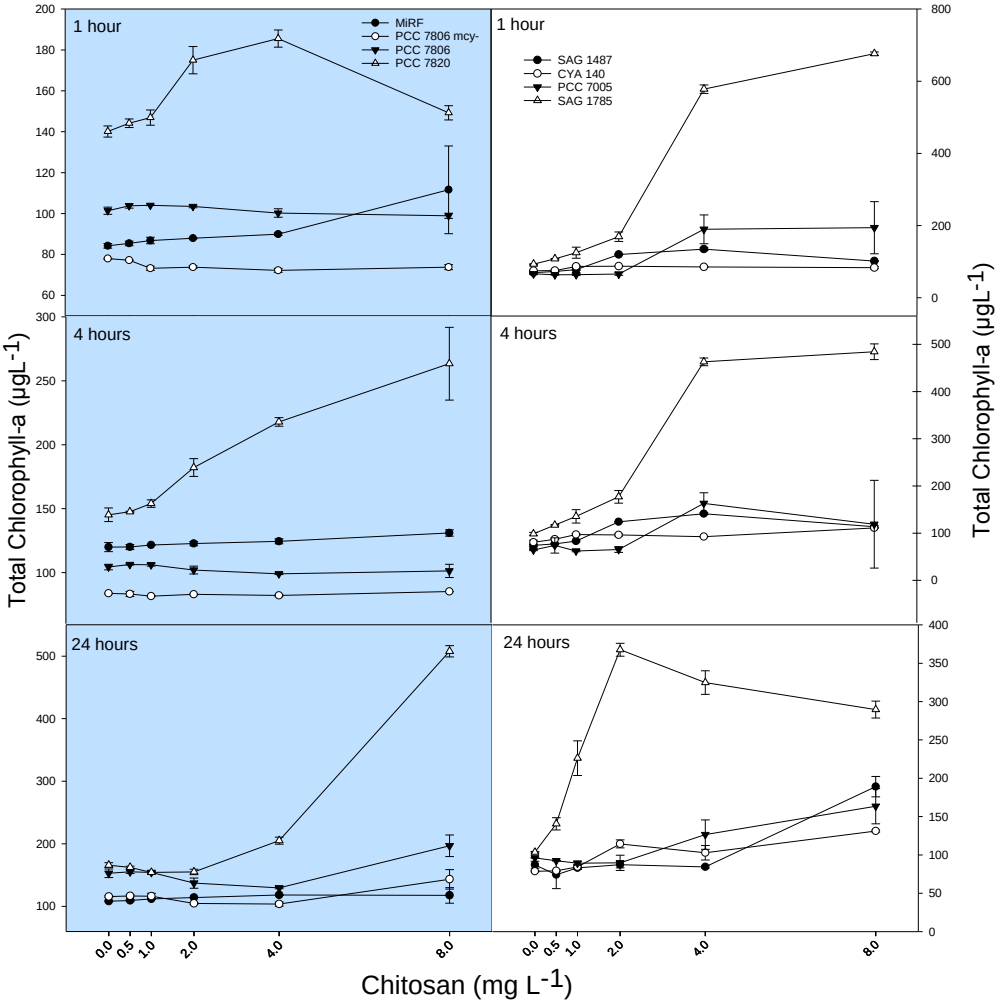

Figure 5.S1: Total chlorophyll-a concentration for all 8 strains tested after 1 hour, 4 hours and 24 hours exposure to different concentrations of chitosan (0 to 8 mg l^{-1}). Error bars indicate standard deviation (*n*=3). Graphs on the left shows strains with late response (MIRF, PCC 7806 ΔmcyB, PCC7806 and PCC 7820) and graphs on the right shows strains with early response (SAG 1487, CYA 140, PCC 7005 and SAG 1785).

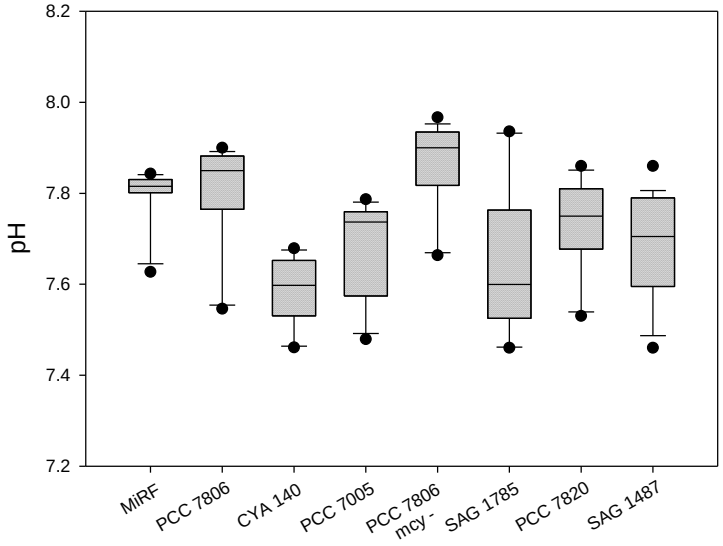

Figure 5.S2: Box plot showing pH median between the treatment, 10th, 25th, 75th and 90th percentiles with error bars (*n*=18) for each strain.

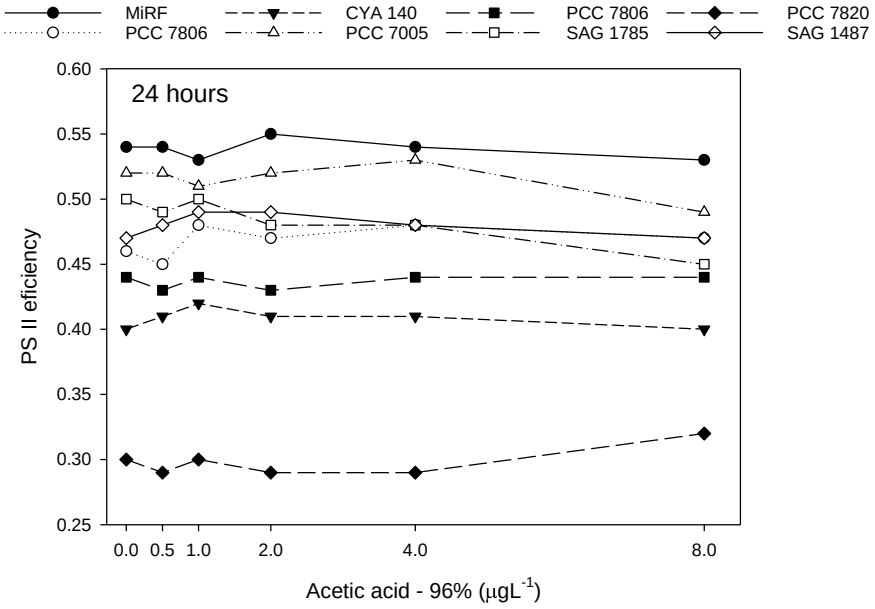

Figure 5.S3: PS II efficiency of all the eight strains tested after 24 hours exposure to different acetic acid concentration, the same used for the chitosan treatments.

Table 5.S1: Scientific papers available that studied chitosan coagulation efficiency or/and the effect on cyanobacteria/dinoflagellate. – means negative effect on the biota target, 0 means no negative effect observed and NR means no effect reported. FChl-a means filterable chlorophyll-a.

Literature available	Endpoints	Effect
Mucci et al., 2017	PS II efficiency and Membrane permeability (MP)	-
Pei et al., 2014	K^+ release and dissolved toxins	-
Shao et al., 2012	Phycocyanin and allophycocyanin release	-
Wang et al., 2015	K^+/M^{2+} release and dissolved toxins	-
This study	PSII efficiency, dissolved toxins, MP and FChl-a	-
de Magalhães et al., 2016	PSII efficiency	0
Guo et al., 2015	PSII efficiency	0
Li and Pan, 2015	Dissolved toxins	0
Li and Pan, 2013	Cell viability and recovery	0
Lürling et al., 2017	PSII efficiency	0
Ma et al., 2016a	K^+ release	0
Rojsitthisak et al., 2017	Growth	0
Noyma et al., 2017	PS II efficiency	0
Noyma et al., 2016	PS II efficiency	0
Miranda et al., 2017	PS II efficiency and dissolved toxins	0 and -
Capelete and Brandão, 2013	NR	NR
Huang et al., 2015	NR	NR
Lama et al., 2016	NR	NR
Li and Pan, 2016	NR	NR
Li et al., 2015	NR	NR
Ma et al., 2016b	NR	NR
Rakesh et al., 2014	NR	NR
Wang et al., 2016	NR	NR
Yan et al., 2009	NR	NR
Yuan et al., 2016	NR	NR
Zou et al., 2006	NR	NR
Pan et al., 2012	NR	NR
Pan et al., 2011b	NR	NR
Pan et al., 2006	NR	NR
Pandhal et al., 2017	NR	NR

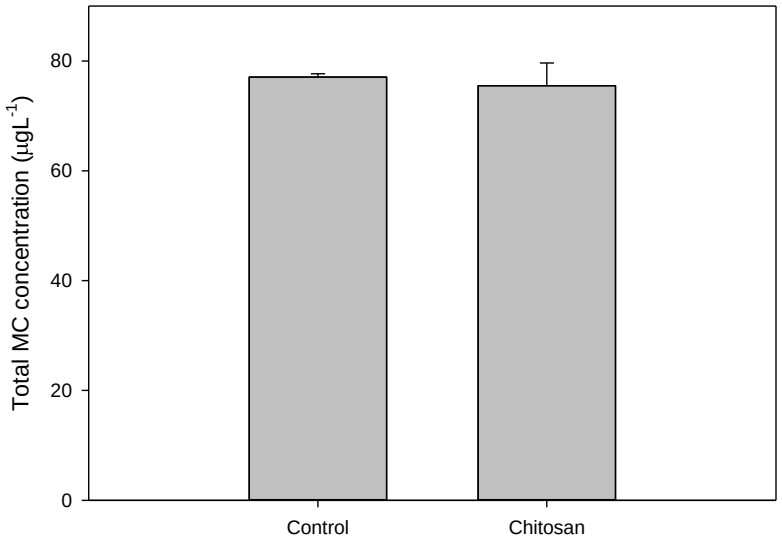

Figure 5.S4: Total MC incubated for 24 hours with only WC medium (Control) and with WC medium plus 8 mg chitosan l^{-1} (Chitosan).

6

Whole lake combined PAC-Phoslock® treatment to manage eutrophication and cyanobacterial bloom

Abstract

Lake De Kuil Tthe Netherlands, 6.7 ha, maximum depth 9m) suffered from cyanobacterial blooms since the early 1990s as a consequence of eutrophication. A system analysis revealed that the internal loading was the main contributor to the problem. To control internal loading and cyanobacteria blooms, the lake was treated in 2009 with a low dose of flocculant and a solid-phase-phosphate sorbent (Phoslock®). This Flock and Lock treatment successfuly increased water quality. Ongoing diffuse P-inputs, however, have gradually moved the lake back towards an eutrophic state with *Plankthotrix rubescens* dominance. Thus, a re-application of flocculant and Phoslock® was performed in 2017. The lake has been monitored before, during, and after this second application. We showed that Phoslock® reduced sediment phosphorus (P) release from 9.63 to 0.05 mg P $m^{-2}d^{-1}$, however, due to the ongoing diffuse P input, repeated interventions will be necessary in the future. The Flock and Lock treatment was successful in reducing total phosphate, turbidity, chlorophyll-a, and increasing water quality. Secchi depth also increased and microcystin concentration reduced. Two weeks after the intervention, cyanobacteria accumulation was observed near the shore of the lake, which spontaneously disappeared after 15 days. After the intervention, the lake was open for the bathing season, without any swimming bans during the summer of 2017. Experimental tests were done to understand why the scums occurred, and also to test a possible measure to avoid such nuisance. The results showed that the ballast and flocculant dose used in the intervention was enough to keep the biomass in the bottom for 1h, however, after 24h the *P. rubescens* escaped from the flocks and floated back to the surface. A promising approach to avoid biomass accumulation might be to damage the cell first with peroxide and settle them later using the Flock and Lock technique, however, more upscaled tests must be performed before field application can take place.

Introduction

Lake De Kuil is a small urban lake located in the southwestern of The Netherlands (Fig 6.1A), it was created as a result of a sand excavation in the 1950s. The lake is relatively deep, maximum 9 meters depth and its mean water depth is 4 meters (Fig 6.1B). With an area of 6.7 ha and a volume of 268000 m^3, the lake has been used by citizens as a bathing site. The local water authorities, according to the Bathing Water Directive 2006 (Council of the European Union, 2006), have to preserve, protect and improve the water quality to protect human health. However, as many urban ecosystems, cyanobacterial blooms have occurred annually in the lake from the 90s on (Waajen et al., 2016a).

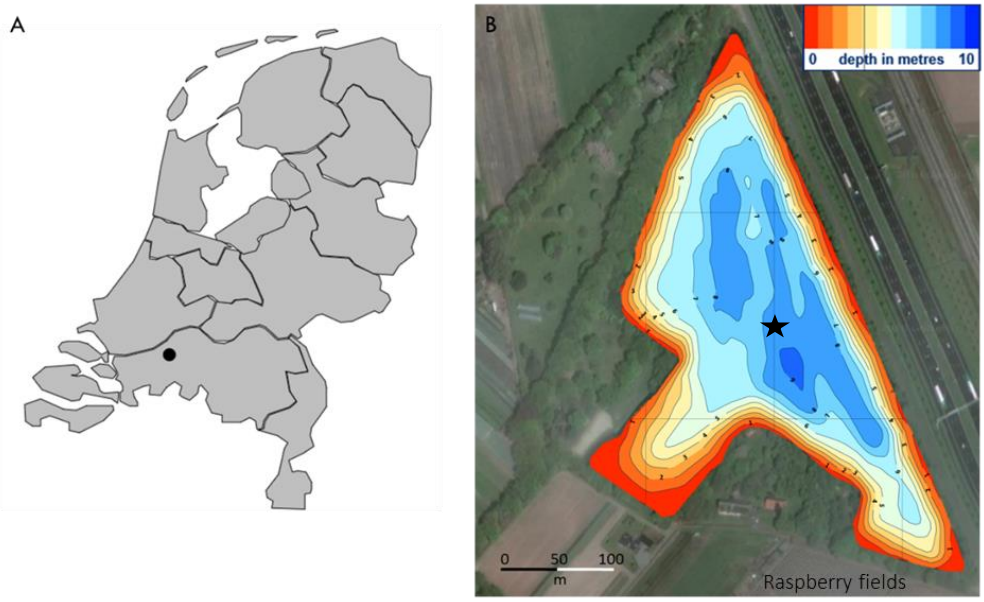

Figure 6.1: Panel A shows the map of The Netherlands and circle represent the Lake De Kuil location. Panels B shows the bathymetry of the lake and the star represents our sampling location.

Urban waters have been suffering from water quality issues, such as eutrophication, as a result of intense anthropogenic pressures (Teurlincx et al., 2019 and references therein). As a consequence, many urban waters encounter cyanobacteria nuisance (Noble and Hassall, 2015; Waajen et al., 2014), which pose a major threat to the functionality of the ecosystem as well as for human and animal health (Azevedo et al., 2002; Codd et al., 2005; Lürling and Faassen, 2013). Urban waters, however, can potentially contribute to biodiversity at a landscape-level

(Hassall, 2014, see table 2) and improve individual and community health (Lee and Maheswaran, 2011). In addition, those systems can be a bridge to reconnect cities to the biosphere, increasing people`s awareness of their actions impact in nature, mainly in young generations (Anderson and Moss, 1993; Andersson et al., 2014). Thus, to restore and protect ecosystem services in cities are extremely needed not only from an ecological point of view but also socially.

The first step to managing eutrophication and cyanobacterial blooms is a proper system analysis (SA) (Lürling et al., 2016), which will lead to the most promising set of measurements. In 2008, a SA in Lake De Kuil revealed that approximately 95% of the phosphorus input was being released from the sediment and the lake was also suffering from an *Aphanizomenon* sp. bloom (Waajen et al., 2016a). The output of the SA guided the water authorities to choose for a Flock and Lock technique. This technique consists of applying a low dose of coagulant together with a ballast/P-sorbent material, that will target not only the cyanobacterial biomass and Filterable Reactive Phosphate (FRP) in the water column but also the high internal loading in the lake (Lürling and Van Oosterhout, 2013).

A first intervention was done in 2009 which a low dose of iron chloride (coagulant) was added together with lanthanum modified bentonite (LMB), commercially known as Phoslock®. LMB has been applied in more than 200 water bodies and extensively tested in laboratory and mesocosms (Copetti et al., 2016). Its success lays on the strong affinity for phosphate (PO_4^{3-}). Once LMB is in contact with PO_4^{3-}, it forms rhabdophane ($LaPO_4 \cdot nH_2O$), a mineral with low solubility, which is stable over a wide pH range and under different redox conditions (Firsching and Brune, 1991; Jonasson et al., 1988). Over time, rhabdophane is converted to monazite ($LaPO_4$), a mineral even more stable than rhabdophane (Cetiner et al., 2005; Dithmer et al., 2016a). This is an advantage over aluminium hydroxides, which may lose their initial phosphate binding capacity when aged for a short period such as six months (Berkowitz et al., 2006).

The details from the first Flock and Lock application in Lake De Kuil have already been published by Waajen et al. (2016a). In a nutshell, the treatment was successful in reducing total phosphate, chlorophyll-a and increasing water quality. Ongoing diffuse P-inputs, however, have gradually moved the lake back towards an eutrophic state. Eight years after the first intervention, the lake suffered from a *Planktothrix rubescens* bloom. Thus, on May 2017, a re-application of Phoslock® and flocculent (polyaluminium chloride, PAC) was done. The lake was monitored before, during, and after the intervention.

In this study, we aimed at gaining insight in the efficacy of the Flock and Lock treatment to manage eutrophication and cyanobacterial bloom in Lake De Kuil. In addition to evaluating field results, a set of experiments was done to understand the reason why not all the cyanobacterial biomass was kept in the sediment after the application. We hypothesized that the intervention would improve water transparency right after the treatment. In addition, we expected a reduction in the internal loading due to the Phoslock® application.

Methods

Application

On May 9, 10.5 tons of Phoslock® were applied to the surface of the lake to act as a ballast and also to bind FRP available in the water column. On May 10, in the morning, 6 tons of polyaluminium chloride – PAC (Calflock P-14; 7.2% Al, a specific gravity of 1.31Kg L^{-1}) buffered with calcium carbonate ($CaCO_3$) was applied to the surface to act as a coagulant to target the biomass. In the afternoon of the same day, additional 22.05 tons of Phoslock® were injected in the hypolimnion layer (4 meters depth) only in the deeper part of the lake to target the internal loading (Fig. 6.2). The Phoslock® dose was calculated according to the immediately and potentially releasable and bioavailable P in the upper 10 cm of the lake sediment and the TP in the water column. PAC dose was estimated based on a Jar test done on the same day of application. The application and dose calculation were done by Phoslock Europe GmbH and the monitoring by the Aquatic Ecology and Water Quality Management Group from Wageningen University.

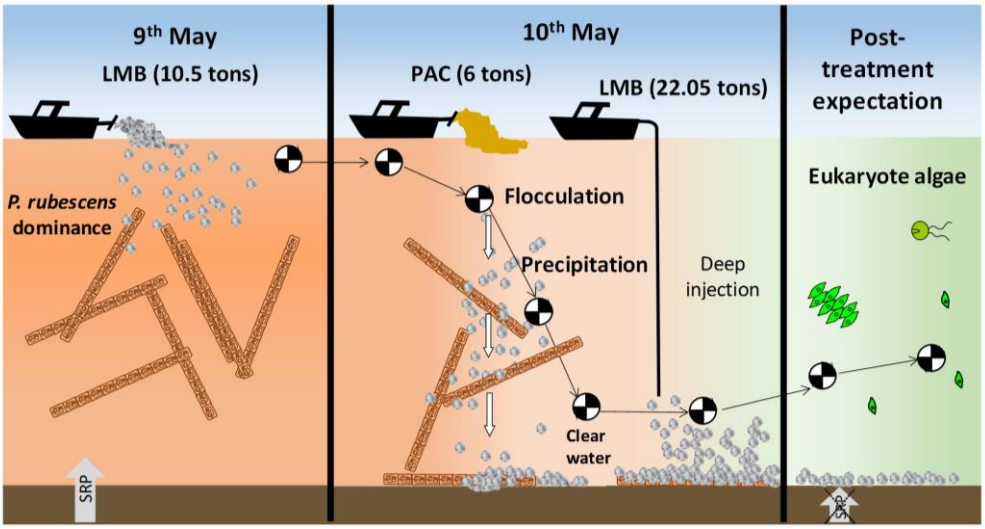

Figure 6.2: Scheme of the treatment done in Lake De Kuil in 2017. On May 9, lanthanum modified bentonite (LMB, Phoslock®) was applied at the surface of the lake. On May 10, in the morning, 6 tons of polyaluminium chloride – PAC was applied at the surface, and in the afternoon of the same day, more 22.05 tons of Phoslock® was injected in the hypolimnion layer. Secchi disk represents expected water transparency.

Lake sampling

Water sample were taken every one meter in the middle of the lake (indicated by a star in Fig. 6.1B), to analyze phosphorus concentration (FRP and TP), total and filterable metals (lanthanum and aluminium), chlorophyll-a, turbidity, intracellular microcystins, and pH. In situ, Secchi depth was measured. The lake was sampled monthly four times before the treatment, every day during the application, one day after the treatment, biweekly from May until July, and monthly until December, totalizing 15 sampling events in 2017.

FRP and TP concentrations were analyzed using a Skalar SAN+ segmented flow analyzer following the Dutch standard NEN-EN-ISO 15681-2:2005 (NNI, 1986) or by ICP-MS after destruction using HNO_3-HCl (aqua regia). Total and filterable metals (Al and La) were also analyzed through ICP-MS. For filterable nutrients and metals, water samples were filtered through glass-fiber filters (Whatman GF/C, Whatman International Ltd.). Chlorophyll-a concentration was measured using PHYTO-PAM

phytoplankton analyzer (Heinz Walz GmbH, Effeltrich, Germany). Turbidity was measured through Hach 2100P turbidity meter and pH using WTW-pH320.

Water samples were also filtered through glass-fiber filters (Whatman GF/C, Whatman International Ltd.); the filter was rolled and placed in an 8 ml glass tube to analyze intracellular microcystin. The filters, after the drying process in the freeze dryer (Alpha 1-2 LO), were extracted three times at 60°C in 2.5 ml 75% methanol: 25% Millipore water. The extract was dried in a Speedvac concentrator (Savant™ SPD121P, Thermo Fisher Scientific), and then, reconstituted in 900 µl methanol solution (J.T. Baker®, 97%). After that, the reconstituted samples were transferred to a 1.5 ml tube with a cellulose-acetate filter and centrifuged for 5 min at 16.000 x g. The filtrates were transferred to amber glass vials and analyzed for eight MC variants (MC - dmRR, RR, YR, dmLR, LR, LY, LW, and LF) using LC-MS/MS according to Lürling and Faassen (2013). The counterplot graphs were created using the software Surfer 13 and bars/dots plots using Sigma Plot 13.

Core experiment

Before the field application (April, 2017), 17 sediment cores were taken using a UWITEC core sampler to test the efficiency in target the P release from the sediment of the proposed treatment. Four cores were left untreated, four were treated with solo PAC (2mg L^{-1}), four with solo Phoslock®, and the last four were treated with the combination of PAC and Phoslock®. One sediment core was used to quantify the potential releasable P in the sediment in order to determine the Phoslock dose®. For that, a sequential extraction protocol modified from Paludan and Jensen (1995) and used by Cavalcante et al. (2018) was done. There were around 6.5 mg P in the upper 6 cm to be immobilized, thus a dose of 650 mg (100:1 ratio) was added in each core treated with Phoslock® to immobilize the upper 6 cm of the sediment. The cores were naturally anoxic and after received the treatments were placed in the laboratory at 7°C in the dark. Ten millilitres of water from each core were taken before the application, after 1 day, 7, 14, 23, and 640 days and filtrated using glass-fiver filters (Whatman GF/C). The filtrated were analysed for their FRP concentration as mentioned before.

Sediment P-release

Sediment cores were taken before the application (3rd April 2017), one month after the in-lake application (14th June 2017), and around one year after the application (10th April 2018) using a UWITEC core sampler. The overlying lake water

from the cores was removed and filtered through glass-fiber filters (Whatman GF/C). Afterward, the filtered lake water was gently placed back into the respective cores. Eight cores were taken before the application, in which 4 were bubbled with N_2 to evaluate P- release under anoxia and 4 were left naturally under oxic conditions. After one month, eight cores were taken and one year after, 4 cores. Since all the cores taken after the intervention were naturally anoxic no N_2 bubbling was needed and they were kept naturally under anoxic condition. All the cores were placed for 4 weeks in a dark chamber at 7°C. Weekly, 10 ml samples were taken and measured for their FRP concentration as mentioned before, at the end of each sampling event 10 ml of nanopure water was added. The data from the previous years (2009 to 2015) used in the graph (Fig 6.10) has been already published by Waajen et al., 2016a.

Experimental approach

Two weeks after the application, a biomass accumulation was observed near the shore of the lake, thus, some additional experiment was done to understand why part of the *Planktothrix rubescens* was not retained on the sediment. Five hypotheses were tested:

1) The PAC dose applied was too low to produce sufficient flocks to settle the cyanobacteria.
2) The way PAC was dosed in the field created too low pH at the onset of flock formation therewith preventing good development of flocks.
3) The ballast dose (Phoslock®) was too low to settle the flocks.
4) The cyanobacterial density was too high to be effectively flocked and precipitated.
5) The Phoslock® did not adsorb any FRP but released FRP.

In addition, we also performed an extra experiment to test if a combined treatment, using hydrogen peroxide (H_2O_2) with PAC and Phoslock®, would have avoided the cyanobacteria biomass accumulation.

To test each hypothesis, on May 30, 2017, water samples from Lake De Kuil were taken in different locations where the biomass was accumulated. The general set-up of the assays was that 100 ml of lake water was transferred to 125ml glass tubes and treated with the designed compound(s) or left untreated (control). After 1 hour, 10 ml samples from the top and from the bottom of the tube were taken to measure chlorophyll-a and Photosystem II efficiency (PSII) using PHYTO-PAM

phytoplankton analyzer. Also, pH was measured in the middle of the tubes. When necessary, microcystin concentration was also measured as previously described. Difference in chlorophyll-a concentration between different treatments was analyzed by one-way ANOVA or *Kruskal-Wallis* One Way Analysis of variance on Ranks when normality tests failed (*Shapiro-Wilk*) in the tool pack Sigma Plot 13.0

To test our first hypothesis, a fixed Phoslock® dose of 200 mg L^{-1} was added and followed by PAC in a range of 0, 1, 2, 4, 6, and 8 mg Al L^{-1}. After one hour, samples were taken and analyzed as mentioned before. The experiment was performed using one single experimental units per treatment.

During the in-lake intervention, PAC was first mixed with lake water (1:5) and afterwards sprayed at the surface of the lake, thus, pH value could have been lower than the optimal for flocculation process. Thus, our second hypothesis was tested making different ratios (1:5, 1:10, 1:20, 1:50 and 1:100) of PAC: Lake De Kuil water. After mixing for 15 seconds, these were dosed at 2 mg Al L^{-1} to lake water with 200 mg L^{-1} Phoslock® as a ballast. The experiment was run in triplicate and sampled as outlined above.

The total amount of Phoslock® applied in the lake (32.5) yielded a dose of 125 mg L^{-1}. The ballast, however, will surely have been less as only 31% of the Phoslock® has been added at the surface. Therefore, the effect of different ballast doses on the efficiency of *P. rubescens* removal was studied to test our third hypothesis. The PAC dose was kept at 2 mg Al L^{-1} while Phoslock® as ballast was dosed at 0, 50, 100, and 200 mg L^{-1}. The experiment was performed in triplicates and further conducted similarly to the previous one.

To test our fourth hypothesis, an experiment using different concentrations of cyanobacteria and ballast was performed. Different concentrations of Phoslock® (50, 100 and 200 mg L^{-1}) in combination with PAC (2 mg L^{-1}) were added to various cyanobacteria concentrations (24, 50, 100, and 200 µg chlorophyll-a L^{-1}). One tube per cyanobacteria concentration was left untreated. After 1 hour, samples from top and bottom were taken. In addition, an extra series was left standing for 24 hours. The experiment was performed in triplicates and samples were taken and analyzed as previously described.

Our fifth hypothesis was tested through an adsorption experiment. Eight FRP solutions containing 0, 5, 10, 20, 40, 80, 120 and 160 mg P L^{-1} were prepared by dissolving KH_2PO_4 in nanopure water. A total volume of 40 mL FRP solution and 80 mg of Phoslock® was brought in 50 mL falcon tubes; the bottles were closed with a screw cap. The experiment was conducted in triplicate. The suspensions were continuously

mixed on a shaker at 180 rpm for 24 hours at 22°C, whereafter samples were taken and centrifuged for 10 minutes at 2500 rpm. The supernatant was filtered through unit filters (Aqua 30/0.45CA, Whatman, Germany). Filtrates were analyzed for their FRP concentration as mentioned before. The same method was followed to assess the maximum adsorption capacity under anoxia, however, this time the tubes were bubbled with N_2 gas for around 5 minutes.
In order to calculate the maximum SRP adsorption we fitted the data in the isotherm Langmuir model:

$$\frac{\mathrm{Ce}}{\mathrm{Qe}} = \frac{1}{\mathrm{qmKL}} + \frac{\mathrm{Ce}}{\mathrm{qm}}$$

in which *Ce* is the equilibrium concentration of the adsorbate (FRP, mg L^{-1}), *Qe* is the adsorption capacity at equilibrium (mg g^{-1}), qm is maximum adsorption capacity (mg g^{-1}), and KL is the Langmuir adsorption constant. Plotting $\frac{\mathrm{Ce}}{\mathrm{Qe}}$, versus C_e the slope will give $\frac{1}{\mathrm{qm}}$ and the intercept $\frac{1}{\mathrm{qmKL}}$ from which it is possible to calculate qm and KL. Ce and Qe will be obtained from the adsorption experiment:

$$\mathrm{Qe} = \frac{(\mathrm{C_0} - \mathrm{Ce})\mathrm{V}}{\mathrm{M}}$$

Where Qe is the amount of SRP adsorbed at equilibrium (mg g^{-1}), C_0 is the initial concentration of SRP in solution (mg L^{-1}), Ce is the concentration of FRP in solution at equilibrium (mg L^{-1}), M is the adsorbent mass used (g) and V is the volume of FRP solution (Langmuir, 1918).

An extra experiment using a combined treatment adding, first, hydrogen peroxide to damage the cells, followed by PAC and Phoslock® to remove the biomass from the water column was carry out to evaluate the efficiency of such treatment. This experiment tested the hypothesis that peroxide would damage *P. rubescens* cells enough to hamper photosynthesis and buoyancy, which would keep filaments aggregated in flocks at the bottom of the experimental units.

Based on the results of the hydrogen peroxide exposure assay, a working dose of 5 mg L^{-1} was chosen (data not shown). Aliquots of 100 mL concentrated *P. rubescens* from Lake De Kuil were transferred to 12 glass tubes of 125 ml. Three tubes of each series remained untreated (controls), three tubes per series were treated with coagulant (PAC, 2 mg L^{-1}) and ballast (Phoslock®, 200 mg L^{-1}), and the remaining six tubes were treated with hydrogen peroxide (5 mg L^{-1}). After three hours, three of the

peroxide treated tubes were treated with a coagulant (PAC, 2 mg L^{-1}) and ballast (Phoslock®, 200 mg L^{-1}). The tubes were incubated for 24 hours in the laboratory, where after 2 mL samples from the top of the test tubes and from the bottom of each tube were collected. These samples were analyzed for chlorophyll-a and PSII as mentioned above. In this experiment, 8 ml samples were filtered through glass fiber filters (GF/C, Whatman®, Germany) and placed in glass tubes for dissolved microcystin analysis. The samples were dried, reconstituted and analyzed as previously mentioned.

Results

Water Quality

Before the treatment, the lake was suffering from a *Plankthotrix rubescens* bloom, which was distributed through the water column but it was more concentrated in the upper 5 meters. One month before the application, the mean cyanobacteria chlorophyll-a concentration over the whole water column was 40.5 (±SD 9.8) µg L^{-1} and in the day before the intervention, the mean was 19 (±SD 2.9) µgL^{-1}. Already during the intervention, after PAC application, the mean chlorophyll-a reduced to 4.1 (±SD 0.6) µg L^{-1} and one day after the treatment reduced to 2.6 (±SD 0.6) µg L^{-1} (Fig. 6.3A). Two weeks after the application the mean was 7.2 (±SD 1) µg L^{-1} in our sampling location (Fig. 6.1B); however, near the shore of the lake, a biomass accumulation was observed at the surface (around 170 µg L^{-1}) (Fig. 6.4). After 15 days, this biomass accumulation disappeared without any intervention.

Turbidity, as well as chlorophyll-a concentration, was high in the day before the application (36.4 ±SD 15.1 NTU) and decreased during the application to 26.9 (±SD 4.3) NTU. On the first day after the application, turbidity was reduced to 12.6 (±SD 1.7) (Fig. 6.3B). The day before the application, Secchi depth was around 0.4 m and increased to 0.95 m in the first day after application. Two weeks after, it increased to 2.0 m and after six months to around 3.5 m (Fig. 6.3B).

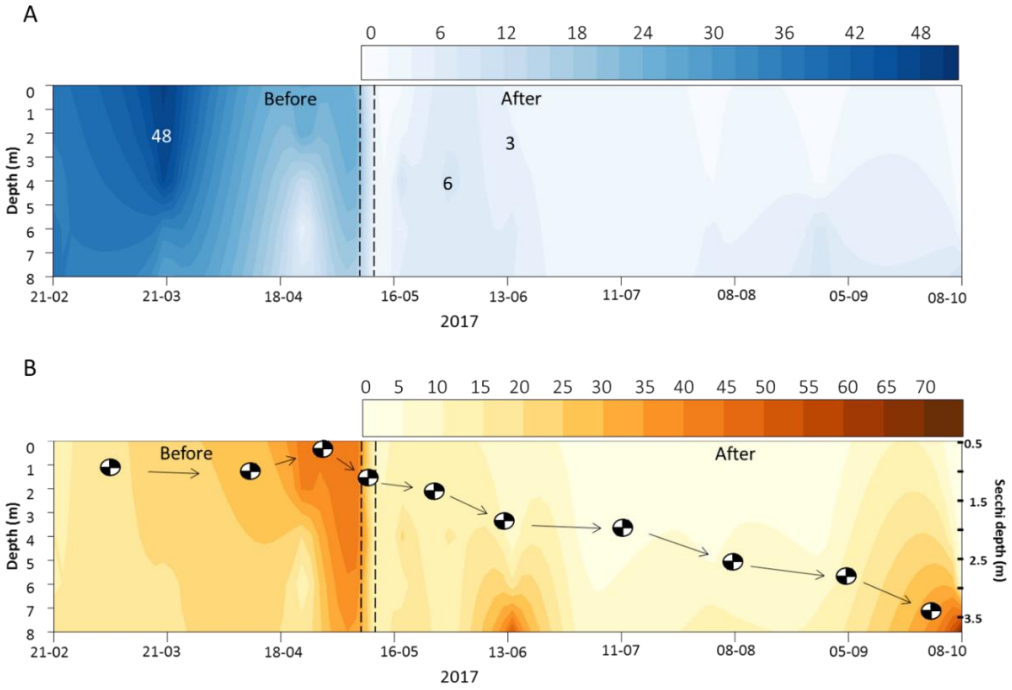

Figure 6.3: Contour plots of cyanobacteria chlorophyll-a (µg L^{-1}) (Panel A) and turbidity (NTU) (Panel B) over time. Secchi disks represent secchi depth over time (Panel B). Dashed lines represent the moment during the application.

Figure 6.4: *Plankthotrix rubescens* accumulation at the shore of the lake two weeks after treatment.

During the treatment, the lake was suffering from a *P. rubescences* bloom, which is a toxic cyanobacterium, so microcystin (MC) concentration was also analyzed. The highest intracellular MC concentration was found before the application: 146 µg L^{-1} at the surface of the lake. During the application and after one

day the values were below 20 µg L^{-1} (Fig. 6.5). However, after seven and twenty days, MC concentrations had increased again to 31.1 (±SD 14.2) and 23.3 (±SD 10.4) µg L^{-1}, respectively. Nonetheless, one month after application the values were below 5 µg L^{-1} and remained below 0.2 µg L^{-1} during 2017.

TP was also reduced from 134 (±SD 12) µg L^{-1} to 72 (±SD 57) µg L^{-1}, one day after treatment. After one month, the values were reduced to 53 (±SD 27) µg L^{-1} and to 23.8 (±SD 14) µg L^{-1} five months after the intervention (Fig. 6.6A). FRP concentration was low before the application (15.7 ±SD 1.9 µg L^{-1}) and increased mainly at the surface to a maximum value of 45 µg L^{-1} one week after the application. One month after the treatment, FRP concentrations were below or equal to 10 µg L^{-1} (Fig. 6.6B).

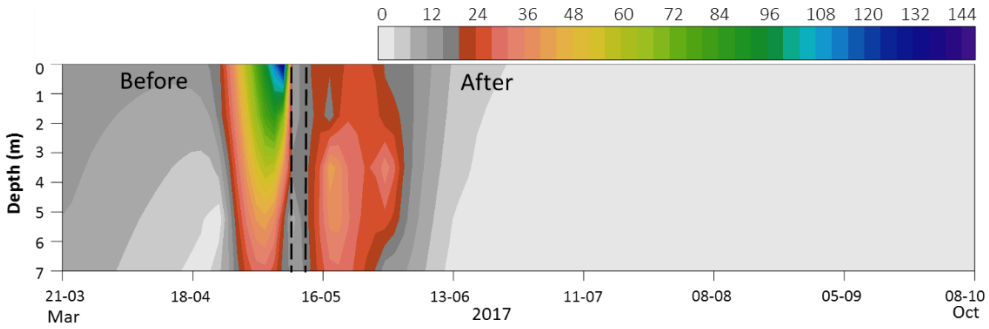

Figure 6.5: Contour plots of intracellular microcystins (µg L^{-1}) over time. Gray color represents values below the guidance values for Dutch bathing waters (<20 µg L^{-1}). Dashed lines represent the moment during application.

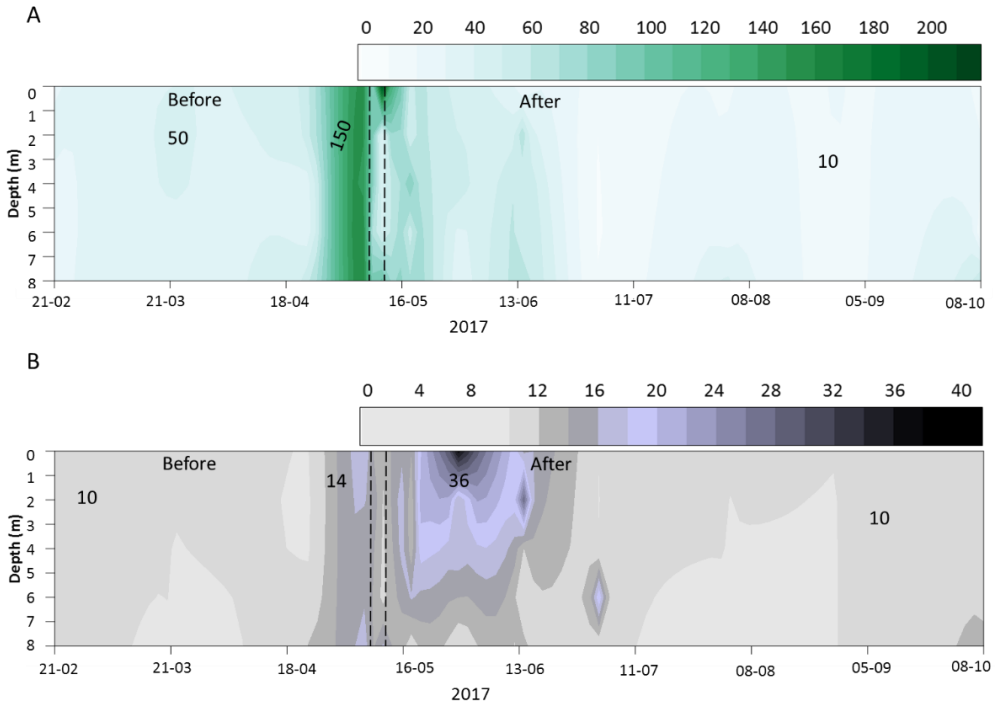

Figure 6.6: Contour plots of total phosphorus (Panel A) and filterable reactive phosphorus (Panel B) (µg L^{-1}) over time. Dashed lines represent the moment during application.

Total Lanthanum (TLa) and Total Aluminium (TAl) concentration before the application were 0.0166 (±SD 0.0125) µg L^{-1} and 0.0007 (±SD 0.000625) mg L^{-1}, respectively. TLa and TAl concentrations were high during the application, 167 (±SD 76) µg L^{-1} and 0.26 (±SD 0.04) mg L^{-1}, respectively (Fig. 6.7). One day after the treatment, TLa and Tal concentrations declined sharped, 46% and 60%, respectively. This sigmoidal decrease continued and, after 37 days, TLa concentrations were more than 11 times less than during the application and TAl 5 times less (Fig. 6.7). Filterable Lanthanum (FLa) concentrations were between 1.7 and 5.8 µg FLa L^{-1} during the application and remained low over time. The maximum value found was 7.2 µg FLa L^{-1}, 22 days after application in the deeper layers (Fig. 6.1S). Filterable aluminium (FAl) varied from 0.04 to 0.01 mg FAl L^{-1} during the application and the highest concentration was 0.06 mg FAl L^{-1} after one day of PAC application (Fig. 6.1S).

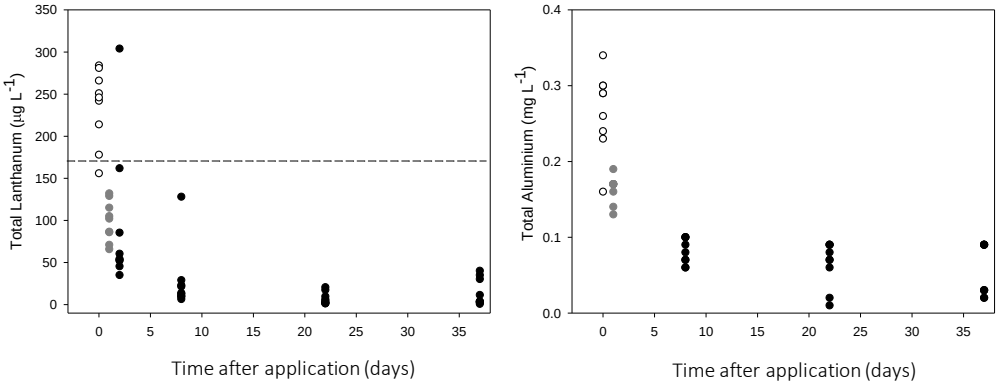

Figure 6.7: Total Lanthanum (left graph) and Total Aluminium (right graph) concentrations. Dots represent metals concentration for each water depth during the application (empty dots), one day after the application (grey dots), and two, eight, twenty-two and thirty-five days after treatment (black dots). Dash line in lanthanum graph signifies maximum allowed concentration for Dutch waters (<150 µg L^{-1}).

pH value decreased during the application from 8.4 to 6.6 at the surface of the lake (Fig. 6.8). During the application the minimum pH value was 6.6. One day after the application, the pH value increased to 7.4 at the surface and 7.6 at the 1 meter depth. At the surface, pH remained between 8.5 and 7.6 until the end of 2017. At the bottom of the lake, pH was lower than at the surface, mainly in two weeks after the application, and varied between 6.9 and 7.8 (Fig. 6.8).

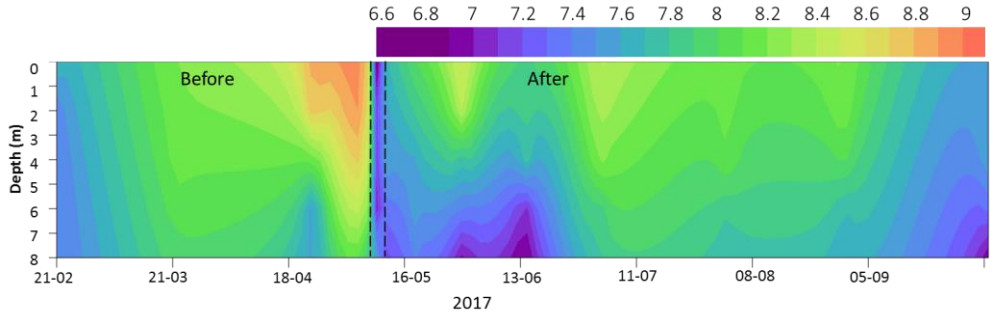

Figure 6.8: Contour plots of pH over time. Dashed lines represent the moment during application.

Core experiment

FRP concentrations in the sediment cores before the application (time 0) were similar between treatments, however, after the treatments, the cores that were left untreated (control) and the ones treated with solo PAC showed an increase in FRP concentrations over time (Fig. 6.9). The maximum value found were 1432 ±SD 1109

(control) and 2210 ±SD 1176 µg L^{-1} (PAC treatment), both after 640 days of incubation (Fig. 6.9). Contrary, the cores treated with solo Phoslock® and with Phoslock® and PAC showed a reduction in FRP concentration after one day of treatment and FRP values remained low after 640 days of incubation (11.5 ±SD 7.1 and 22.7 ± 14 µg L^{-1}, respectively) (Fig. 6.9).

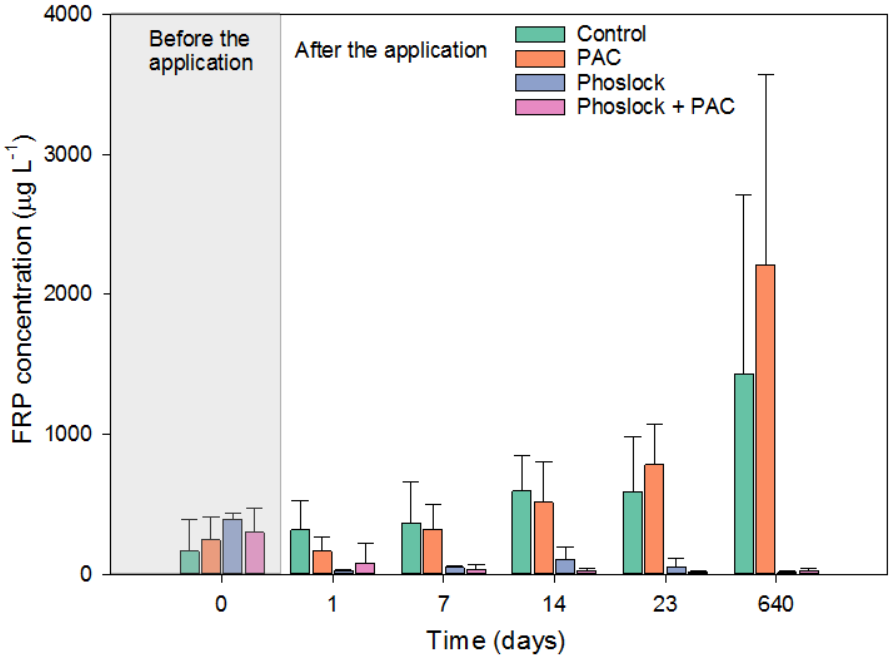

Figure 6.9: FRP concentration over time from sediment cores from Lake De Kuil which were left untreated (control) or treated with PAC (2 mg Al L^{-1}), or with solo Phoslock® (650 mg), or with PAC and Phoslock® combined (*n*=4). Error bars indicate SD.

Sediment P-release

Before the second application, May-2017, the anoxic sediment P release was approximately two times higher than oxic sediment P-release (Fig. 6.10). The anoxic P release from the sediment reduced from 9.63 (±SD 4) mg P m^{-2} d^{-1} before the second application to 0.05 (±SD 0.03) mg P m^{-2} d^{-1}, one month after the second application (Fig. 6.10). One year after the application, anoxic P release from the sediment was 1.33 ±SD 0.7 mg P m^{-2} d^{-1}, which was lower than before the application but higher than one month after the application (Fig. 6.10).

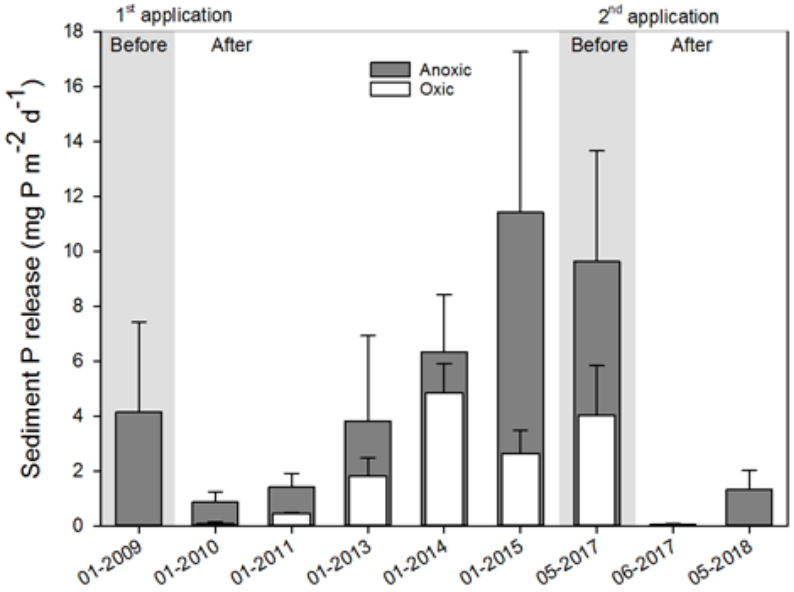

Figure 6.10: Sediment phosphate release over time (month-year) before and after the first application (data from Waajen et al., 2016a) and also before and after the second application under oxic and anoxic conditions. Error bars indicate SD.

Experimental approach

In the first experiment, in which we tested different PAC concentrations with a fixed dose of Phoslock®, *P. rubescens* accumulated within one hour at the water surface in the control, while at each PAC dose combined with 200 mg L^{-1} Phoslock® virtually all *P. rubescens* were precipitated (Fig. 6.11A). In the second experiment, the way PAC was dosed in the field was tested and similar results as in the first experiment were observed: in the control, the *P. rubescens* biomass accumulated within one hour at the water surface, while at each PAC: water ratio the corresponding PAC dose (2 mg L^{-1}) combined with 200 mg L^{-1} Phoslock® precipitated virtually all cyanobacteria biomass (Fig. 6.11B). A one-way ANOVA on log-transformed chlorophyll-a concentrations in the top of the tubes indicated significant differences between treatments ($F_{5,17}$ = 662.9; p <0.001). Holm-Sidak Pairwise Multiple Comparison revealed that the chlorophyll-a concentration in the top of the control tubes was significantly higher than that in the treatments. Likewise, chlorophyll-a concentrations in the bottom of the tubes were significantly different between treatments and control ($F_{5,17}$ = 124.1; p < 0.001).

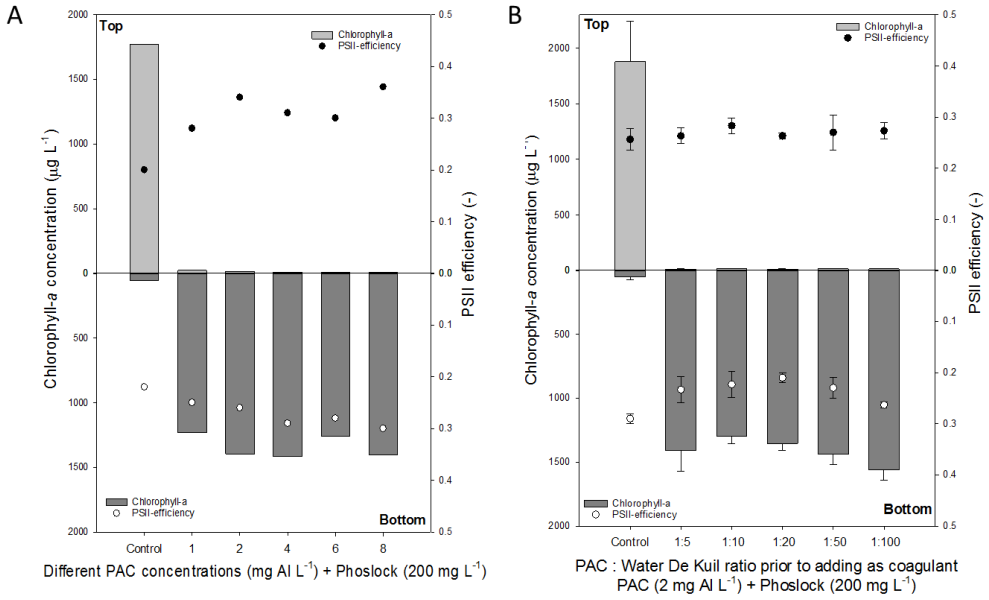

Figure 6.11: Chlorophyll-a concentrations in the top 2 mL (top light grey bars) and bottom 2 mL (lower dark grey bars) of 100 mL cyanobacteria suspension from De Kuil incubated for 1 h. Filled circles represent the Photosystem II efficiencies (PSII) of the cyanobacteria collected at the water surface and open circles at the bottom. Panel A shows cyanobacteria suspension in the absence or presence of different concentrations of PAC (1–8 mg Al L^{-1}) combined with a ballast (Phoslock® 200 mg L^{-1}). Panel B shows cyanobacteria suspension in the absence or presence of a ballast (Phoslock® 200 mg L^{-1}) combined with PAC (2 mg Al L^{-1}) from different pre-mixtures with lake water. Error bars indicate SD (n = 3).

In the third experiment, we tested the effect of different ballast doses on the efficiency of *P. rubescens* removal. Each ballast dose was capable of settling the cyanobacteria and in the control - after one hour - the chlorophyll-a concentration at the top of the tube was significantly higher than in the treatments ($F_{3,11}$ = 495.8; p < 0.001), while at the bottom it was lower ($F_{3,11}$ = 835.1; p < 0.001) (Fig. 6.12A). After 24 hours, one-way ANOVA also indicated significant differences in chlorophyll-a concentrations at the top ($F_{3,11}$ = 19.3; p < 0.001) and at the bottom (log-transformed data; $F_{3,11}$ = 45.8; p < 0.001) of the test tubes (Fig. 6.12B). However, after 24 hours, the majority of the settled flocks had risen again and accumulated at the water surface (Fig. 6.12B).

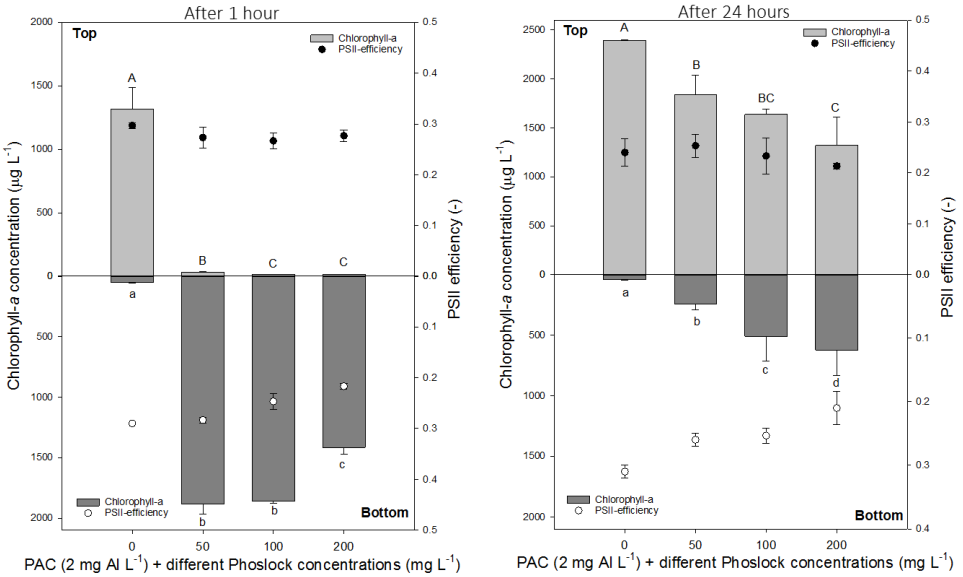

Figure 6.12: Chlorophyll-a concentrations (µg L^{-1}) in the top 2 mL (top light grey bars) and bottom 2 mL (lower dark grey bars) of 100 mL cyanobacteria suspension from De Kuil incubated for 1 hours (left graph) and 24 h (right graph) in the absence or presence of different concentrations ballast (Phoslock 50, 100, and 200 mg L^{-1}) combined with the flocculant PAC (2 mg Al L^{-1}). Also included are the PSII efficiencies of the cyanobacteria collected at the water surface (filled circles) and at the bottom (open circles). Error bars indicate SD (n = 3). Similar letters indicate homogeneous groups at the $p < 0.05$ level.

The fourth experiment was performed to check whether the cyanobacteria biomass in the lake was too high to be removed by the used treatments. After 1 hour, 100 mg Phoslock® L^{-1} was enough to settle even the highest biomass, while lower biomass could still be removed effectively using around 50 mg Phoslock® L^{-1} (Fig. 6.13A). However, the results after 24 hours showed much lower removal and strongly hampered efficiency; even at low biomass high amounts of ballast could maximally remove 76% (Fig. 6.13B). The fifth experiment tested if Phoslock® release FRP instead of adsorbing it. In this experiment Phoslock® (lot 20161102) did not release a detectable amount of FRP under oxic or anoxic conditions, yet instead showed high SRP adsorption capacity of 8.3 and 7.9 mg SRP g^{-1} under an oxic and anoxic condition, respectively (Fig. 6.2S).

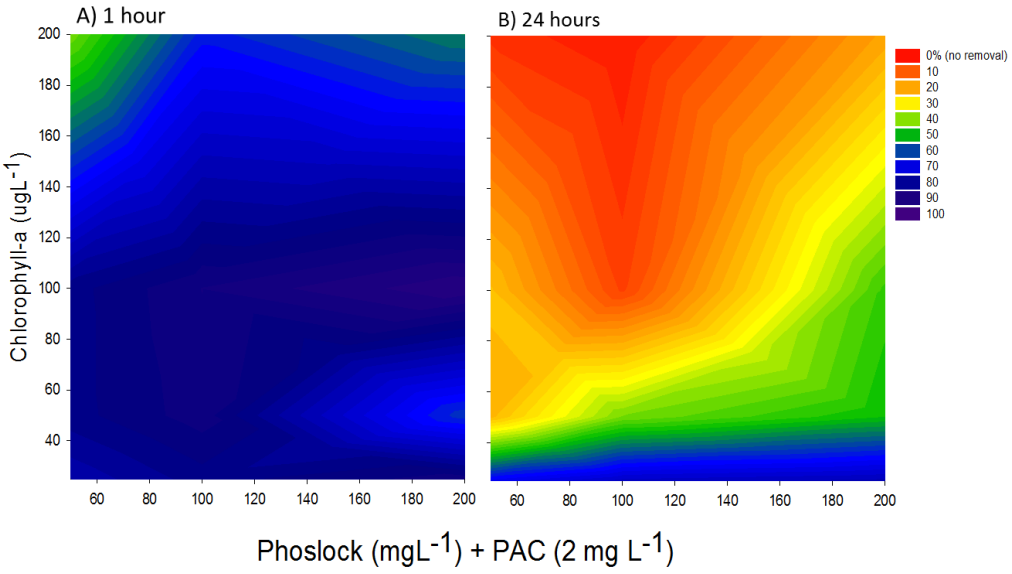

Figure 6.13: Percentage of cyanobacterial biomass (µg L^{-1}) removal using different Phoslock® concentrations in presence of PAC (2 mg Al L^{-1}). Left panel after 1 hour and right panel after 24 hours.

In the extra experiment to test the efficiency of hydrogen peroxide followed by a coagulant and ballast on the removal of cyanobacteria, the chlorophyll-a concentrations in the top water layer of the tube were significantly different between treatments after 24 hours ($F_{3,11}$ = 149.5; p < 0.001). At the top of the tubes, the chlorophyll-a concentration in the H_2O_2 with Flock and Lock treatment was significantly lower compared to the other treatments (Fig. 6.13A). Contrary, at the bottom of the tubes, chlorophyll-a concentrations were lowest in the control and sole peroxide treatment; significantly higher (log-transformed data; $F_{3,11}$ = 401.8; p < 0.001) in the Flock and Lock treatment, and highest in the combined treatment – H_2O_2 and Flock and Lock (Fig 6.13A). The Holm-Sidak post hoc pairwise comparison revealed for both top and bottom water samples three homogenous groups: 1) the controls and sole peroxide treatment, 2) Flock and Lock treatment and 3) combined treatment (Fig 6.13A). The PSII in the top of the test tubes was significantly different among treatments ($F_{3,11}$ = 206.9; p < 0.001), as was PSII in the bottom samples ($F_{3,11}$ = 70.2; p < 0.001). The Holm-Sidak post hoc pairwise comparison for PSII efficiency revealed for both top and bottom water samples two homogenous groups: 1) the controls and sole flock and lock treatments, and 2) both peroxide treatments. Extracellular microcystin concentrations were significantly different between the

treatments ($F_{3,11}$=77.98 p <0.001) (Fig. 6.13B). The Holm-Sidak post hoc pairwise comparison divided MC concentrations into 3 homogenous groups from lower to highest concentration 1) control and Flock and Lock treatment, 2) combined treatment (H_2O_2 + Flock and Lock), and 3) only hydrogen peroxide treatment (Fig. 6.14B). The most abundant variant in all the treatments was dmMC-RR followed by MC-YR and dmMC-LR.

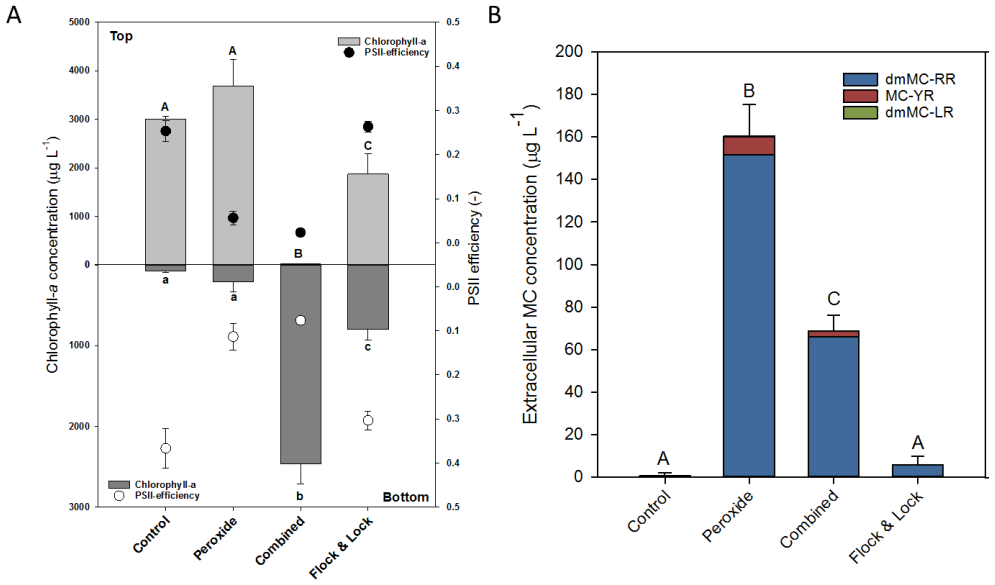

Figure 6.14: Panel A shows chlorophyll-a concentrations in the top 2 mL (top light grey bars) and bottom 2 mL (lower dark grey bars) of 100 mL cyanobacteria suspension from Lake De Kuil after 24 h exposure to peroxide (5 mg L^{-1}), peroxide (5 mg L^{-1}) + coagulant (2 mg Al L^{-1}) and ballast (200 mg Phoslock® L^{-1}) (Combined) or only coagulant (2 mg Al L^{-1}) and ballast (200 mg Phoslock® L^{-1}) (Flock & Lock). Filled circles represent the PSII efficiencies of the cyanobacteria collected at the water surface and open circles at the bottom. Panel B shows extracellular microcystin concentrations after 24 hours exposure to different treatments. Error bars indicate SD (n = 3). Similar letters indicate homogeneous groups at the p < 0.05 level.

Discussion

In agreement with our hypothesis, the Flock and Lock treatment applied in Lake De Kuil considerably improved water transparency. Turbidity and cyanobacterial chlorophyll-a concentration decreased and Secchi depth was increased (Fig. 6.3). Similar results were observed when Flock and Lock was applied in the same lake for the first time in 2009 (Waajen et al., 2016a). The technique also substantially improved the water transparency in another urban bathing site, Lake Rauwbraken

(The Netherlands) (Lürling and Van Oosterhout, 2013). Besides transparency, the treatment also reduced intracellular microcystin (MC) concentration, however, immediately after the intervention MC concentrations were still above the guideline value for Dutch bathing waters (20 μgL^{-1}) (WHO, 2003). Nonetheless, one month after application the values were below the guideline. As microcystin, cyanobacterial chlorophyll-a concentration was also low, thus, according to WHO guideline the lake water was not posing any health risk (WHO, 2003). Hence, the lake facilities were open for the bathing season on June 1st, 2017.

Total Phosphorus was high before treatment, probably due to the high cyanobacterial biomass, which contributes to the particulate phosphorus concentration alone. Phoslock® per se would not reduce TP/chlorophyll-a in the water column (e.g., Lürling and van Oosterhout, 2012; Noyma et al., 2016), it would only reduce FRP. Thus, when a system is suffering from a bloom, to flock the cells and settle them to the bottom seems a better approach to target both TP and FRP, as already described in laboratory experiments (de Magalhães et al., 2016; Miranda et al., 2017; Noyma et al., 2017, 2016) and whole field application (Lürling and Van Oosterhout, 2013; Waajen et al., 2016a and this work). Ideally, P-sorbents alone should be applied during winter/autumn in the temperate systems, where P released from the sediment has not been taken up by cyanobacteria, thus, the P limitation would hamper cyanobacteria growth later in the summer. However, due to administrative reasons, it is not always possible (e.g., Epe et al., 2017; Nürnberg and Lazerte, 2016). Contrary to TP, the FRP concentration was low before the treatment, probably because most of the FRP available was incorporated in the cyanobacterial biomass (e.g. Spears et al., 2007).

Total Lanthanum (TLa) concentration, during the application, was above the maximum allowed concentration of 150 $\mu g\ L^{-1}$ for Dutch waters (Sneller et al., 2000). However, already one day after the treatment concentrations were below the allowed value in the whole water column (grey dots Fig 6.7). After two days, the only value upper the limit was at 7 meters depth, most likely because settled Phoslock® was concentrated in the deeper layer. TLa concentrations serve as a tracer for how fast Phoslock® settled in the water column (Fig. 6.7A). In a laboratory test, without any turbulence, 97% of the LMB added was settled after one day (van Oosterhout and Lürling, 2013). In Lake De Kuil, probably due to water movements, 58% of the LMB was settled after one day, and after one and four weeks most of the LMB added was settled (88% and 94% respectively). Similar TLa reduction over time was observed in a shallow lake where Phoslock® was also applied (Lürling and van Oosterhout, 2012),

and also on other 16 lakes, including the first application in Lake De Kuil (Spears et al., 2013b).

Filterable Lanthanum (FLa) did not exceed the Dutch maximum value allowed of 10.1µg L^{-1}, even during the application. Neither exceeded NOEC (no effect concentration) values for any aquatic organisms so far tested for FLa: *Daphnia carinata* (Barry and Meehan, 2000); *Daphnia magna* (Lürling and Tolman, 2010; Sneller et al., 2000); *Ceriodaphnia dubia* (Stauber and Binet, 2000); *Melanotaenia duboulay* (Stauber and Binet, 2000); *Caenorhabditis elegans* (Zhang et al., 2010), *Hydrocharis dubia* (Xu et al., 2012), and *Hydrilla verticillat*a (Wang et al., 2007). Important to note that not all filterable La found must be seen as bioavailable La^{3+} because of the capacity to form precipitates/complexes with humic acids or filterable colloids, as has been demonstrated by Reitzel et al. (2017). In fact, free La^{3+} might occur only in low alkalinities lake with low FRP concentration (Spears et al., 2013c). La toxicity data is still scarce and with large disparity. Its discrepancy, beside the interspecies differences, occurs due to the different condition each ecotoxicological tests are performed which might define La speciation and consequently La toxicity (e.g., different oxyanions concentration and alkalinity) (Herrmann et al., 2016; Spears et al., 2013b).

During the application pH was kept in a safe range (≥ 6.6), concerning Al solubility and common freshwater pH values (Gensemer and Playle, 1999; IAFG, 2015). In general, pH values were lower at the bottom compared to the surface after application, which might be related to the higher respiration rate and degradation of the high amount of organic matter from the settled biomass (Wetzel, 2001).

Iron (Fe) reduction under anoxia decreases the natural P-binding capacity of the sediment, thus, P release under anoxia is commonly higher than under oxic condition (Smolders et al., 2006), as was observed here (Fig 6.10). Phoslock® caused a reduction of 99% in P-release from the sediment after one month of the treatment and 86% after one year, a similar reduction has been described before (Meis et al., 2013; Waajen et al., 2016a and De Magalhães., et al 2018). The increase in sediment P-release one year after the application (1.33 mg P m^{-2} d^{-1}) compared to one month after the application (0.05 g P m^{-2} d^{-1}) may be attributable to the ongoing P diffuse source entering in the lake. The experimental cores treated with Phoslock®, were dosed for the upper 6 cm of the sediment, which was a lower dose than in the field application in which Phoslock® was dosed for the upper 10 cm. The fact that the FRP concentration, in the cores treated with Phoslock®, remained lower after 640 days of incubation even with a lower dose compared to the field application underpins that

the increase in P flux from the sediment comes from an external P load and not from the sediment, since there is enough phosphate binding capacity in the upper layer of the lake sediment as the core experiment showed (Fig. 6.9).

The SA from 2008 revealed an external input of 0.23 mg P m^{-2} d^{-1} (Waajen et al., 2016a), which by itself might not fully explain an increase of 1.28 mg P m^{-2} d^{-1} released from the sediment after 11 months of the intervention. The second Flock and Lock intervention was based on the SA done in 2008, however, in 2013, a Raspberry farm has settled about 20 meters from the lake (Fig 6.1B). The farm pumps water from the lake for irrigation purpose and it is unknown exactly how much nutrients are running off from the fields to the lake. Yet, water leakage from one of the berry pot, sampled once on September 2018, had an extremely high concentration of 6.8 mg SRP L^{-1}. Thus, the external contribution estimated in 2008 might be underestimated for 2017 as the land use has changed. This might interfere in the longevity of the treatment.

Two weeks after the treatment, part of the settled *P. rubescens* migrated towards the surface and accumulated near the shore (Fig. 6.4). The lab experiments showed that the PAC dose used in the intervention was high enough to form flocks that settled. Also, the ratio PAC : Lake water used did not interfere in the flock's formation. The Phoslock® batch used was able to adsorb FRP under oxic and anoxic condition, similar adsorption values were found in the literature (Chapter 2; Noyma et al., 2016; Ross et al., 2008). PAC dose, mode of application and the applied Phoslock® batch, therefore, all seemed fit for purpose. Also, the Phoslock® dose applied in the lake was high enough to sink the cells for a short period, however, after 24 hours the cells escaped again towards the surface (Fig. 6.12), and this also happened at higher Phoslock® doses (Fig. 6.S3).

The biomass accumulation observed in the field possibly occurred because after the improvement in water transparency (Fig 6.3), light was able to achieve deeper into the water column allowing intact *P. rubescens* sank to survive due to its capacity to grow under low light condition (Halstvedt et al., 2007), and to subsequently float back up to the surface. Similarly, after a long restoration program, Jacquet et al. (2005) observed a shift in phytoplankton community to *P. rubescens* which seems as a result of an increase in transparency. Thus, a different approach to avoid *P. rubescens* accumulation was tested. The idea was to damage the cells first with H_2O_2, so they would not be capable to photosynthesize, and later sink them to the bottom (Fig. 6.S4). Our experiment showed that this treatment kept most of the biomass at the bottom of the tubes after 24 hours.

Methods to manage cyanobacteria blooms must be efficient safe, cheap and easy to apply, thus materials that cause toxins release must be applied carefully or avoided (Merel et al., 2013). Here, the application of only hydrogen peroxide released MCs (Fig 6.14B), as previously observed in the literature (Barrington et al., 2013; Lürling et al., 2014a; Yang et al., 2018). Although less than the treatment with only peroxide, the combined treatment also released mycrocystins. Lysis of settled cells reduces the risk of recolonization, as observed in our experiment, while near the sediment the cyanotoxins can be degraded by decomposing bacteria faster than in the water column (e.g., Holst et al., 2003; Grützmacher et al., 2010; Li and Pan, 2015). Thus, the combined treatment with H_2O_2, PAC, and Phoslock® seems a promising approach to avoid biomass accumulation and recolonization, however, more upscale tests should be performed before considering it for field application.

The Flock and Lock technique was already applied in a eutrophic urban lake in The Netherlands, Lake Rauwbraken. The treatment shifted the lake from a eutrophic to a mesotrophic state, however, differently from Lake De Kuil, the *Aphanizomenon* sp. biomass was kept effective in the sediment without any biomass accumulation at the surface (Lürling and Van Oosterhout, 2013). Water authorities must be aware that a mere copy-paste of methods to manage eutrophication and cyanobacterial blooms will not always lead to the same output or to a successful restoration. Each water body is unique and a mitigation strategy should always consist of a proper updated system analysis and experimental tests on different scales (small tubes, a microcosm, and enclosures) before a field intervention can be performed (Lürling et al., 2016).

In Lake De Kuil, the Flock and Lock technique successfully increased water transparency, reduced intracellular microcystin concentration, and decreased total phosphorus concentration in the water column, as well as the P-release from the sediment. Thus, Lake the Kuil could be open on time for the bathing season. The first Flock and Lock treatment in Lake De Kuil cost €140,000 (€17,500 per year) and the second treatment cost €100,000, considering the costs a long-term monitoring program is extremely important to shed light on the longevity and efficiency of the technique.

Conclusion

- The Flock and Lock treatment increased water transparency and precipitated part of the cyanobacterial biomass in Lake De Kuil. After two weeks, biomass accumulation

was observed near the shore of the lake. The scums spontaneously disappeared after 15 days and the Lake facilities were open on July 1st, 2017 without any swimming bans in 2017.

- *P. rubescens* is capable of increasing buoyancy and migrating towards the water surface. Increase ballast or coagulant dose will not avoid the biomass accumulation. Yet, to damage the cells first by using hydrogen peroxide, and then settle the filaments seems a feasible approach to avoid biomass accumulation after the treatment, but needs to be tested in *in situ* enclosures.
- Flock and Lock treatment reduced 99% the sediment P-release after one month of intervention and 86% after one year. Due to the ongoing diffuse P input, repeated interventions might be necessary in the future.
- In-lake measures to manage eutrophication and cyanobacterial blooms, even if the effect is not perpetual, are necessary to speed up recovery and to reduce nuisance.

Acknowledgements

We thank Wendy Beekman, John Beijer and Frits Gillissen from Wageningen University for their assistance. M. Mucci PhD scholarship was funded by SWB/CNPq (201328/2014-3).

Supplementary information

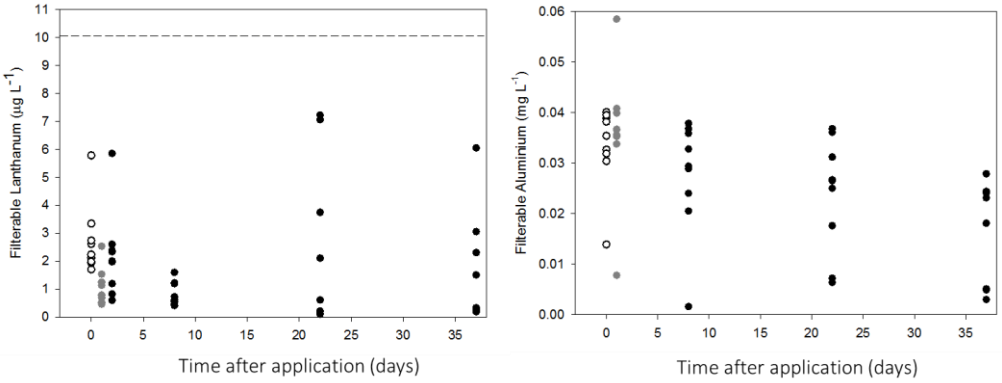

Figure 6.S1: Filterable Lanthanum (left graph) and Filterable Aluminium (right graph) concentrations. Dots represent metals concentration for each water depth during the application (empty dots) and one (grey dots), two, eight, twenty-two and thirty-five days (black

dots) after the treatment. Dash line at lanthanum graph represents allowed concentration for Dutch waters (<5µg L^{-1})

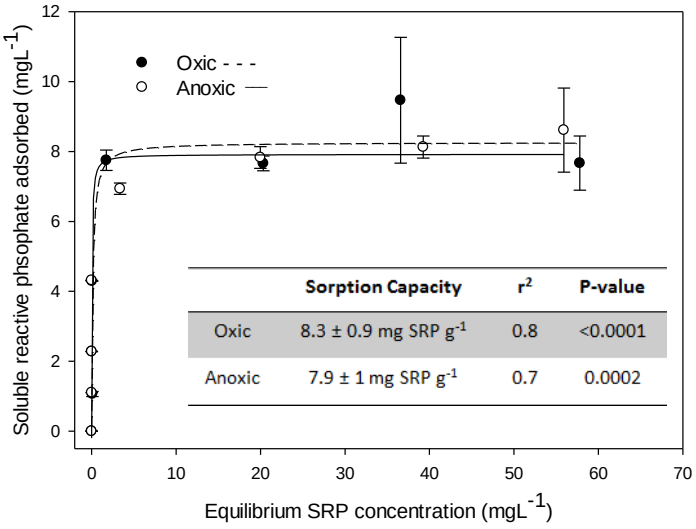

Figure 6.S2: Langmuir adsorption isotherm of Phoslock® under oxic and anoxic condition. Table inside the figure shows maximum soluble reactive phosphorus adsorption capacity, the r square of the model and P-value of both regressions. ± represents SE (*n*=3).

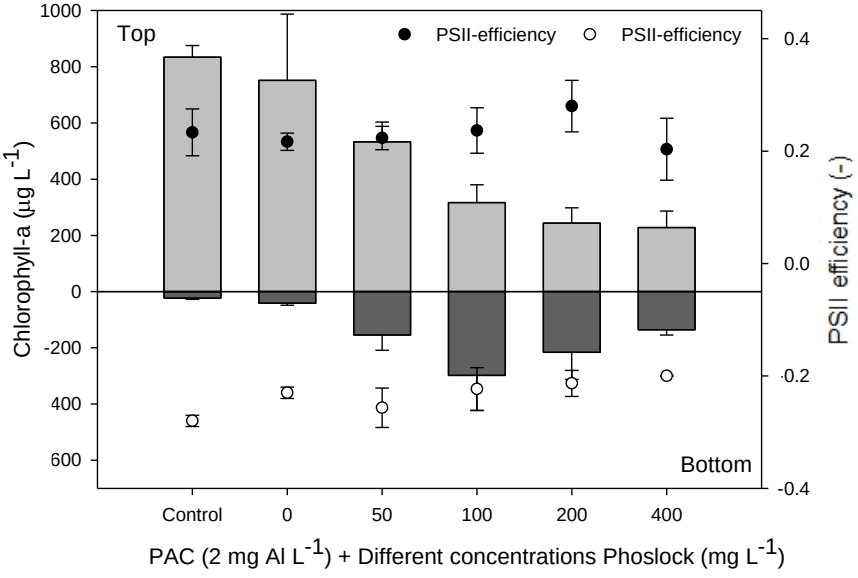

Figure 6.S3: Chlorophyll-a concentrations (µg L^{-1}) in the top 2 mL (top light grey bars) and bottom 2 mL (lower dark grey bars) of 100 mL cyanobacteria suspension from Lake De Kuil incubated for 24 hours in the absence or presence of different concentrations ballast (Phoslock 50, 100, 200, and 400 mg L^{-1}) combined with the flocculant PAC (2 mg Al L^{-1}). Also

included are the PSII efficiencies of the cyanobacteria collected at the water surface (filled circles) and at the bottom (open circles). Error bars indicate SD (n = 3).

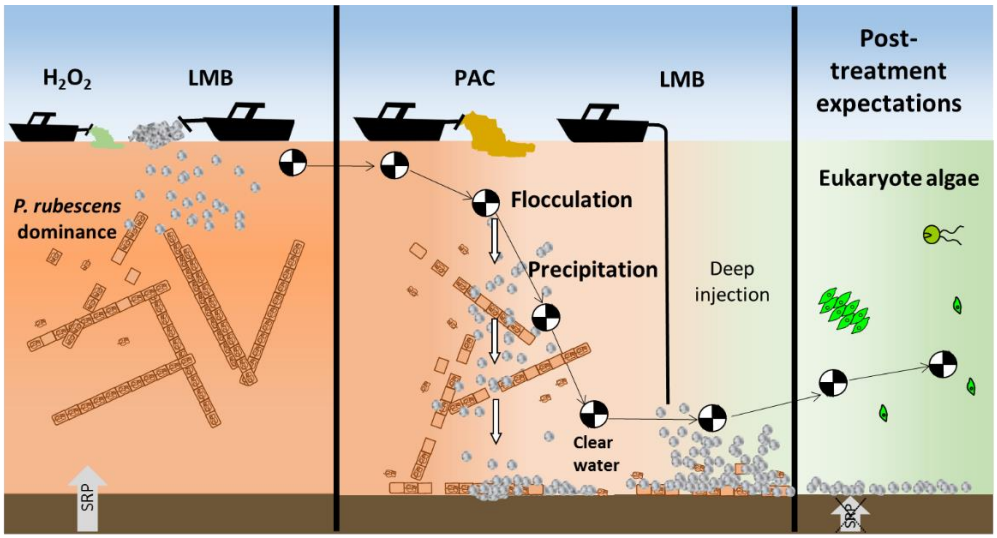

Figure 6.S4: Scheme showing the principle of the combined treatment: first hydrogen peroxide is added, to damage the cells, followed by the ballast (LMB) and a low dose of flocculant (PAC) to flock and settle the damaged cells. Finally, LMB is injected in the deep layer to reduce SRP release from the sediment.

7

General reflection and synthesis

Geo-engineering in lake restoration

Eutrophication and harmful cyanobacterial blooms are increasing worldwide and despite being studied for almost a century (Naumann, 1919), mitigation remains a huge challenge. In this context, geo-engineering and in-situ measures have gained attention as a quick fix to the problem. The aim of this thesis was to study potential geo-engineering materials and in-site techniques to manage the problem through experimental approach and whole-ecosystem intervention. Here, I will reflect and connect the main findings of this thesis highlighting that a thorough system analysis should be followed up by experimental tests before an in-situ intervention can be performed.

Geo-engineering has been considered as an attractive approach, mainly due to the pressure to rapidly improve water quality. For example, the European Union Water Framework Directive dictates that water bodies should have a good ecological and water quality status at 2027. These pressures, also occur in countries in transition. For instance, in Brazil, the municipality of Belo Horizonte had to turn a hypertrophic lagoon (the Pampulha Modern Ensemble) which was constantly being fed by external nutrients source to a "good" water quality status to get it included in the World Heritage List by UNESCO (whc.unesco.org/list/1493/). Also, when a population`s only drinking water source is suffering from a cyanobacterial blooms a quick solution is appealing and highly needed. As a relatively new topic, geo-engineering in lake restoration has risen many questions concerning safety, longevity, and efficacy. A straight answer to whether geo-engineering meets these demands is rather difficult. It relies on which material is being used and under which conditions. One of the reasons behind the challenge in mitigating the problem successfully is to understand and accept that there is no single magical solution. Each system is unique and the cyanobacterial bloom may have different causes in different water bodies. Also, the available techniques such as P-inactivation, biomanipulation, dredging, algaecides, and "Flock and Lock", have their limitations in improving water sources. Thus, a broad-scale generalization will not always lead to successful restoration; an ideal mitigation plan should always include a proper system analysis followed by experimental tests on various scales before a field application can be performed.

Geo-engineering, thus, is one of the several tools available to manage the problem, and should not be seen as a silver bullet to quickly solve water quality issues (Mackay et al., 2014) neither is it an excuse to not reduce nutrient pollution. Control of the external nutrient load is crucial, and tackling point sources such as treating

collected waste water is the most logic first step. Nonetheless, even in countries like the Netherlands where point sources have been dealt with adequately, cyanobacterial blooms occur frequently. This has everything to do with legacies stored in the lake bed and with ongoing diffuse loading, which makes in certain case in-lake interventions needed to speed-up recovery and minimize the nuisance. Particularly in cases where the only source of drinking water is suffering from a bloom (Fig. 7.1) a fast and safe solution is needed, even if it is not long-lasting and needs repeating.

This thesis explored some geo-engineering materials looking specifically into solid phase P sorbents (chapter 2 and 3), a flocculant (chapter 4 and 5), and a combination of those (chapter 6).

Figure 7.1: 4.1 million people affected in Bahia state (Brazil) without potable water in 2017. (A) shows the only water source a family had for six months; (B) they filtered the water with cloths and boiled before consumption. (C) shows the difference in color between the water they were drinking and the potable one. The family reported constant diarrhea and afterwards the woman discovered a small nodule in her liver. Although they do not know if the symptoms were caused by the water, it can be a case of cyanotoxin ingestion. (Source: http:// g1.globo.com/bahia/noticia/2017/04/ pior-seca-em-73-anos-traz-fome-e-faz- populacao-dividir-agua-com-animais.html)

Chemical P inactivation through solid-phase P sorbents

The demand for materials, which are easy to apply, safe, cheap, and effective in turning bioavailable phosphorus into insoluble forms has been increasing due to environmental policies concerning improvement in water quality all over the world. Thus, the first step was to evaluate ten natural and modified materials on their capacity to chemically inactivate P. We observed that environmental conditions (such as pH and anoxia) might affect the efficiency of the tested materials. Also, the natural materials (e.g. locas Fe-rich soils) did not perform as good as modified materials (e.g. Al modified zeolite and La-modified bentonite) (chapter 2). Nonetheless, considering

the price difference and the importance to look for and tests affordable solutions, natural materials should still be considered for tests/applications. For instance, locals soils can be available close to a water bodies (e.g. Noyma et al 2016; Pan et al., 2006a) in contrast to the commercially available LMB, (Phoslock®), which costs between 2000 - 2500 euros per ton (Traill 2019, personal communication).

In chapter 2, we found that Phoslock® was the best performing material, thus, we continued to study it. Phoslock® has been applied successfully in around 200 freshwater systems (Copetti et al., 2016; Chapter 6) and its efficiency has been tested in a broad range of pH, humic acids concentrations and oxygen conditions (chapter 2; Dithmer et al., 2016a; Lürling et al., 2014; Reitzel et al., 2017; Ross et al., 2008). However little is known about its performance in saline environments. The requirement to mitigate eutrophication in coastal areas prompted us to evaluate Phoslock® efficiency and behaviour in saline waters in chapter 3 . We showed that Phoslock® was effective in removing phosphate in all salinities tested from brackish to seawater, whilst filterable lanthanum (La) concentrations remained very low. La was disassociated from the clay matrix, however, free La^{3+} released in synthetic seawater was removed by complexation with carbonate and/or phosphate. We concluded that the use of Phoslock® in saline environments could be considered, yet, that ecotoxicological studies must be performed before field applications in estuarine and marine environments can take place.

In chapter 2 and 3, we argued that materials should be tested under a realistic conditions not only looking at efficiency but also on the possibility to be applied in a large scale. For instance, if we had used solo NaCl instead of sea salt to imitate seawater in chapter 3, a mistaken conclusion would be drawn concerning Free La^{3+} concentration and thus potential toxicity. Similarly, in chapter 2, we selected a realistic range of pH (6 to 9) found in the environment (Wetzel, 2001). In contrast, certain studies, selected materials based on performances at a pH of 5 or under a temperature of 65°C (Peleka and Deliyanni, 2009); conditions that are unlikely to be found in a eutrophic water body. Hence, tests under unrealistic conditions provide no predictive value for performance under field applications. Along the same lines, some synthetic materials (e.g. layered double hydroxides) have shown a 5 to 10 times better performance than LMB, however, these materials are complex and expensive to produce on a large scale, which hampers their use in a field application (Jiang and Ashekuzaman, 2015). Thus, it is essential to reflect not only on the efficiency of modified materials in adsorbing P but also on their price and availability, a point that is often overlooked in literature.

Here, Phoslock® was deeper studied due its good performance under different conditions, and its availability to be used at larger scales. However, there are also other materials, mainly Al, Ca, Fe based compounds, that are able to adsorb P (chapter 2; Berg et al., 2004; Funes et al., 2016; Gibbs and Özkundakci, 2010). A proper system analysis (chapter 1) will guide to the most promising material, in addition, controlled experiments and chemical equilibrium modeling under realistic conditions, such as done in chapters 2 and 3, will enlighten the implications and effectiveness of the proposed intervention/material before in-situ applications can be executed. Besides the existing materials and the improve of current materials, there is also a need to develop new ones. For instance, materials that are capable of simultaneously adsorbing ammonium or metals. Certainly, to succeed in such tasks, a multidisciplinary and international collaboration between research groups is essential.

The impact of the biodegradable flocculant, chitosan, on cyanobacteria

When an aquatic system is already suffering from cyanobacterial blooms, the application of solid phase P sorbent only, will not bring a quick improvement in water transparency. To overcome this problem, an addition of a metal-based coagulant together with a solid-phase P sorbent/ballast has been used to target phosphate in the water column, the P flux from the sediment, and the ongoing bloom, a technique called Flock and Lock (e.g. chapter 6; Lürling and Van Oosterhout, 2013; Waajen et al., 2016a). In this context, chitosan has been proposed as an "eco-friendly" flocculant as an alternative to inorganic flocculants, such as polyaluminium chloride (PAC) and iron chloride (Renault et al., 2009). In addition, a chitosan modified soil has been tested also to flock and sink the cyanobacterial biomass as an alternative to algaecides, which can cause toxins release from the cells, while chitosan-aided flocking and sinking is thought not to have these unwanted side effects (Pan et al., 2012). This assumptions finds, however, no support in literature and given the well-known antibacterial properties of chitosan (Kong et al., 2010; Sudarshan et al., 1992), broader studies were required to test chitosan proposed eco-friendliness.

Hence, in chapter 4, the effect of chitosan on several cyanobacterial species was tested and showed that chitosan may cause rapid cell lysis and leakage of cell content. In chapter 4, we also learned that the effect of chitosan might be strain dependent and one species in specific, a common bloom-forming cyanobacteria (*Microcystis aeruginosa*), seems to be rather resistant, which was also observed by

Miranda et al., 2017. Thus, in chapter 5, we looked more closely at strain variation using *M. aeruginosa*, as a model species, whilst also measuring cyanotoxin release. We showed that chitosan was able to damage *M. aeruginosa* cells causing cell lysis and, consequently, cyanotoxins release. These effects were, however, strain dependent. Hence, chapters 4 and 5 revealed that chitosan, contrary to what has been claimed, might have unwanted side-effect and should not be used as a coagulant without testing its effects on the cyanobacterial assemblage being targeted to avoid unwanted rapid release of cyanotoxins. Such tests should not only include coagulation efficiency but also cell viability. Our studies also pave the road for further in-depth studies to chitosan formulations that may have a delayed cell lysing effect. Cell lysis of settled cells might reduce the risk of recolonization, which can be viewed as beneficial especially in shallow water bodies or in cases such as described in chapter 6 that have a risk of resuspension and recolonization of settled cyanobacterial cells (Preston et al., 1980).

Besides its negative effect in certain cyanobacteria species/strains, our group also showed that chitosan flocculation efficiency is hampered by high pH values and also high alkalinity (Lürling et al., 2017). Thus, depending on the system`s conditions, the use of chitosan, may be a disadvantage compared to metal-based coagulants. In addition to being effective, as earlier mentioned, materials should also be affordable. Chitosan can be 80 to 120 times more expensive than PAC, which might also be a drawback (Lürling et al., 2017). A holistic understanding of the aquatic system will guide to the most promising flocculant and as previously highlighted, controlled experiments are essential to understand the implication and efficiency of materials (flocculant or solid-phase P sorbent) used to mitigate eutrophication and cyanobacterial blooms.

Whole-ecosystem application

In chapter 6, we monitored a small swimming lake in the Netherlands that was treated with a low dose of flocculant (PAC) together with a ballast/solid-phase P sorbent (LMB, Phoslock®) to target the phosphate in the water column, P-release from the sediment, and the ongoing cyanobacterial bloom. This approach (Flock and Lock) was chosen because the lake was already suffering from a cyanobacterial bloom, and as mentioned previously, solid-phase P sorbent application only, as Phoslock®, will reduce only phosphate in the water column and the internal loading, but it will not target the biomass. Ideally, for an easier and cheaper application, solo

Phoslock® application should be done before the cyanobacteria build up biomass. The enforced system P limitation would then avoid development of a cyanobacterial bloom without the need to add any flocculent. However, there is a wide gap between theory and practice, in the case of Lake De Kuil a typical winter bloom-forming organism was present, *Planktothrix rubescens*, and due to bureaucracies or economical reasons, it is not always possible to have all permits in time for a solo solid-phase P sorbent application (e.g. Epe et al., 2017; Nürnberg and Lazerte, 2016; chapter 6). In addition, certain tropical systems rather commonly have cyanobacterial blooms all year round or for long periods (e.g., Magalhães et al., 2018; Miranda et al., 2017). In this context, the addition of a low dose of flocculant together with a solid-phase P sorbent, the Flock and Lock technique, is a powerful approach (e.g., Chapter 6; Lürling and Van Oosterhout, 2013; Waajen et al., 2016a).

The proposed Flock and Lock treatment was tested first in sediment cores collected from the lake, in which revealed to be efficient in reducing phosphate release from the sediment. In the lake, The Flock and Lock treatment was successful in reducing total phosphorus, turbidity, chlorophyll-a and increasing water transparency in Lake De Kuil. Phoslock® also effectively reduced P release from the sediment, as was previously observed in the core experiment. After two weeks of treatment, a biomass accumulation of the cyanobacteria *Planktothrix rubescens* was observed near the shore of the lake, which spontaneously disappeared after two weeks. From experimental tests, using the cyanobacteria accumulation found in the lake, we learned that a higher dose of PAC and LMB would not have prevented this reoccurrence. Thus, a new approach was tested using hydrogen peroxide to first damage the cells and later flock and sink them using PAC and Phoslock®. This approach was successful in keeping the biomass on the bottom of the test tubes. Surely, tests in a more complex system, such as enclosure, have to be done to better understand the technique`s limitations and efficiency. This combined approach (hydrogen peroxide or another algaecide with a flocculant and a ballast) might be beneficial in shallow lakes, where there is a risk of resuspension of flocks and cyanobacterial recolonization of the water column (Fig. 7.1) (Preston et al., 1980).

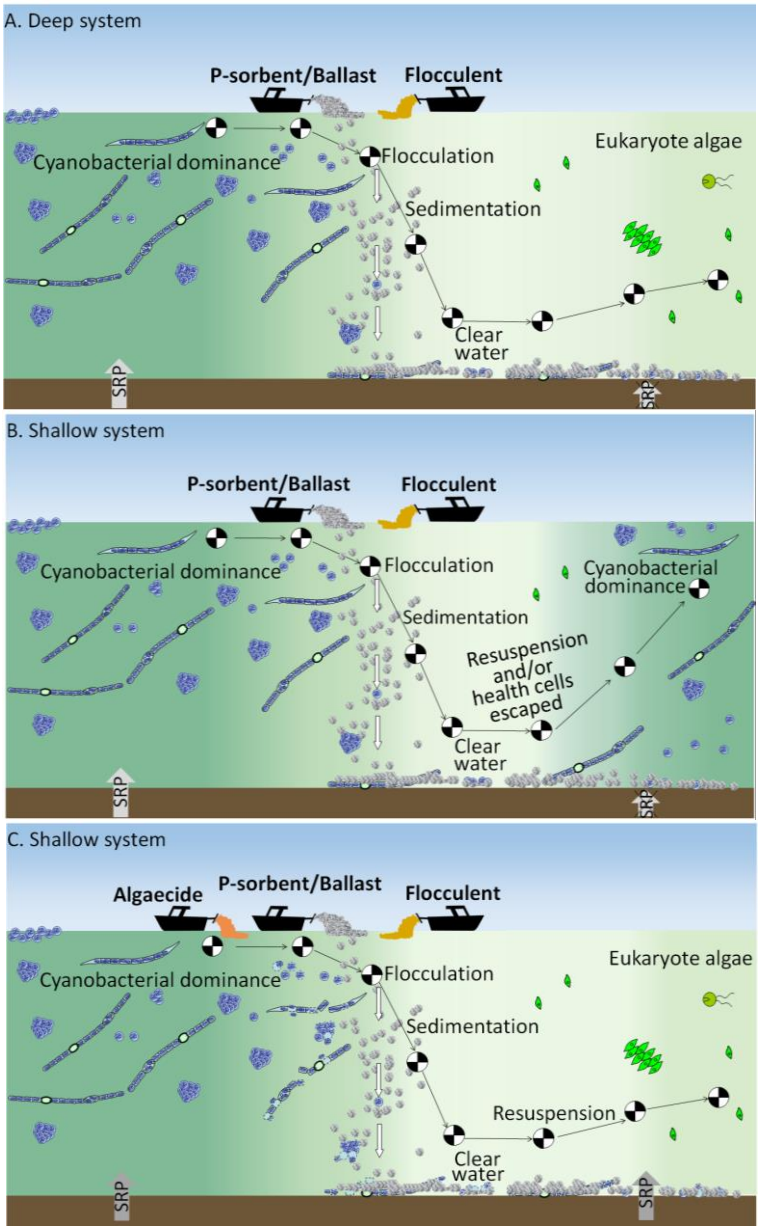

Figure 7.1: Possible geo-engineering approaches for different types of aquatic system. Panel A shows, from left to right, a deep system with a cyanobacterial bloom being treated with a P sorbent/ballast and a flocculant. The cells are flocculated and sink to the bottom, and after the treatment clear water with eukaryote algae is expected, as is a reduction in the P-release from the sediment. Panel B shows the effect of similar treatment on a shallow system. In this type of system settled cyanobacteria tend to ressuspended, so after the treatment only a short period of clear water is expected. Panel C shows a promising approach to avoid cyanobacterial reinoculation in shallow systems by adding algaecides (e.g. H_2O_2) to first damage the cells and later a P-sorbent/ballast and a flocculant to flock and sink the cells. In doing so, cyanobacteria recolonization is unlikely to occur. Secchi disks represent water transparency.

Positively, cell lysis of settled cells will reduce the risk of recolonization, while close to the sediment the cyanotoxin released can be faster decomposed (e.g. Grützmacher et al., 2010; Holst et al., 2003; Li and Pan, 2015). According to **chapters 3 and 4**, chitosan might be a candidate able to damage the cells and to flock at the same time, however, there is no information available on the effect of chitosan on a natural population of *P. rubescens*, which makes it certainly an interesting topic to explore further.

In Lake De Kuil the estimated external P load in 2008 was 5% compared to the internal loading, thus a P-sorbent (i.e. Phoslock®) was used to target P-release from the sediment. However, in certain systems, where the external P load is high and its control is not feasible, the use of modified P-sorbent materials might be too expensive since re-application will be needed more often. In those cases, the use of a natural ballast with lower P-sorption capacity than modified materials (as studied in **chapter 2**) together with a flocculant to flock and sink the cells seems a more practical approach (Fig. 7.2). As always, a proper system analysis followed by experiments must guide to the best approach for each system.

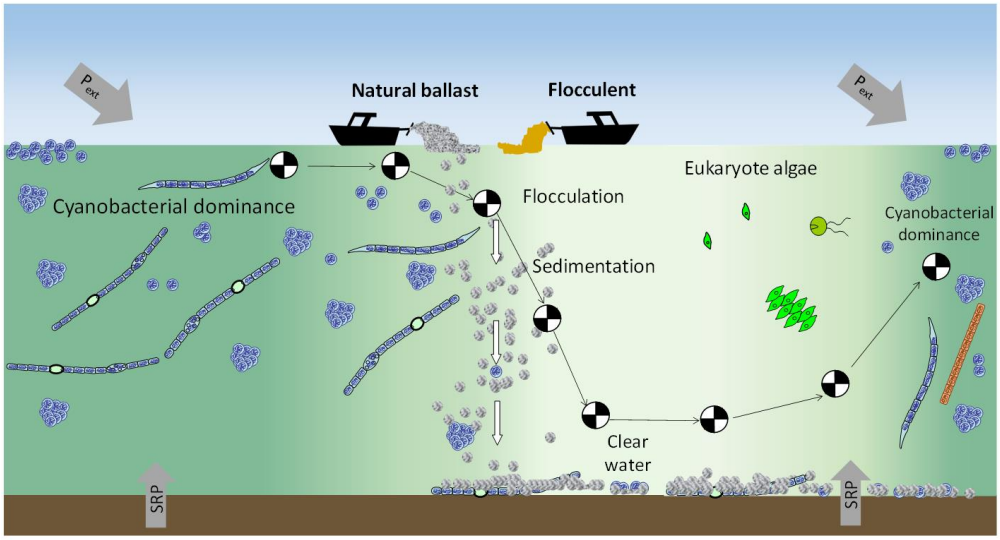

Figure 7.2: Scheme showing, from left to right, a system constantly receiving P from an external source (P_{ext}) and with the cyanobacterial bloom being treated with a natural ballast and a flocculant. The cells, are flocculated and sink to the bottom, and after the treatment, clear water with eukaryote algae is expected. However, after some time, due to the constant source of P from external source and from the sediment (SRP), reoccurrence of cyanobacteria is expected. Thus re-application might be needed from time to time.

It was the second time that an intervention was done in Lake De Kuil to manage eutrophication, and it is likely that another intervention will be needed in the future due to the ongoing diffuse P input, which is low compared to the internal loading, but, over time will lead to severe internal loading (Fig. 6.10). The impact of diffuse sources of nutrients in water bodies is challenging to control and tends to increase even more due to the rapid urbanization and increasing food production (FAO, 2017). Land management procedures, known as best management practices (BMPs), such as constructed wetlands/ponds, are regarded as a potential way to diminish the pollution, however such approach needs space which in urban regions is a limiting factor (Cooke, 2005; Coveney et al., 2002; Yin and Shan, 2001). Moreover, effectivity of BMPs has been challenged (Osgood, 2017). Thus, if the diffuse nutrient pollution is continuous, as in Lake De Kuil, managing eutrophication will also be a continuous process. The period of time between one intervention and another will depend on how much of P is flowing in to the system. In Lake De Kuil, after 8 years a second treatment was needed. On the contrary, in Lake Rauwbraken after more than 10 years of treatment the lake water is still in a good condition (van Oosterhout 2018, personal communication). Lake Rauwbraken was treated using the same approach (Flock and Lock) as Lake De Kuil, both lakes resulted in water quality improvement, however, no biomass accumulation was observed in lake Rauwbraken after the treatment (Lürling and Van Oosterhout, 2013). This exemplifies that each lake is unique and that a copy paste of methods will not always lead to the same result. Water authorities must be aware that there is no panacea to manage eutrophication and cyanobacterial blooms. Also they need to be critical in implementing anything without a proper and updated SA, since there are, many self-claimed curative measures on the market promising “magic solutions,” yet without scientific underpinning and unclear mechanisms. In Lake De Kuil, the intervention was based on the SA made for the first treatment in 2008, yet, some aspects in the lakes surroundings have been changed. For instance, the raspberry farming next to the lake is operating since a few years and such activities cannot a priori be viewed as having no impact.

Experimental tests are important to explore materials limitations, however, only with a whole-ecosystem intervention and a strong monitoring program before, during and after a treatment it is possible to entirely understand the longevity and efficacy of techniques (Schindler, 2012). Thus, it is essential to continue monitoring a restored system and reporting the results. Typically, often only the successful stories are shared. A failed restoration plan should be seen as much as important as a

successful one, because we could certainly learn from it and step forward to solving water quality issues.

Outlook

There is no single solution to manage eutrophication and cyanobacterial blooms. In accepting this statement, we are in a "learn as you go" process (Fig. 7.3). The first step proposed is to understand the aquatic system through a system analysis (Cooke, 2005; Lürling et al., 2016), which will guide the selection of the most promising tool(s) available, which can also be a "do nothing" approach. Once the tool(s) is (are) selected, the next phase is to test it (them), ideally, at different scales from laboratory experiments to enclosures. To be considered as a potential useful tool ideally needs to meet four criteria: it should be (1) effective, (2) easy to be manufactured and applied, (3) affordable and (4) safe. If the tools meet these requirements, an intervention would be the next step. Here, monitoring programs are extremely important not only to shed light on the efficacy and longevity of the intervention but also to learn more about the limitations of chosen technique(s). Over time, if nutrient pollution was not entirely stopped the problem might return and an update of the system analysis is needed following the same stages as in the previous intervention. System analysis and experimental tests before a field application are crucial for a successful restoration story.

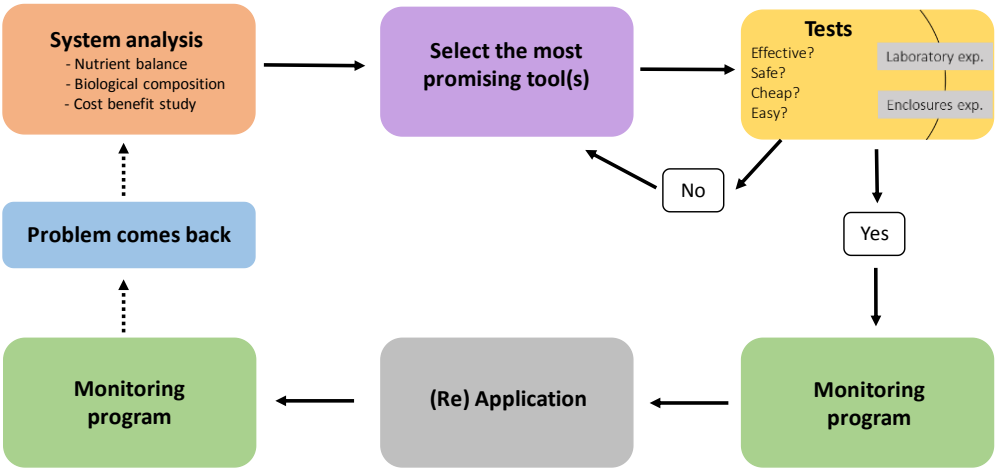

Figure 7.3: Simplified overview of proposed steps to manage eutrophication and cyanobacterial blooms

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Summary

Eutrophication and cyanobacterial blooms are increasing worldwide. Despite being studied for almost a century, mitigating eutrophication remains a challenge. Motivated by this challenge, we studied potential geo-engineering materials and in-site techniques to manage the eutrophication and cyanobacterial blooms in controlled experiments and a whole-ecosystem intervention.

As phosphorus (P) control is essential to manage eutrophication, this thesis started evaluating natural and modified clays and soils for their capacity to adsorb P (chapter 2). We showed that four out of ten materials were able to adsorb P, and that P adsorption differed under varying abiotic conditions. The modified materials (lanthanum (La) modified bentonite, commercially called Phoslock® and Aluminium modified zeolite, commercially called Aqual-P®) were able to adsorb more P than the naturals ones such as Fe-rich soils

The need to mitigate eutrophication in coastal areas prompted us to evaluate Phoslock® efficiency and behaviour in saline waters in chapter 3. Phoslock® was able to adsorb P in all salinities tested from brackish to seawater, whilst filterable La concentrations remained very low. We concluded that the use of Phoslock® on saline waters should be considered, yet, ecotoxicological studies must be performed before field applications in saline environments.

Beside solid-phase P sorbents, flocculants have also been used in lake restoration. In this context, chitosan has been proposed as an "eco-friendly" flocculant as an alternative to metal based flocculant, such as polyaluminium chloride (PAC). In chapter 4, we tested the effect of chitosan on several cyanobacterial species and showed that chitosan may cause rapid cell lysis. In chapter 5, we looked closer into strain variation whilst also measuring cyanotoxin release. We showed that chitosan was able to cause cyanotoxins release. These effects were, however, strain dependent. Chitosan application might therefore cause toxin release in the water column, and it should not therefore be used without testing its effects on the cyanobacterial assemblage being targeted to avoid unwanted rapid release of cyanotoxins.

In chapter 6, we showed field results from a whole-lake treatment with PAC and Phoslock®. This technique called Flock and Lock aimed to target P from the water column, P-release from the sediment and the ongoing cyanobacterial bloom. The intervention was successful in improving water quality in Lake De Kuil. After two

weeks of the treatment, however a surface scum was observed near the shore of the lake, which disappeared spontaneously after two weeks. The lake was open in time for the bathing season without any swimming bans during 2017. Tests to why the scums occurred, and how to avoid their occurrence showed that promising approach to avoid biomass accumulation is to damage the cell first using hydrogen peroxide and later settle them with the Flock and Lock technique. Larger scales tests need still to be performed to shed light on possible limitations of this technique.

In chapter 7 I reflected that there is no single magical solution to manage eutrophication and cyanobacterial blooms. Each system is unique and each material/technique (P immobilization, chitosan, Flock and Lock, peroxide) has its limitations. Thus, a broad-scale generalization (copy-paste of methods) will in most cases not lead to a successful restoration. A mitigation plan must always include a proper system analysis and experimental tests under realistic condition on various scales before a field application can be performed.

Resumo

Essa tese de doutorado foca em modos de mitigar/controlar dois dos maiores problemas de água no mundo - a eutrofização e as florações de cianobactérias. A eutrofização ocorre quando o corpo d`água recebe nutrientes em excesso, principalmente fósforo e nitrogênio. Esses nutrientes são oriundos de atividades humanas. Por exemplo quando um excesso de fertilizantes é usado na agricultura, eles são lixiviados para os sistemas aquáticos. A agropecuária e os dejetos dosméticos e industriais (esgoto) diretamente despejados nos sistemas também contribuem com o problema. A quantidade excessiva dos nutrientes fazem com que as microalgas (fitoplâncton) se multipliquem rapidamente transformando o sistema áquatico que estava, anteriormente, em um estado claro para um estado túrbido e nas maiorias das vezes verde. Pode ocorrer também uma mortandade de peixes devido a falta de oxigênio. Além disso, na maioria das vezes as microalgas que crescem excessivamente são cianobactérias, microorganismos capazes de produzir toxinas que podem causar danos e até a morte de animais domésticos e humanos. Portanto, a eutrofização e as florações das cianobactérias diminuem drásticamente a qualidade da água impedindo o uso dos sistema aquáticos como fonte de água potável, também para irrigação, pesca, e para uso recreático em geral.

A forma mais direta de combater a eutrofização é reduzir os nutrientes que entram nos corpos d`água. Entretanto, muitas vezes reduzir a carga externa não é suficiente, pois o sedimento dos lagos acumulam nutrientes ao longo do tempo, contribuindo, assim, para a continuação do problema mesmo quando a carga externa é reduzida. Além disso, diminuir a carga externa de nutrientes, muitas vezes, não é possivel. Por exemplo imagine quanto tempo vai levar até que todas as cidades do Brasil tenham coleta e tratamento de esgoto adequado. Isso não significa, entretanto, que a população tenha que esperar até que todas as infraestruturas sejam implementadas para que qualidade da água melhore. Intervencões nos ecossistemas aquáticos podem ser feitas para imediatamente mitigar e controlar eutrofização e cianobactérias, melhorando assim, a qualidade da água.

Apesar de ter sido estudada por quase um século, a mitigação da eutrofização continua sendo um desafio. Motivada por este desafio, nessa tese foram estudadas possíveis técnicas e materiais para controlar a eutrofização e as florações de cianobactérias por meio de experimentos laboratoriais e uma aplicação real em um

lago. Nesse contexto de restauração, técnicas de geo-engenharia são uma boa ferramentas para o combate contra a eutrofização. A geo-engenharia é definida como uma técnica que altera o ciclo dos nutrientes, principalmente de fósforo, pois é o elemento mais fácil e barato, até o momento, de ser controlado/reduzido. Uma vez que o fósforo é quimicamente inativado, as algas não conseguem usá-lo e portanto não conseguem se multiplicar exageradamente. Portanto, esta tese começa avaliando argilas e solos naturais e modificados em sua capacidade de adsorver fósforo (P) – químicamente inativar o P (capítulo 2). Mostramos que quatro entre dez materiais foram capazes de adsorver P e que a adsorção de P diferiu sob diferentes condições abióticas. Os materiais modificados (bentonita modificada com lantânio (La), comercialmente denominada Phoslock® e zeólita modificada com alumínio, comercialmente denominada Aqual-P®) foram capazes de absorver mais fósforo do que os materiais naturais, como os solos ricos em ferro.

A necessidade de mitigar a eutrofização nas áreas costeiras nos levou a avaliar a eficiência e o comportamento do Phoslock® em águas salinas no capítulo 3. O Phoslock® foi capaz de adsorver fósforo em todas as salinidades testadas de água salobra à marinha, enquanto as concentrações de lantânio filtráveis permaneceram muito baixas. Conclui-se que o uso de Phoslock® em águas salinas pode ser considerado. No entanto, estudos ecotoxicológicos devem ser realizados antes da aplicações nesses ambientes.

Além dos adsorventes de fase sólida, os floculantes também têm sido usados na restauração de lagos. Neste contexto, a quitosana tem sido proposta como um floculante "ecologicamente amigável", uma alternativa a floculantes à base de metais, como o cloreto de polialumínio (PAC). No capítulo 4, testamos o efeito da quitosana em várias espécies de cianobactérias e mostramos que a quitosana pode causar uma rápida lise celular. No capítulo 5, analisamos mais de perto a variação da resposta de diferentes cepas de cianobactérias e medimos a liberação de cianotoxina. Nós mostramos que a quitosana foi capaz de causar liberação de cianotoxinas. Estes efeitos foram, no entanto, cepas dependentes. A aplicação de quitosana pode, portanto, causar a liberação de toxinas na coluna d'água e não deve, ser usada sem antes testar seus efeitos sobre a população natural de cianobactérias para evitar uma indesejada liberação rápida de cianotoxinas.

No capítulo 6, mostramos os resultados de um tratamento feito em um lago holândes (De Kuil) com PAC e Phoslock®. Essa técnica chamada Flock and Lock visa adsorver o P disponível na coluna de água, diminuir a liberação de P do sedimento e também a biomassa de algas presente na coluna d`água. A intervenção foi bem sucedida e houve uma melhoria significativa da qualidade da água no Lago De Kuil. Após duas semanas do tratamento, no entanto, uma acumulação de cianobactérias foi observada perto da margem do lago, que desapareceu espontaneamente após duas semanas, e portanto o lago foi aberto a tempo para a temporada de banho sem quaisquer proibições de natação durante 2017 e 2018. Testes para compreender melhor a acumulação de cianobactérias e como evitar sua ocorrência mostraram que uma abordagem promissora é danificar primeiro a célula usando peróxido de hidrogênio e depois adicionar PAC e Phoslock® (Flock and Lock). Testes em escalas maiores, como em mesocosmos, ainda precisam ser realizados para esclarecer possíveis limitações dessa técnica.

No capítulo 7, foi refletido que não existe uma solução mágica ou única para controlar a eutrofização e a proliferação de cianobactérias. Cada sistema aquático é singular e cada material ou técnica (por ex. imobilização de P, quitosana, Flock e Lock, peróxido) tem suas limitações. Assim, uma generalização em larga escala (“copia e cola” de métodos) na maioria dos casos não levará a uma restauração bem-sucedida. Um plano de restauração deve sempre incluir uma análise adequada do sistema e testes experimentais sob condições realistícas em várias escalas, antes que uma real aplicação ecossistêmica possa ser executada.

Acknowledgements

Here we go to the section that everyone reads. Please, also take a look on the content of the thesis. There are colourful graphs on page 129 and shinning cyanobacteria on page 86. :)

Science is team work and this thesis was not different. Although I am the only one getting a diploma, there are many, many, wonderful people behind it. Here, I would like to express my deep gratitude to all who have contributed in the thesis and participated in my life during this journey.

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About the author

Maíra Nunes Teixeira Mucci was born on March 16th 1990 in a region full of mountains and waterfalls (Ponte Nova, Minas Gerais, Brazil). In 2008, she started the BSc in Biology at Federal University of Juiz de Fora. Aquatic ecology appeared earlier on her path and, in the first year of university, she started as an intern at LEA (Laboratory of Aquatic Ecology) assisting in different projects from monitoring programs to allopathic interactions between algae. In 2012, she graduated as a bachelor, and in 2014 she got the teaching diploma in Biology. During her studies, she got a personal grant to study for half year at Colorado College – Colorado Spring, USA (2012) and one year at Wageningen University and Research (WUR), the Netherlands (2013). After graduating, the idea of a PhD abroad started and in 2015 she got a personal grant to do the full PhD in Wageningen. Since them she has been focusing on ways to turn green waters to a clear state. Although physically away from Brazil, during her PhD she collaborated with researchers from Rio de Janeiro State University (UERJ) and Federal University of Rio de Janeiro (UFRJ), partnership that she hopes continue lasting long. After the PhD, more questions than answers were raised and she is still inspired to continue working on the field of aquatic ecology and water quality issues.

List of publications

Noyma, N.P., de Magalhães, L., Furtado, L.L., **Mucci, M.,** van Oosterhout, F., Huszar, V.L.M., Marinho, M.M., Lürling, M., 2016. Controlling cyanobacterial blooms through effective flocculation and sedimentation with combined use of flocculants and phosphorus adsorbing natural soil and modified clay. Water Res. 1–13. doi:10.1016/j.watres.2015.11.057

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D I P L O M A

For specialised PhD training

The Netherlands Research School for the
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Maíra Nunes Teixeira Mucci

born on 16 March in Ponte Nova, Brazil

has successfully fulfilled all requirements of the
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Wageningen, 18 April 2019

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Selection of SENSE PhD Courses

- Environmental research in context (2015)
- Aquatic Ecology - Robustness of aquatic ecosystems in the face of global change (2018)
- Research in context activity: 'Actively communicating on research subject in the form of accessible video and in the form of contributions to Wikipedia page on 'Geo-engineering in Lakes'(2018)

Selection of Other PhD and Advanced MSc Courses

- Project and Time management, Wageningen Graduate School (2015)
- Scientific Publishing, Wageningen Graduate School (2015)
- The essential of writing and presenting, Wageningen Graduate School (2016)
- Reviewing scientific paper, Wageningen Graduate School (2016)
- Teaching and supervising thesis Students, Wageningen Graduate School (2016)
- Scientific Writing, Wageningen Graduate School (2017)
- Alum for phosphorus control in lakes and ponds, North America Lake management Society, USA (2018)

External training at a foreign research institute

- Experiments on the effects of soil additions to ammonium and phosphate fluxes in lagoon sediment, Rio de Janeiro State University, Brazil (2016-2017)

Management and Didactic Skills Training

- Supervising four MSc, and four BSc students with thesis (2016-2018)
- Assisting practicals of the MSc courses 'Practical Aquatic Ecology and Water Quality' (2015-2018) and "Advanced Limnology, theory & practice- Lake Lesser Prespa" (2016)

Selection of Oral Presentations

- *Effect of salinity on adsorption of phosphate and ammonium by natural and modified zeolite.* 36th International Symposium of the North American Lake Management Society, 1-4 November 2016, Banff, Canada
- *Chitosan as a coagulant on Cyanobacteria may cause rapid cell lysis.* 10th Symposium for European Freshwater Science, 2-7 July 2017, Olomouc, Czech Republic.
- *In-lake treatment with PAC and Lanthanum Modified Bentonite to manage eutrophication.* 34th Congress of the International Society of Limnology, 19-25 August 2018, Nanjing, China

SENSE Coordinator PhD Education

Dr. Peter Vermeulen

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