Absence of vertical migration across a halocline for the cyanobacteria *Nodularia spumigena*

MALIN LUNDIN 2024
MVEK12 THESIS FOR BACHELOR'S DEGREE 15 HP
ENVIRONMENTAL SCIENCE | LUNDS UNIVERSITY

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Lund 2024

Abstract

Algae blooms, induced by eutrophication, have been an increasingly prevalent environmental concern in the Baltic Sea since the 1970s, causing ecosystem degradation and hypoxia in bottom layers. One predominant harmful algae bloom-forming species in the Baltic Sea is the cyanobacteria *Nodularia spumigena*. *N. spumigena* produces hepatotoxin nodularin and is a diazotrophic alga; hence, it should be phosphorus-limited. However, looking at the large bloom masses *N. spumigena* can sustain in the Baltic Sea, this does not seem to be true. Furthermore, excess phosphorus is usually trapped below the halocline in the Baltic Sea. In this study, we conducted a laboratory experiment to investigate whether *N. spumigena* has the ability to migrate over a halocline. We also explored whether this migration was triggered by phosphate depletion above the halocline and evaluated the impact of phosphate addition on *N. spumigena's* average chlorophyll concentration. The experiment was conducted in artificial water columns with two different phosphate concentrations above the halocline (phosphate-depleted and phosphate-enriched). Measurements of chlorophyll- and phosphate concentration at different depths were taken once during the day (10:00) and once during the night (22:00), over a 96-hour sampling period. The results showed no clear vertical migration of *N. spumigena* over the halocline at any point during the 96-h sampling period. This pattern was consistent in both the phosphate-enriched and phosphate-depleted water columns, contradicting our hypotheses that the vertical migration of *N. spumigena* might be induced by phosphate deficiency. Furthermore, our results suggest the unexpected finding that *N. spumigena* might not be as phosphate-dependent as initially thought. However, the reason behind this is still unsure. Despite the fact that *N. spumigena* does not seem to have the ability to migrate over a halocline, we believe that this research will, in the long term, contribute to the understanding of *N. spumigena* and other cyanobacteria in the Baltic Sea bloom-forming mechanisms, as well as to a comprehensive understanding of necessary mitigation efforts to battle ongoing eutrophication effects in the Baltic Sea.

Popular Sciences Summary

Imagine a hot summer's day, and you are walking down to the water for a swim. However, instead of the usually glittering blue water, you are met by a green, thick, foul-smelling mass. This is something that is becoming more and more common for summer guests on the east coast of Sweden. And one of the biggest culprits is the blue-green algae *Nodularia spumigena*. *Nodularia spumigena* is an alga that forms massive green blooms in the late summer in the Baltic Sea, and these blooms are not only unpleasant but can be toxic to both humans and other animals, as well as hurtful to the Baltic Sea's ecosystems.

All algae need nutrients, specifically nitrogen and phosphorus, to form these blooms. *Nodularia spumigena* is a nitrogen-fixating alga, meaning it can collect nitrogen from the air and water but needs to get phosphorus from somewhere else. Excess phosphorus is usually trapped in the deep water in the Baltic Sea. Therefore, in this study, we ask ourselves if *Nodularia spumigena* might swim all the way to the deep water in the Baltic Sea to get this phosphorus.

Our study disclosed that this does not seem to be the case and that *Nodularia spumigena* does not seem to have the ability to swim down to the deeper, more saline water in the Baltic Sea. The study also found that adding phosphorus to the water had a smaller impact on the growth of *Nodularia Spumigena* than originally believed. However, the reason behind this is still unclear.

We hope this study will be useful in understanding and managing these large algae blooms in the Baltic Sea and that, in the long run, this will lead to less environmental degradation in the Baltic Sea area.

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Introduction

Extensive blooms of toxic cyanobacteria in the Baltic Sea can be harmful to both human and animal life and are a precursor for anoxia and hypoxia in the bottom layers (Chorus & Welker, 2021; Löptien & Dietze, 2022). *Nodularia spumigena* is one of the most common harmful algae blooms (HABs) forming cyanobacterium in the Baltic Sea (Sivonen et al., 1989). The largest blooms of *N. spumigena* occurs during mid-late summer, and the cyanobacterium produces the hepatotoxin nodularin (Repka et al., 2004; Sivonen et al., 1989). *N. spumigena* is diazotrophic, meaning it is N_2-fixating (Löptien & Dietze, 2022; Sivonen et al., 1989). By converting atmospheric nitrogen to bioavailable nitrogen, substantial amounts of N can be added to an already over-fertilized system such as the Baltic Sea, fuelling further algae blooms (Löptien & Dietze, 2022; Wannicke et al., 2012). Phosphorus should, therefore, be the primary limiting nutrient for *N. spumigena*, in accordance with the Redfield ratio (Lilover & Stips, 2008; Tanioka et al., 2022). However, the large biomass *N. spumigena* forms in the summer in the Baltic Sea makes one question whether the species might get inorganic phosphorus from somewhere else (P. Carlsson, personal communication, May 05, 2024).

Excess phosphorus is usually trapped below the halocline in the deepwater of the Baltic Sea. A halocline is a salinity stratification formed in the Baltic Sea by lighter fresh water from rivers and lakes floating on heavier salt water from the Atlantic (Finnish Meteorological Institute., 2023, September 1). The large difference in density between the fresh and saltwater layers prevents mixing between these water masses (Finnish Meteorological Institute., 2023, September 1). The vertical placement of the halocline varies with location within the Baltic basement but usually occurs at depths around 40-80 meters and is around 10-20 meters thick (Finnish Meteorological Institute., 2023, September 1; Leppäranta & Myrberg, 2009; Lilover & Stips, 2008). Extensive phytoplankton growth and increased organic matter transportation to deepwater has, in turn, an increased effect on phosphorus realised from bottom sediment as they become anoxic (Conley et al., 2002; Löptien & Dietze, 2022).

Vertical migration is a functional mechanism thought to be beneficial to some cyanobacteria as it allows them to move between light-abundant surface layers of a water body and lower more nutrient-rich layers (Overman & Wells, 2022). This movement is achieved through buoyancy regulation, either by gas vesicles or accumulation of carbohydrate ballast (Brookes & Ganf, 2001). Carbohydrates are accumulated during high irradiance due to photosynthesis and cause a decrease in

buoyancy and, hence, subsequent sinking. Once the irradiance decreases and the cell slows down or stops photosynthesising, the carbohydrates are consumed, and the buoyancy increases again (Walsby et al., 2006). These changes are also modulated by availability of mineral nutrients (Walsby et al., 2006). The uses of vertical migration have been shown for cyanobacterial species in freshwater, such as *Microcystis aeruginosa*, *Planktothrix rubescens*, *Oscillatoria agardhii* and *Anabaena flos-aquae* (Belov & Giles, 1997; Den Uyl et al., 2021; Kromkamp & Walsby, 1990; Reynolds et al., 1987; Walsby et al., 2006). Furthermore, vertical migration over a halocline of 6 psu and 11 psu has been shown for the Baltic Sea dinoflagellate species *Heterocapsa triquetra* (Jephson et al., 2011). However, *H. triquetra* did not migrate over a salinity gradient of 16 psu.

This bachelor's thesis aims to study whether the diazotrophic cyanobacteria species *N. spumigena* migrates through a halocline to deeper waters in order to take up phosphorus for cell growth.

A laboratory experiment was carried out to see whether *N. spumigena* conducts vertical migration. We hypothesise that *N. spumigena* conducts vertical migration and will travel past the halocline in the experiment with phosphorus depletion in the top layer to reach the phosphorus-enriched water further down. However, we furthermore hypothesise that vertical migration of *N. spumigena* might not occur in the control experiment when the phosphorus concentration is equal throughout the artificial water column.

Additionally, the impact phosphate addition has on the growth of *N. spumigena* will also be addressed.

Relevance for Environmental Sciences

The Baltic Sea has gone through eutrophication due to excessive inputs of nutrients from surrounding areas since the 1950s (Helsinki Commission [HELCOM], 2023). Higher nutrient levels result in excessive phytoplankton growth and biomass accumulation (HELCOM, 2023). Large-scale algae blooms have been occurring increasingly in the Baltic Sea since around 1970, some of which, as blooms caused by *N. spumigena,* are toxic to humans and animals (Karlson et al., 2021; Sivonen et al., 1989). This affects not only the composition and function of the Baltic Sea ecosystem, but also the recreational and functional values of the system as a whole, as bathing water becomes foul and organisms' health degrades (HELCOM, 2023; Karlson et al., 2021). Algae blooms reduce water clarity and degrade the light conditions throughout the water column (HELCOM, 2023). They are furthermore a precursor for bottom death, as large biomass influx to the seafloor results in high oxygen consumption below the halocline (HELCOM, 2023). The area covered by bottom death in the Baltic Sea in 2021 spanned up to ~31% of the total bottom area in the Baltic proper, where up to 21% are areas affected by anoxia (Hansson & Viktorsson, 2023). An expansion of hypoxic areas (oxygen conc <0.2 mg/L) from around 10,000 km² before 1950 to

over 60,000 km² since 2000 has been seen and is attributed to increased algae blooms and eutrophication (Carstensen et al., 2014; HELCOM, 2023; Meier et al., 2019). In order to battle the environmental degeneration of the Baltic Sea, the Helsinki Commission (HELCOM) was formed following the Helsinki Convention in 1974. A key goal of HELCOM is to "Reach a Baltic Sea unaffected by eutrophication", and a part goal of this is to have a "Natural level of algal blooms" (HELCOM, 2023). Furthermore, in their report "*HELCOM Thematic assessment of eutrophication 2016-2021"*, HELCOM (2023) states that:

> "We /../ require a better understanding of the linkages between eutrophication effects and the conditions of pelagic habitats and in particular an understanding of how nutrient ratios influence the composition and succession of plankton /../"- (HELCOM, 2023)

Moreover, Löptien and Dietze (2022) states in their article that:

> "Despite their critical role, the controls on cyanobacteria blooms are not comprehensively understood yet. This limits the usability of models-based bloom forecasts and projections into our warming future." – (Löptien & Dietze, 2022)

As *N. spumigena* is one of the two dominating bloom-formatting cyanobacteria in the pelagic Baltic Sea, knowledge of its physiological adaptions to obtain nutrients is of extra interest (Repka et al., 2004). As hypoxia is spreading across the Baltic Sea, phosphate stored in the sediment is released into the bottom water as a consequence of anaerobic conditions (HELCOM, 2023; Löptien & Dietze, 2022). If *N. spumigena* can migrate over the halocline into deep water to obtain this phosphate, large-scale algae blooms of *Nodularia* can be expected to continue occurring even for an unforeseeable future long after we manage to control the nutrient influx to the Baltic Sea. Extensive growth of the diazotrophic *N. spumigena* also adds bioavailable nitrogen to the Baltic Sea, continuing the eutrophication effects we already see today (HELCOM, 2023; Löptien & Dietze, 2022). In addition to this, vertical migration of *N. spumigena* would also transport inorganic phosphate from the bottom areas to the epipelagic zone, hence fuelling extensive blooms of other phytoplankton species by adding both bioavailable nitrogen and phosphorus.

Ethical reflection

This thesis was conducted in accordance with ALLEA The European Code of Conduct for Research Integrity (ALLEA, 2019). Phytoplankton used for this project are the cyanobacteria *Nodularia spumigena*. Cyanobacteria are prokaryote living

organisms. Currently, no regulations or conduct for the uses of wild cyanobacteria are in place (P. Carlsson, personal communication, May 07, 2024). As of general knowledge today, cyanobacteria do not hold any organelles that could convoy pain or distress. The project will be conducted based on this knowledge. However, we recognise that this understanding might change in the future, and that the ethical aspect of the project should then be reconsidered.

Materials and Method

A laboratory experiment was performed to study if the cyanobacterium *Nodularia spumigena* conducts vertical migration over a halocline. The experiment was carried out over four consecutive days and nights.

Cultivation of cells

Filaments of *N. spumigena* were obtained from surface water in Öresund, Sweden (55°55'27.2"N 12°44'35.2"E) on the 22nd of October, 2023. Single filaments were picked with capillaries, rinsed five times in sterile seawater and incubated with autoclaved F/20 medium (Guillard & Ryther, 1962) without nitrogen addition. The cultures were grown at a constant temperature of 20°C in a 1212 h light/dark cycle. Midday was adjusted to 10:00 and midnight to 22:00 one week before the experiment, by adjusting the light period to between 04:00 and 16:00, and the dark period to between 16:00 and 04:00. Medium was prepared using natural seawater filtered through Whatman GF/F glass fibre filters. The salinity was adjusted to 8 ‰ with deionised water.

Experimental setup

The vertical migration pattern of *N. spumigena* was studied in stratified water columns created in six PVC cylinders (c1,c2,c3,c4,c5,c6) (2 m high and 0.144 m diameter). All sides except the top of the cylinder were covered to replicate the light inhibition of a natural water column. Figure 1. represents the light conditions in the cylinders with light intensity as a function of depth [m]. Daylight fluorescent lamps (OSRAM L 36W/21-840) were positioned 0.2 m above the water surface level in the cylinders, giving light at the same 1212 h light/dark cycle as mentioned above (midday adjusted to 10:00 and midnight to 22:00). The temperature was kept at 20 °C during the whole sampling period of 96-hours. All six cylinders had a halocline at around 1-meter depth, and initial salinity conditions in the cylinders can be seen in Figure 1. Three cylinders were only phosphorus-enriched below the halocline (c1,c2,c3), while the other three control cylinders were P-enriched both above and below the halocline (c4,c5,c6).

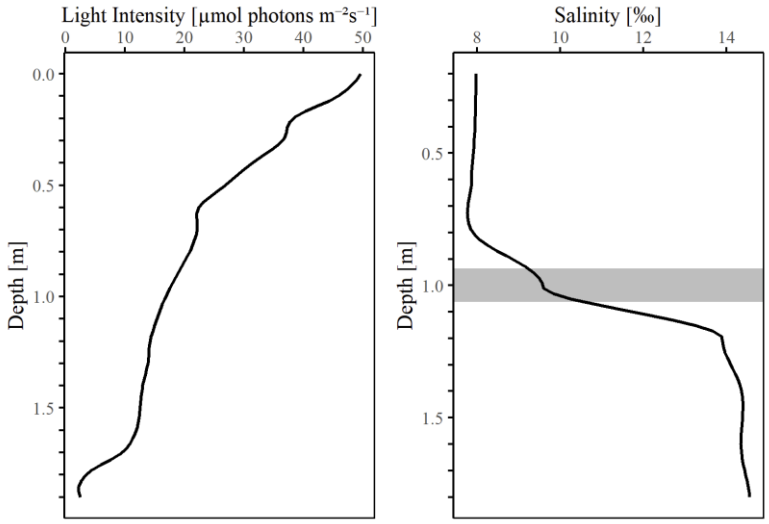

Figure 1.
Initial vertical profiles of light intensity [µmol photons m^{-2} s^{-1}] and salinity [‰] over a depth gradient [m] in the cylinders. The position of the halocline is marked in light grey at around 1-meter depth in the salinity graph. Homogeneity between the cylinders was assumed, and measurements were carried out only on cylinder c5. (n=3 (c5) for light intensity and n=1 (c5) for salinity).

Seawater collection

Around 200 Liters of surface water (~8.9‰ salinity) was collected from Ålabodarna Marina, Landskrona, Sweden (55°56'20.5"N 12°46'20.9"E). The water was filtered through a Pall Type A/E Glass Fiber filter (142 mm, 25/PK) using a peristaltic pump. 100 L of water was adjusted to a salinity of 15‰ by mixing with natural sea salt, and 100 L of water was adjusted to a salinity of 8‰ by dilution with deionised water.

Creation of halocline

Collected seawater was distributed in three 100L bins. One (b1) containing 100L of water with 15‰ salinity, and two bins (b2,b3) containing 50L of water with a salinity of 8‰. Phosphorus was mixed into the bin with a salinity of 15‰ and one of the bins with a salinity of 8‰ (b1,b2) to reach a concentration of 10 µmol P/l. Equal amounts of *N. spumigena* were added into both bins with 8‰ salinity (b2,b3) and thoroughly mixed. Phosphorus-enriched 8‰ salinity water (b2) was filled into control cylinders (c4,c5 and c6), while phosphorus-depleted 8‰ salinity water (b3) was filled into remaining cylinders (c1,c2 and c3). 15‰ salinity water (b1) was pumped into the bottom of all six cylinders, and the water with 8‰ salinity was then pushed upwards in the cylinders to create a halocline with a 7‰ salinity difference at ~1-meter depth.

Measurements

Samples were taken at midday (10:00) and midnight (22:00) for 96-h from nine horizontally placed polystyrene tubes (1 mL serological pipettes), reaching into the centre of each cylinder. The tubes were evenly distributed at 0.2 m distance (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 m) along the cylinders, and all tubes were equipped with external valves to which a syringe could be connected. ~25 mL of water was taken from each tube (0.2-1.8 m) for measurements of chlorophyll concentration, and 20 mL of water was taken from tubes at depths 0.2, 1.0 and 1.8 meters and filtered through a Sarstedt (0.2 μm) syringe filter for phosphate analysis. After each sampling event, new water of salinity 15‰ and 8‰ was pumped in through tubes at 0.2- and 1.8-meters depth, respectively, to maintain a regular water level. The new water added was phosphorus-enriched or depleted as earlier but contained no new addition of *N. spumigena* cells.

Chlorophyll content

Total chlorophyll concentration and concertation of blue-green algae were determined for each sample (c1.0.2-1.8 m, c2.0.2-1.8 m, c3.0.2-1.8 m, c4.0.2-1.8 m, c5.0.2-1.8 m, c6.0.2-1.8 m.) using a bbe ALA Algal Lab Analyser.

Phosphate analysis

The 20 mL of water taken for phosphate analysis was separated into two 10 mL test tubes, and Phosphate analysis was carried out in accordance with Valderrama 1995. A DR. LANGE CADAS 100 spectrophotometer was used to measure absorbance at a wavelength of 882 nm.

Results

Vertical migration of *N. spumigena*

The vertical distribution of *N. spumigena* was observed in artificially stratified water columns over a 96-h period, both during day (10:00) and night (22:00). Half of the water columns were depleted of phosphate above the halocline (-P) while the other half were phosphate-enriched above the halocline (+P). All cylinders were phosphate enriched below the halocline. No clear migration of *N. spumigena* over the halocline can be seen in either of the treatments (-P/+P) at any of the sampling events during the 96-h sampling period (see Figures 2 and 3).

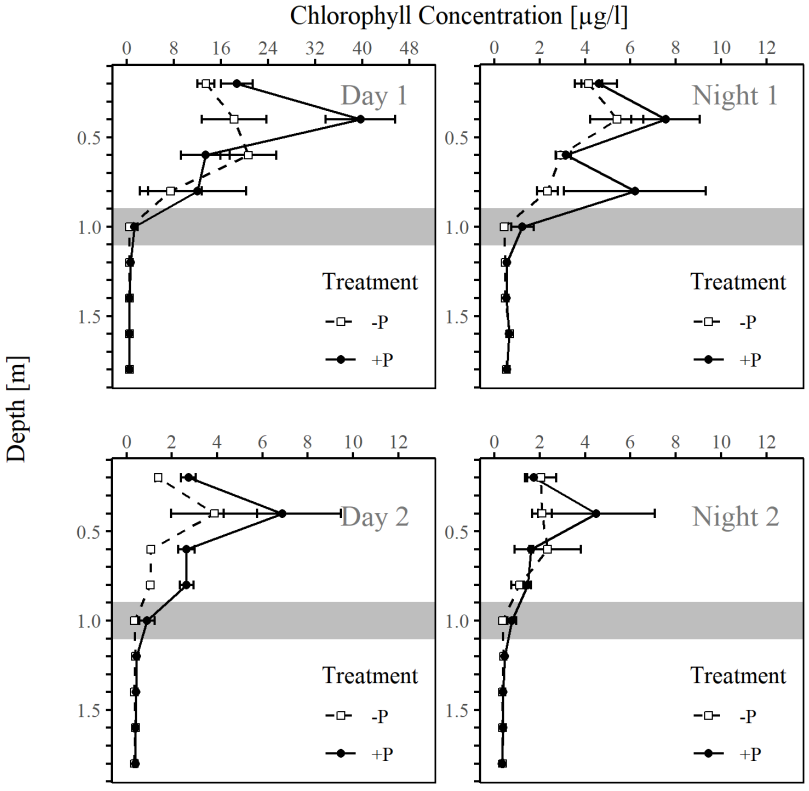

Figure 2.
Chlorophyll Concentration [µg/l] of *N. spumigena* (mean ± SE, $n = 3$) as a function of depth [m] in the six cylinders during the first 48h sampling hours (Day 1 through Night 2). Open squares represent phosphorus-depleted cylinders (c1, c2, c3), closed circles represent phosphorus-enriched cylinders (c4, c5, c6), and halocline position is marked with light grey bars at ~1-meter depth. Note that the y-axis for Day 1 differs from the y-axis for the following days.

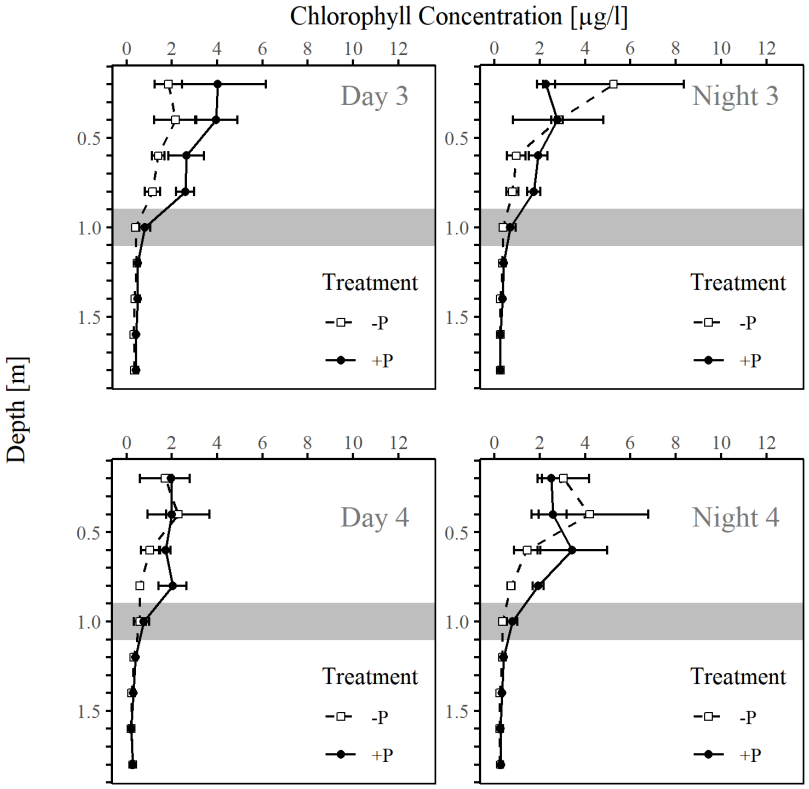

Figure 3.
Chlorophyll Concentration [µg/l] of *N. spumigena* (mean ± SE, $n = 3$) as a function of depth [m] in the six cylinders during the last 48h sampling hours (Day 3 through Night 4). Open squares represent phosphorus-depleted cylinders (c1, c2, c3), and closed circles represent phosphorus-enriched cylinders (c4, c5, c6). The halocline position is marked with light grey bars at ~1 meter depth.

The vertical distribution of *N. spumigena* suggests that the cyanobacteria consistently prefer to remain above the halocline. In Figures 2 and 3, it can be seen that the mean chlorophyll concentration at all depths below the halocline remains consistently below 0.7 µg/l for both the -P and +P treatments (see Appendix Table 1 and 2). Indicating that there has been no strong migration from above the halocline to below the halocline. Additionally, Figure 4 shows that the chlorophyll concentration for depths below the halocline (1.2-1.8 m) remains relatively stable, even as the chlorophyll concentration for depths above the halocline (0.2-0.8 m) varies for both treatments (-P/+P).

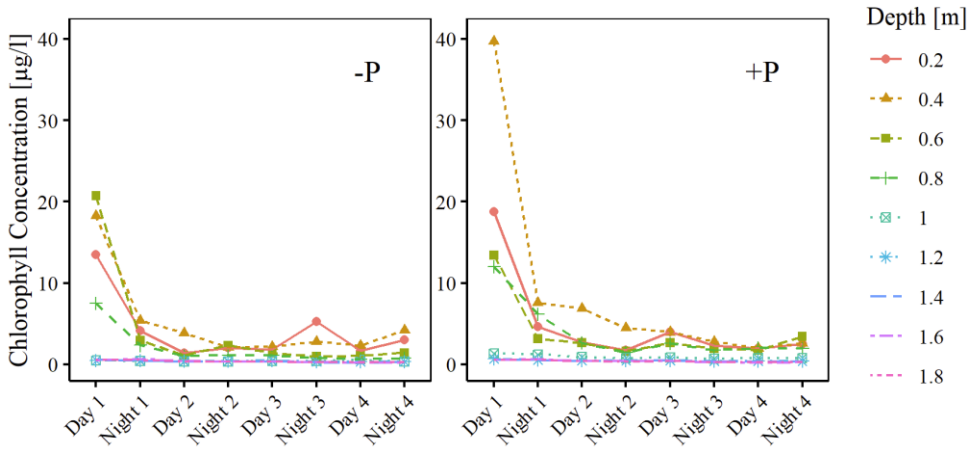

Figure 4.
Chlorophyll Concentration [µg/l] of *N. spumigena* (mean, n = 3) during the 96-h sampling period (Day 1 through Night 4). Each depth [m] is represented separately, in different colours. Depths of 0.2-0.8 are above the halocline, and 1.2-1.8 are below the halocline. The left graph represents the phosphorus-depleted cylinders (c1, c2, c3), and the right graph represents the phosphorus-enriched cylinders (c4, c5, c6).

In Figures 2 and 4, it can be seen that the highest chlorophyll concentration for the sampling period occurs for both the -P and +P treatment on Day 1 above the halocline, reaching 20.71 µg/l at 0.6 m and 39.73 at 0.4 m, respectively (see Appendix Table 1 and 2). From Day 1 to Night 1, a step decrease of chlorophyll concentration from a maximum of 39.73 µg/l to 7.55 µg/l at 0.4 m for the +P treatment and from a maximum of 20.71 µg/l at 0.6 m to 5.40 µg/l at 0.4 m for the -P treatment can be seen. After Night 1, chlorophyll concentrations above the halocline (0.2-0.8m) fluctuated for both treatments but remained consistently below 5.00 µg/l for -P and 7.00 µg/l for +P.

Below the halocline, the highest chlorophyll concentration was recorded at Night 1 (0.67 µg/l at 1.6m) for the -P treatment and at Day 1 (0.69 µg/l at 1.2m) for +P. The chlorophyll concentration in the halocline (1m) exhibited a slight decrease with the +P treatment over time (Mann-Kendall trend test z = -1.9948, *P-value* = 0.04606), while no clear trend was observed in the -P treatment (z = -0.24935, *P-value* = 0.8031) (see Figure 4).

Moreover, a slight decrease in <u>average</u> chlorophyll concentration (ACC) below the halocline was evident for both the -P and +P treatments (z=-2.3506, *P-value*= 0.01874) over the 96-h sampling period (see Figure 5). In contrast, above the halocline, a slight decrease was observed for the +P treatment (z=-2.3506, *P-value*= 0.01874), while no significant trend was identified for the -P treatment (z=-1.3609, *P-value*= 0.1735) (see Figure 5).

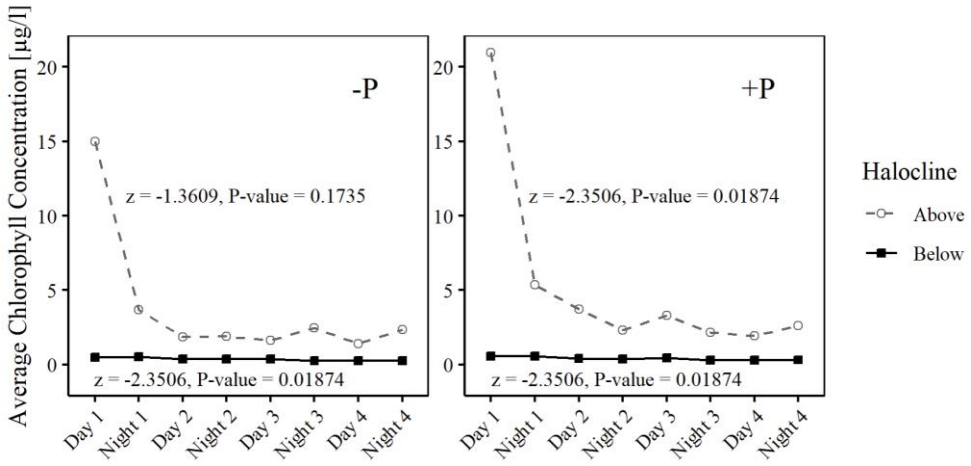

Figure 5.
Average Chlorophyll concentration [µg/l] (*n*=3x4), Above (0.2-0.8m) and Below (1.2-1.8m) the halocline over the 96-h sampling period (Day 1 through Night 4). Open circles and a dotted grey line denote ACC above the halocline. Closed squares and solid black lines denote ACC below the halocline. The left graph represents the phosphate-depleted cylinders (c1, c2, c3), and the right graph represents the phosphate-enriched cylinders (c4, c5, c6). z and *P-value* from the Mann-Kendall trend test placed next to each data series (Above (-P), Below (-P), Above (+P), Below (+P)).

A Welch Two-sample t-test (unpaired and unequal variance) was used to test if the ACC [µg/l] above the halocline differed for the -P and +P treatment. No significant differences between ACCs above the halocline were found at any of the sampling events (Day 1 through Night 4) (*P-value*>0.05), except for Day 3 (t (15.98) = -2.6149, *P-value*=0.01876) (see Appendix Tabel 3).

Phosphate Analysis

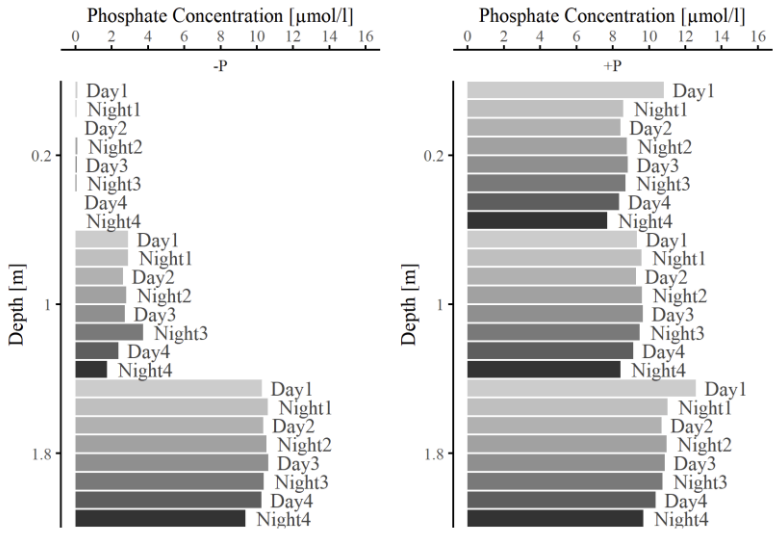

Figure 6.
Phosphate Concentration [µmol/l] (mean, *n*=3) as a function of depth [m] above (0.2 m), in (1 m), and below (1.8 m) the halocline. Each sampling event is represented as a grey-scale bar (Day 1 through Night 4). The left graph represents the phosphate-depleted cylinders (c1, c2, c3), and the right graph represents the phosphate-enriched cylinders (c4, c5, c6).

Figure 6 shows the phosphate concentration [µmol/l], above, in and below the halocline for the -P and +P treatment.

In the -P treatment, phosphate concentrations of less than 0.1 µmol/l were observed above the halocline (0.2m) throughout the 96-h sampling period. Phosphate concentrations between ~2 and 4 µmol/l were observed in the halocline (1m), with the highest concentration recorded on Night 3 and the lowest on Night 4. Phosphate concentrations between ~9 and 11 µmol/l were observed below the halocline (1.8m) throughout the sampling period, with the lowest value at ~9.37 µmol/l for Night 4.

The phosphate concentration for the +P treatment remains relatively consistent across the artificial water column at depths of 0.2m, 1m, and 1.8m and throughout the 96-h sampling period. However, there is a slight increase in concentration range with each depth, with measurements ranging from approximately 8-9 µmol/l at 0.2m, 9-10 µmol/l at 1m, and 10-11 µmol/l at 1.8m. Notably, higher concentrations are observed on Day 1, with levels reaching approximately 10.83 µmol/l at 0.2m and 12.58 µmol/l at 1.8m, both above and below the halocline, respectively. The lowest measured phosphate concentrations were recorded on Night 4 across all three depths (0.2m, 1m, and 1.8m).

Discussion

Algae blooms have been an increasingly prevalent environmental concern in the Baltic Sea since the 1970s. One of the most common species contributing to harmful algal blooms in the Baltic is the cyanobacteria *N. spumigena*. To better understand the mechanisms behind *N. spumigena* bloom outbreaks, a laboratory experiment using artificial water columns was performed to investigate the potential for *N. spumigena* to migrate across a halocline (7‰ salinity gradient). Additionally, the study explored whether this migration was triggered by phosphate depletion above the halocline. Furthermore, the effect of phosphate addition on the average chlorophyll concentration (ACC) of *N. spumigena* was also evaluated.

The results showed no clear vertical migration of *N. spumigena* over the halocline at any point during the 96-h sampling period (see Figures 1 and 2). This pattern is consistent for both the phosphate-depleted (-P) and phosphate-enriched (+P) water columns, contradicting our hypotheses that the vertical migration of *N. spumigena* might be induced by phosphate deficiency. That *N. spumigena* does not seem to conduct vertical migration over a halocline can also be seen in Figure 4, as the chlorophyll concentration for depths below the halocline (1.2-1.8 m) remained stable, even as the chlorophyll concentration for depths above the halocline (0.2-0.8 m) varies for both treatments (-P/+P). If *N. spumigena* had conducted vertical migration, we would have expected to see an increase in chlorophyll concentration at depths below the halocline as the concentrations at depths above the halocline decreased, and vice versa, but this was not the case.

However, the absence of a significant incline in the Mann-Kendall trend test for the ACC above the halocline in the -P treatment (z=-1.3609, *p-value*= 0.1735) may suggest an oscillating curve (or a stable ACC throughout the sampling period, although the latter can be more or less discarded based on figure 5). An oscillating curve could indicate potential vertical migration of *N. spumigena*. However, the anticipated increase or non-significant incline in the ACC below the halocline, expected if vertical migration had occurred, was not observed. Instead, the ACC below the halocline (-P) declined over the sampling period (z=-2.3506, *p-value*= 0.01874), discarding the likeliness of migration of *N. spumigena* to below the halocline.

On the other hand, the chlorophyll concentration *in* the halocline (-P) showed no clear incline over time (z = -0.24935, *p-value* = 0.8031). In combination with the absence of a significant incline for ACC above the halocline, this could suggest potential vertical migration of *N. spumigena* to within the halocline. However, upon

observing Figure 4, it is evident that the decline of chlorophyll concentration above the halocline is consistently greater than the increase in chlorophyll *in* the halocline. Our results cannot completely rule out the possibility of small-scale migration of *N. spumigena* from above the halocline to *within* the halocline for the -P treatment. However, neither does it provide strong evidence for large-scale migration to *within* the halocline for the -P treatment.

In addition, the phosphate analysis (Figure 6) also supports the conclusion that there was no vertical migration of *N. spumigena* over the halocline. If there had been vertical migration of *N. spumigena*, we would have expected a decrease in phosphate levels at some or all of the sampling events below the halocline (1.8m) due to increased P uptake by the greater number of *N. spumigena* cells. Contrary to the rather stable concentration we can see now (~9 and 11 µmol/l). The low value observed on Night 4 (1.8m (-P)) may suggest an increased phosphorus uptake due to higher cell numbers below the halocline, but there is no corresponding increase in chlorophyll concentration for the -P treatment at 1.8m on Night 4 (see figure 3), making this unlikely to be due to migration of *N. spumigena*.

Looking at the impact of phosphorus addition on the ACC of *N. spumigena* (above the halocline), the study disclosed an unexpected finding. The differences in ACC between the -P and +P treatments were non-existent or marginal (t-test; $p>0.05$) for all sampling events except day 3 ($p=0.01876$). A visual indication of differences between ACC in the treatments can still be seen in Figure 5, as ACC above the halocline for the +P treatment was consistently larger than ACC above the halocline for the -P treatment (except for night 4). However, the fact that no statistically significant differences were found is an unanticipated result. Phosphorus is believed to be the limiting nutrient for *N. spumigena*, and much greater growth rates of *N. spumigena* were expected to be seen in the +P treatment than in the -P treatment throughout (Lilover & Stips, 2008).

However, Vahtera et al. (2007) demonstrated in their article that *N. spumigena* can "form and sustain bloom biomasses [by] relying on cellular phosphorus storage and effective remineralisation of organic phosphorus compounds". Additionally, Vahtera et al. (2007) also noted that "*N. spumigena* is a superior competitor for phosphorus at low concentration" compared to other cyanobacteria species. All this suggests that *N. spumigena* may not be as highly phosphate-limited as previously believed. Moreover, Raven (1988) stated in his article that many diazotrophic cyanobacteria almost exclusively grow in areas with high trace metal availability, particularly iron. Building on this, Stolte et al. (2006) found in their study that *N. spumigena* growth rates substantially increased with the addition of DOM-bound iron. This raises the question if the growth of *N. spumigena* in both the -P and +P treatments might be iron-limited, hence explaining why the addition of phosphorus had a marginal effect. However, further testing would have been necessary to confirm this.

Another important thing to notice with the result is the steep decrease in chlorophyll concentration between Day 1 and Night 1 for both treatments (-P/+P), indicating a substantial die-off of *N. spumigena* cells between these sampling events. The reason behind this is unclear and could be attributed to many different factors. It might be due to sub-optimal physicochemical conditions, such as insufficient light irradiance. Furthermore, it could also be because something toxic to cyanobacteria was present in the artificial water columns. The experiment cylinders were glued with epoxy glue, which is toxic to water-living animals, raising the possibility that it may have contributed to the high mortality rates if it had leaked into the water during the sampling period. However, a more substantial die-off of *N. spumigena* would then have been expected. Furthermore, and maybe most likely, the sharp decline between Day 1 and Night 1 of *N. spumigena* might have been due to unsuccessful cell incubation in the starting cultures. It is plausible that the starting cultures had depleted their phosphorus reserves before the experiment began, leading to the *N. spumigena* colonies being weakened at the experiment's start. Consequently, they may not have survived the transition to the new and harsh environment the artificial water columns represent. Alternatively, it could also be due to insufficient levels of trace nutrients, as iron mentioned earlier, in the artificial water columns. Once again, more testing and a re-do of the experiment would be needed to verify this.

The reason why *N. spumigena* did not migrate vertically over the halocline could be due to experimental flaws such as sub-optimal conditions in the artificial water columns or too low sampling frequency and a too short sampling period. Which were restricted to once during the day and once at night for four days and nights, and could result in possible overlooked migration patterns. To develop the experiment, a higher sampling frequency (e.g. sunrise, midday, sunset, midnight) and a longer sampling period could be of interest. It would also be important to ensure that the starting cultures are healthy and to evaluate and eliminate possible sub-optimal conditions in the artificial water columns. Furthermore, to completely rule out the possibility of vertical migration of *N. spumigena* over a halocline, no *N. spumigena* cells should be present below the halocline at the start of the experiment. In this study, the 15‰ salinity water was added through pumping after the 8‰ salinity water, most likely resulting in a slight mixing of *N. spumigena* cells from the 8‰ salinity water into the 15‰ salinity water at the beginning of the experiment. To bypass this, *N. spumigena* colonies could be added only from the top of the cylinders after filling up the cylinders with both the 8‰ and 15‰ salinity water.

However, it is important to note that even if it was of interest to test if *N. spumigena* vertically migrated over a halocline, it might not have been the most likely outcome. Vertical migration of cyanobacteria has only been found for species in freshwater, and no vertical migration of cyanobacteria over a halocline has, to our knowledge, thus far, been shown (Belov & Giles, 1997; Den Uyl et al., 2021; Kromkamp & Walsby, 1990; Reynolds et al., 1987; Walsby et al., 2006). Still, as *N.*

spumigena is one of the dominating HAB-forming species in the Baltic Sea, the mechanism of its bloom outbreaks is of extra interest to study further. Especially if, as our study suggests, they are not as phosphate-dependent as initially thought.

Currently, large efforts to lower nitrogen and phosphate input to the Baltic Sea are active and are expected to, in the long-term, also result in fewer large-scale algae blooms (HELCOM, 2023). This is crucial for the future health of the Baltic Sea. However, it is important to consider that these efforts may not have the desired impact on *N. spumigena* if, as this study suggests, the cyanobacterium is less limited by phosphate than previously believed. A better understanding of cyanobacteria and other phytoplankton in the Baltic Sea is a crucial step to efficient management of the area, both for the preservation of the ecosystem's health and to prevent further environmental degradation, such as the widespread hypoxia. As well as for the recreational and functional values of the Baltic Sea area as a whole. It is also essential for achieving the goals set by HELCOM, such as "A Baltic Sea unaffected by eutrophication" and "Natural level of algal blooms" (HELCOM, 2023). We suggest that further studies should be conducted on both *N. spumigena* and other Baltic phytoplankton to gain an even more comprehensive understanding of the mechanisms behind large-scale phytoplankton blooms in the Baltic Sea. Furthermore, we also suggest that more studies on *N. spumigena's* ability to recycle cellular and organic phosphate compounds, as well as *N. spumigena's* iron dependency, should be conducted.

Even though *N. Spumigena* does not seem to have the ability to migrate over a halocline, the result of this study can be of importance and a part of the greater puzzle to understand the mechanisms of bloom outbreaks of *N. spumigena* and other cyanobacteria in the Baltic Sea. The results will also, hopefully, contribute to the comprehensive understanding of necessary mitigation efforts to battle ongoing eutrophication effects in the Baltic Sea area.

Conclusions

The results did not show any reliance for that *N. spumigena* conducts vertical migration over a halocline to take up phosphate for cell growth. This result was consistent in both the phosphate-enriched and phosphate-depleted water columns, contradicting our hypotheses that the vertical migration of *N. spumigena* might be induced by phosphate deficiency.

Furthermore, the result showed that the impact of phosphate addition on the growth of *N. spumigena* was non-existent or much more marginal than initially expected. What this depends on is unsure, but it might be due to that *N. spumigena* can remineralise cellularly stored- and organic phosphate or that the cyanobacterium is highly iron-dependent.

To confirm this and to further develop knowledge in this field, we suggest that further studies should be conducted both, on *N. spumigena*'s different nutrient dependencies as well as on other key phytoplankton in the Baltic Sea's bloom-forming mechanisms.

We believe that this research will be instrumental in uncovering the mechanisms behind the bloom outbreaks of *N. spumigena* and other cyanobacteria species in the Baltic Sea. The results will also, hopefully, contribute to a comprehensive understanding of necessary measures to address the persistent eutrophication effects in the Baltic Sea region in the long term.

Acknowledgements

First of all, I would like to thank my amazing supervisor, Per Carlsson, Functional Ecology, Lund University. Who indefatigable was there to answer questions, texts, and emails about the experiment and everything around it, who helped with the collection of both *N. Spumigena* filaments and seawater and who, as early as in February, sent me articles about *N. Spumigena* and other cyanobacteria when I said I was interested in doing this project. A better supervisor is hard to wish for.

Furthermore, I would like to thank my lab partner, Ebba Wahlberg, who made the middle-of-the-night lab sessions much more bearable and was there to share the disperse and laughter of broken PVC cylinders, leaking taps, and dying algae.

I would also like to thank my group supervisor, Peter Olsson, with whom I could share the confusion about how to get any statistical result out of the result at all, as well as for making me feel proud about my accomplishments in R. Lastly, I would like to thank everyone else at Lund University who made this thesis possible.

Thank you all.

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Appendix

Table 1.
Chlorophyll concentration [µg/l] (mean, *n*=3) for each depth [m] during the 96-h sampling period (Day 1 – Night 4) for the phosphate-depleted (-P) cylinders (c1, c2, c3).

-P								
Depth [m]	**Day 1**	**Night 1**	**Day 2**	**Night 2**	**Day 3**	**Night 3**	**Day 4**	**Night 4**
Above halocline:								
0.2	13.49	4.15	1.40	2.04	1.85	5.25	1.70	3.03
0.4	18.28	5.40	3.87	2.09	2.16	2.81	2.29	4.21
0.6	20.71	2.92	1.08	2.34	1.40	0.96	1.03	1.44
0.8	7.52	2.34	1.05	1.09	1.14	0.78	0.59	0.73
In halocline:								
1	0.47	0.42	0.34	0.36	0.39	0.39	0.58	0.35
Below halocline:								
1.2	0.49	0.47	0.40	0.39	0.46	0.35	0.33	0.35
1.4	0.52	0.47	0.35	0.34	0.36	0.25	0.22	0.23
1.6	0.47	0.67	0.40	0.36	0.33	0.25	0.21	0.23
1.8	0.53	0.53	0.35	0.35	0.36	0.25	0.27	0.24

Table 2.
Chlorophyll concentration [µg/l] (mean, *n*=3) for each depth [m] during the 96-h sampling period (Day 1 – Night 4) for the phosphate-enriched (+P) cylinders (c4, c5, c6).

+P								
Depth [m]	**Day 1**	**Night 1**	**Day 2**	**Night 2**	**Day 3**	**Night 3**	**Day 4**	**Night 4**
Above halocline:								
0.2	18.75	4.62	2.73	1.72	4.02	2.27	1.97	2.51
0.4	39.73	7.55	6.88	4.49	3.96	2.76	1.99	2.57
0.6	13.40	3.15	2.65	1.62	2.64	1.92	1.72	3.43
0.8	12.01	6.20	2.65	1.46	2.59	1.73	2.04	1.93

In halocline:								
1	1.33	1.23	0.90	0.76	0.81	0.70	0.76	0.78
Below halocline:								
1.2	0.69	0.56	0.45	0.45	0.50	0.40	0.39	0.40
1.4	0.53	0.52	0.42	0.38	0.50	0.34	0.30	0.34
1.6	0.54	0.65	0.39	0.37	0.41	0.26	0.21	0.26
1.8	0.53	0.54	0.39	0.34	0.42	0.26	0.27	0.28

Table 3.
Welch Two Sample t-tests of differences in mean chlorophyll concentration above halocline for -P and +P treatment. T-test for each sampling event (Day 1 through Night 4) presented. Significant *p-value* (p<0.05) and the occasion marked in red.

Sampling event	***t***	**df**	***p-value***
Day 1	-1.2416	17.932	0.2304
Night 1	-1.6391	16.393	0.1202
Day 2	-1.969	19.449	0.06336
Night 2	-0.55653	17.762	0.5848
Day 3	-2.6149	15.997	0.01876
Night 3	0.28436	11.856	0.781
Day 4	-1.1466	13.783	0.2711
Night 4	-0.31009	17.017	0.7603