# Seasonality of cyanotoxin concentrations in mussel tissue from a Baltic Sea mussel farm

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# Seasonality of cyanotoxin concentrations in mussel tissue from a Baltic Sea mussel farm

*Säsongsvariation av cyanotoxinkoncentrationer i musslor odlade i Östersjön*

Alma Sandqvist

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## Abstract

*With climate change-induced increases in water temperatures, cyanobacteria and their toxins are becoming increasingly prevalent in the Baltic Sea. Using mussels as a bioindicator, this thesis assesses the seasonality of cyanotoxin production and examines the accumulation of cyanotoxin in mussel tissue. Samples were collected from March to October bi-weekly and weekly, yielding a high temporal resolution from a submerged mussel farm close to Stockholm, at both 3 m and 10 m depth. Mussel tissue was analysed using both enzyme-linked immunosorbent assay (ELISA) analysis and liquid chromatography mass spectrometry (LC-MS) analysis for their microcystin and nodularin concentrations. The ELISA results were unusable due to interference either from the solvent or from dissolved mussel tissue. When analysing the extracted mussel tissue using LC-MS instead, microcystins were under the limit of detection. Nodularins had a base line concentration of around 4µg/kg of mussel tissue, with levels rising in initial tandem with rising levels of the cyanobacteria Nodularia spumigena. The toxin levels in mussels were however not linearly related to the N. spumigena population, but were affected by both accumulation and depuration. Even though Sweden does not have regulation on cyanobacterial toxins in mussels, we can look at those globally available. The mussels collected from the 3 m "surface depth" exceeded the Australian threshold value for safe consumption of 51µg/kg on two occasions during summer. The deep samples were always well below this safety limit. In conclusion, if mussels are harvested between October and May, they would be below the suggested limit for consumption with regards to microcystins and nodularins.*



*Keywords:* cyanotoxins, mussel tissue toxins

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# 1. Introduction

Since the 1970s, increasing anthropogenic pressures has greatly decreased the ecological and environmental status of the Baltic sea (HELCOM 2023). A major sign as well as driver of this negative development is harmful algal blooms, extensive overgrowths of microalgae and cyanobacteria, resulting in ecological disturbances like dead bottom zones and potentially high toxin levels (McGowan 2016). While blooming is part of many taxa of algae as their seasonal periodicity (McGowan 2016), the intensity, size and time range has been increasing in the last decades due to drivers like eutrophication, resulting in blooms that have far more severe effects than "natural" ones (HELCOM 2023, Berdalet 2023, McGowan 2016). Future climate as well as nutrient conditions are predicted to worsen harmful algal blooms (Neumann 2013).

While the blooms are generally referred to as “algal”, this is only half the truth. The summer blooms in the Baltic Sea are mainly caused by cyanobacteria, with the ratio of bacteria to algae differing depending on environmental factors such as nutrient availability, temperature and salinity. The ubiquitously found cyanobacteria are photoautotrophs, and consist of a wide range of morphological, taxonomic and physiological diversity (Gaysina et al., 2019). Like plants, the most famous photoautotrophs, cyanobacteria obtain their energy through photosynthesis and are through this primary production a vital base of food webs, as well as the earth's oxygen supply. In contrast with algae, some cyanobacteria species are diazotroph, meaning they are able to fix nitrogen from the atmosphere, playing an important ecological role in nitrogen cycling (Madigan et al., 2022). Consequently they are less limited by available nitrogen in the water body, benefiting their resource competition against algae (Madigan et al., 2022). Another strategy developed by many cyanobacteria is buoyancy, thanks to air filled vesicles, which tends to limit their blooms to the water surface, but also the light that reaches lower levels. However some Baltic species have been recorded “blooming” up to 10-20m deep (Hajdu et al. 2007). The three most common Baltic species are *Nodularia spumigena, Aphanizomenon flos-aquae*, and the genus *Dolichospermum,* all of which are diazotroph (Mazur-Marzek et al. 2013). Warming sea temperatures are expected to benefit cyanobacteria since they favour warmer temperatures compared to their algal counterparts who in general have adapted to slightly colder water temperatures. Thus, cyanobacteria are expected to dominate the “algal” blooms, calling for more research into their effect on the ecosystem.

One of the major impacts that cyanobacteria have on the ecosystem which awakens concerns, is their potential toxicity. The huge diversity of cyanobacteria unsurprisingly results in a wide array of types of cyanotoxins (Chorus & Welker, 2021; He et al., 2025). Like some micro algae, some, but not all, cyanobacteria produce toxins, believed to be a strategy to deter grazing and thus increase fitness (Lemaire et. al. 2012). The toxins can pose harm to both aquatic organisms as well as humans, and in some cases even be lethal, but this depends on the dose and chemical activity of the toxin (Bartram & Chorus, 1999; Berdalet et al., 2023; Visciano et al., 2016). In this thesis, two cyanotoxin groups are studied: microcystin (or MCs) and nodularins (or NODs). Nodularins, produced by and named after *Nodularia spumigena*, are the most prominent cyanotoxins in the Baltic Sea, while microcystin producers (some *Delichospermum* species) favour lower salinities and are recorded at much lower rates (Olofsson et al. 2025). With regards to the Baltic, these are the two most studied groups of cyanotoxins. MCs are hydrophilic cyclic peptides found in freshwater and brackish water. Human exposure to the toxin can lead to liver damage, liver failure, as well as cancer in cases of high exposure (Shi et al., 2021), with less severe effects being nausea, vomiting and stomach cramps. NODs are pentapeptides of a cyclic structure. Their toxic effects are diverse, causing “allergic reactions, skin rashes, gastrointestinal illness, nausea, liver damage, and bleeding” (Melaram et al., 2024).

The conditions under which the toxins are produced are not as straightforward as simply a high concentration of individuals leading to higher toxin production. The factors that affect toxin production are complex and not yet fully understood, but seem to have a strong seasonal component affected by nutrient availability (Lawson et al., 2025). The effects of nutrient availability have been studied, but the results between studies are at times contradicting and inconclusive (Glibert et al., 2018, Klitzke et al., 2010). Therefore, assessing actual levels of toxins requires a direct measurement; it is not sufficient to measure the abundance of cyanobacteria or single environmental factors like temperature or nutrient availability.

One way to study cyanotoxin levels in water bodies is through directly quantifying toxin abundance from phytoplankton samples. However, this method is limited by that it only gives a snapshot into the toxins that are bound within the bacterial cells at the time, but not to the toxins that are released, nor their accumulation or the effect in the ecosystem. Measuring toxin levels in the water itself gives results with such low concentrations that conclusions are hard to draw. Thus the approach used in this thesis is to use mussels as a bioindicator. The Baltic Sea native blue mussel, *Mytilus edulis*, due to its role as a filter feeder,

accumulates toxins in its tissues (Camacho-Munez et al. 2021, Olofsson et al. 2025). Quantification of these toxin levels gives insight into toxin levels over both time and space, since they represent an accumulation of toxins over time. Since mussels are stationary they also allow for comparison between different sites (Olofsson et al 2025). Lastly, they concentrate the toxins, thus improving detectability and making data easier to work with. For these reasons, this thesis uses mussels as a bioindicator, in order to study accumulation and exposure of toxins over time.

Mussels are relevant to study in this context not only from an ecological point of view but also since mussel aquaculturing may increase in the Baltic Sea in the future. It is currently limited, but if scaled up, envisioned as a way of mitigating eutrophication by removing nutrients through the removal of the mussel biomass, improving water quality through increased filtering activity, while also producing healthy food (Kotta et al. 2020). The effectiveness of this vision has however been questioned, with concerns raised about its efficiency due to the small size of blue mussels in the Baltic due to its low salinity (Hedberg et al., 2018), and potential environmental dangers of large scale farming. In other words, more investigation is needed.

A main concern with mussel farming in the Baltic is the concentration of toxins in the mussels, and if the levels would be so high as to cause adverse effects to human health if ingested. Currently, the mussels sampled in this thesis are not used for human consumption, but as fertilizer and for animal feed. The timing of the harvest of these mussels varies yearly with regards to blooming, but generally takes place around April and around September. There is evidence of mussels purifying themselves of microcystins and nodularins, but it is an incomplete process: some toxin levels remain (Burmester et al., 2012, Camacho-Muñoz et al., 2021). For possible future human food applications, there is a risk of harm. Testing is thus necessary to ensure human, as well as animal health, and such a program is in place on the west coast of Sweden since 2001 (Persson et. al 2020). Such testing has not yet been applied to bivalves harvested in the Baltic. A seasonal analysis of toxin accumulation in mussels would give indications of when toxin concentrations are at their lowest, and harvest would be the safest. One such study was done in 2025, examining three different Baltic Sea mussel farms (Olofsson et al. 2025). There are limited threshold values available for safe consumption of cyanotoxin exposed food and water, especially with regards to the Baltic. The only current threshold value found is from Australia, setting a maximum of 51 µg/kg of microcystins and nodularins (Tetsai et al. 2016). More research is needed if the farmed mussels are to be used as food in the future.

There are different analytical methods for quantification of cyanotoxins in tissue. The two methods chosen for this thesis is liquid chromatography mass spectrometry, or LC-MS, and enzyme-linked immunosorbent assay (ELISA). The widely used ELISA analysis method was chosen for its efficiency, since around 40 samples could be run at once, its rapid results (within one day), and for its relatively low costs (Chorus & Welker 2021). While the ELISA analysis has a low limit of detection, it is not able to classify different types of MCs and NODs. LC-MS analysis on the other hand, can quantify and classify different cyanotoxins, but at higher costs and time consumption (Chorus & Welker 2021). Both methods were chosen due to their recognition as reliable methods, and for their availability with regards to costs, time and equipment availability.

As mentioned, there is a need for further investigation into cyanotoxin production, effects, threshold values and future scenarios. This thesis will try to fill in a few gaps, namely: 1. Assessing the seasonality of cyanotoxin concentrations in mussel tissue, and whether the growth depth of mussels has an effect. 2. Does the nodularin and microcystin toxin concentration in mussel tissue exceed threshold values for safe human consumption? 3. Do cyanotoxin concentrations in mussels correlate to cyanobacteria biovolume, specifically *Nodularia spumigena*. 4. Is LC-MS or ELISA analysis most suitable for analysing mussel tissue toxin levels? To answer these questions, mussels as well as cyanobacteria (through water samples) were sampled at a temporally high resolution from March to October 2024 at depths of 3 m and 10 m at a mussel farm belonging to Ecopelag in the Stockholm archipelago nearby Dalarö on the east coast of Sweden.

# 2. Methods

## 2.1 Mussel and water sample collection

The samples were collected at a mussel farm belonging to Ecopelag in the Stockholm archipelago nearby Dalarö on the east coast of Sweden, where the mussels are grown in cage structures. The samples were collected at two different depths: Surface (3 m) and Deep (10 m) and frozen at -20°C immediately on arrival to the lab. Each sample consisted of around 750 g of mussels. Sampling started in March of 2024 and continued biweekly (spring and autumn) or weekly (summer) throughout October 2024. At each occasion, a water sample (0.75 or 1 l) was collected and filtered in order to have samples of phytoplankton for cyanotoxin analysis, both at deep and surface levels. The filters were frozen for further analysis. For nutrient data, between 12-15 ml of water was additionally filtered through a 0.22 filter using a syringe and the filtrate was frozen at -20°C until further analysis. For the phytoplankton data, around 50 ml of unfiltered water was collected and 4-5 drops of Lugol solution added in order to fix the cells. Until cell counts, the samples were stored at room temperature.

## 2.2 Mussel sample dissection and preparation

This was performed on a total of 45 samples: 30 surface samples from March to October 2024, and 15 samples from the deep level, spanning June to September 2024. Unfortunately one sample was lost, so there is no data for week 19, giving a gap in the time series. The samples were defrosted on the day of dissection. Large and medium mussels were chosen for dissection, and small ones avoided for the sake of time efficiency. Since the size of the mussels vary throughout the year, the length of the largest and smallest dissected individual was measured to establish an average. On a clean and dry wettex tissue the mussels were opened using a scalpel. All the tissue was scraped out and placed in a clean glass beaker. Care was taken to not contaminate the tissue with pieces of shell, since some filamentous algae can grow on larger individuals' shells.

When around 5 g of mussel tissue was gathered, the tissue was homogenized using an Ultra-Turrax bench homogenizer, until no clots of tissue could be detected. The homogenized tissue was then transferred to sterile PETG bottles, and the exact weight measured and noted. The samples were then frozen at -20°C until further analysis.

## 2.3 Cyanotoxin extraction from mussel tissues using methanol

The methanol extraction was performed according to a 2023 paper on LC-MS analysis of nodularins (España Amóregui et al. 2023). The homogenized mussel tissue samples were thawed, and 2 g were transferred into clean 15ml centrifuge tubes. 4ml of 100% methanol was then added to each sample to dissolve the cyanotoxins from the mussel matrix. All tubes were vortexed 2 minutes, after which they were centrifuged for 3 minutes at 3500rpm to separate the solids from the liquid extraction. The extracts were then filtered through a syringe 0.22 µm Whatman filter, removing larger particles and collected in 1.5ml low binding eppendorf tubes. A negative control of pure, likewise filtered methanol, was run as a parallel for each run, of which there were five in total.

For ELISA analysis, 250 µl of the sample extract was transferred out in a low binding eppendorf tube, and left to evaporate covered with a paper tissue in a fume hood, until all methanol was gone. This process took around 5 days. After complete evaporation, 250 µl of lab reagent blank from the ELISA kit was then added to each sample, vortexed for 90 seconds and then centrifuged at 3500 rpm for 5 minutes. Each sample was then diluted in the ratio 1:20 in LRB and stored at -80°C until further analysis.

For the LC-MS analysis 250 µl of the extract from the extraction described above was diluted with 750 µl MilliQ water and stored at -70°C until analysis.

## 2.4 Cyanobacterial biomass toxin analysis

For this method, samples from March to October 2024 were used. This analysis was performed on the cyanobacterial biomass left on the filters from the water samples taken simultaneously as the mussel samples. There are many extraction protocols to choose between when extracting cyanotoxins. This method was chosen due to not being very time consuming, and not needing any strong solvent. The toxins are extracted into water using only heat. The quantification was determined using an ELISA analysis, just like the toxin levels in mussel tissue.

The filters were thawed and placed in a glass tube, where they were “washed” with 3ml of MilliQ water, encouraging separation of the biomass from the filter. The test tubes were then placed in an 80°C water bath for 10 minutes, after which the filters were removed and “wrung out” with metal tweezers. The remaining liquid was thereafter filtered using a 0.22 um PVDF filter to a 2 mL glass vial which was stored at -20°C. After thawing, the extracts were analysed using

ELISA in the same manner as the mussel samples. Since the volume of milliQ water added, as well as the original amount of water filtered was know for each sample, toxin concentrations from biomass per liter of water could be back calculated.

## 2.5 ELISA analysis

The specific ELISA (enzyme-linked immunosorbent assay) analysis chosen for the detection and quantification of microcystins and nodularins in the cyanotoxin extracts from both mussel tissue and cyanobacterial biomass was from Golden Standard Diagnostics, called Abraxis Microcystins-ADDA SAES ELISA. It is highly sensitive and specific, and can be performed with basic lab equipment, which is why it was chosen. While it can detect the total level of NODs and MICs, it is not able to tell the varieties apart. The principle of the assay is the binding of antigens, in this case microcystins and nodularins to specific biotinylated antibodies. A microplate with wells covered with antibodies specific to nodularins and microcystins is used, to which the samples are added as well as a cyanotoxin biotinylated protein analogue, resulting in competition for the binding sites. The higher the concentration of cyanotoxins present in the sample, the less analogue will bind. With the addition of an enzyme conjugate and substrate solution, a blue colour is produced. The intensity of the colour is inversely proportional to the concentration of cyanotoxins, since a higher presence of analogue results in a stronger blue color. Using an ELISA reader, absorbance is measured which using interpolation of the standard curve of each run gives quantitative results. The limit of detection was 0.016 µg/L, while the limit of quantification was 0.05 µg/L.

The analysis was performed according to the kit instructions. 50 µl of standard solutions, controls and samples were added in duplicated into the wells of the microplate. 50 µl antibody solution was added to each well and incubated for 90 minutes, after which the plates were washed and 100 µl of Enzyme conjugate was added. After a second incubation of 30 minutes, the plates were again washed before 100 µl of colour substrate was added. The plate was then left for a 25 minute incubation after which 50 µl of stopping solution was added. The absorbance was then read at 450 nm using a microplate ELISA reader.

## 2.6 LC-MS analysis

The cyanotoxins from mussel tissue extracts were analysed using liquid chromatography tandem mass spectrometry at Lund University, Sweden. Blank controls and recovery standards were used with every analytical batch to ensure sound results. The limit of detection (LOD) was 0.1 ng/ml. The toxins classified were nodularin, microcystin-RR, microcystin-LR, microcystin-YR, microcystin-WR, microcystin-HilR, desmethyl-microcystin LR, desmethyl-microcystin RR and cylindrospermopsin.
A full protocol for the analysis can be found in annex 1.

## 2.7 Phytoplankton analysis

Phytoplankton samples, fixed in Lugol Solution (Calissendorff and Falhammar 2017), were analysed at the University of Gdansk, Poland, at the Department of Marine Biology and Biotechnology, Faculty of Oceanography and Geography (Katedra Biologii Morza i Biotechnologii, Wydział Oceanografii I Geografii).

Using an inverted microscope, Utermöhls method (HELCOM 2021) was used for cell counts. Using appropriate literature, as well as geometrical analysis, phytoplankton was identified at the lowest possible taxonomic. Biovolume was calculated using the geometric analysis. The full protocol can be found in annex 2.

## 2.8 Determination of $PO_4$-P, $NH_4$-N, and ($NO_2$)-+($NO_3$)-N from water samples

Filtered (0.22 µm) and frozen water samples were analysed for inorganic nutrient concentrations $PO_4$-P, $NH_4$-N, and $NO_2$+($NO_3$)-N at Stockholm University, Institute for Ecology, Environment and Botany.
The lowest rate of detection for both $NH_4$-N and $PO_4$-P was 0.5 µg/l, while it was 0.3µg/l for $NO_2$+($NO_3$)-N. The full protocol can be found in annex 3.

# 3. Results

## 3.1 Cyanotoxin levels in mussels analysed by ELISA vs. LC-MS

While ELISA analysis is only able to detect and quantify bulk toxin concentration of microcystins and nodularins, LC-MS analysis is able to classify and quantify the different types. Visualized in table 1, in the LC-MS results microcystins were always below detection, meaning all toxins discovered are nodularins.

*Table 1. Results from the classification of toxins from the LC-MS analysis, and the associated toxin producing cyanobacteria.*

| **Cyanobacteria species or genus** | **Associated toxin** | **Detected in water samples** | **Toxin level found through LC-MS analysis** | **Deemed to affect toxicity in mussels** |
|---|---|---|---|---|
| Aphanizomenon flos aquae | Cylindrospermopsin | 0-714 $mm^3/l$ | Below detection | No |
| Dolichospermum | Microcystin, cylindrospermopsin | 0-73 $mm^3/l$ | Below detection | No |
| Nodularia spumigena | Nodularins | 0-61$mm^3/l$ | 1-62 µg/kg | Yes |

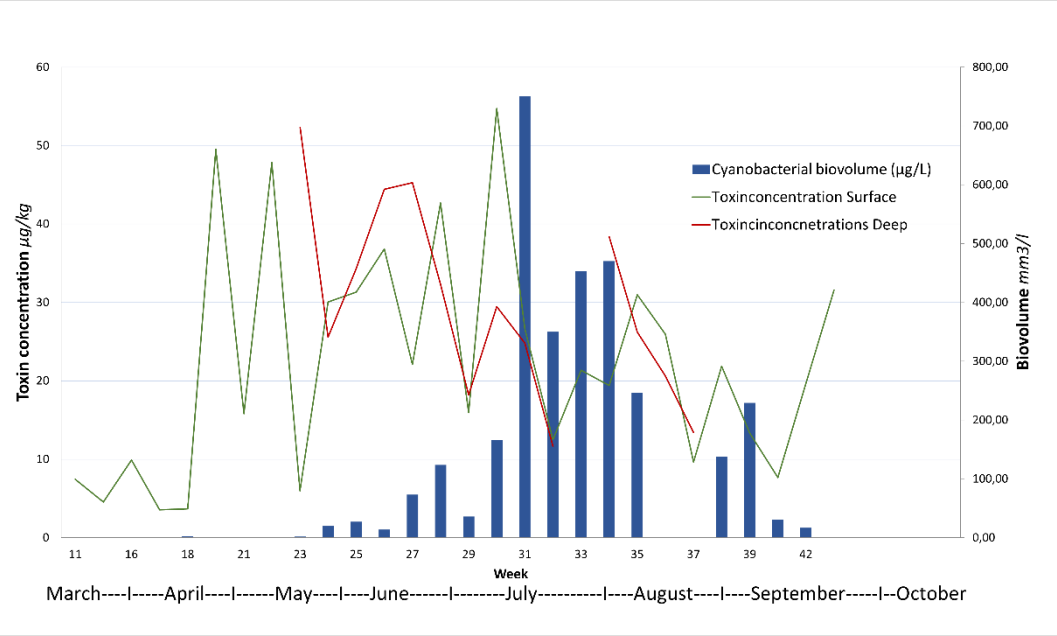


*Figure 1. Results from the ELISA analysis of microcystins and nodularins in mussel tissue, for Deep and Surface samples, in µg/kg, as well as the total biovolume of the species Nodularia spumigena, Aphanizomenon flos aquae, and the genus Dolichospermum in $mm^3g/l$*

The ELISA analysis of the mussel tissue showed strongly undulating levels of cyanotoxin concentrations (Fig.1). The high values of cyanotoxins in the surface samples (3 m) during week 18-23 are in contrast with the very low levels of cyanobacteria recorded during this time. Cyanotoxin concentrations in the mussels from deeper waters show similar high concentrations as the ones found on the surface, with a slightly decreasing trend over time and some variations.

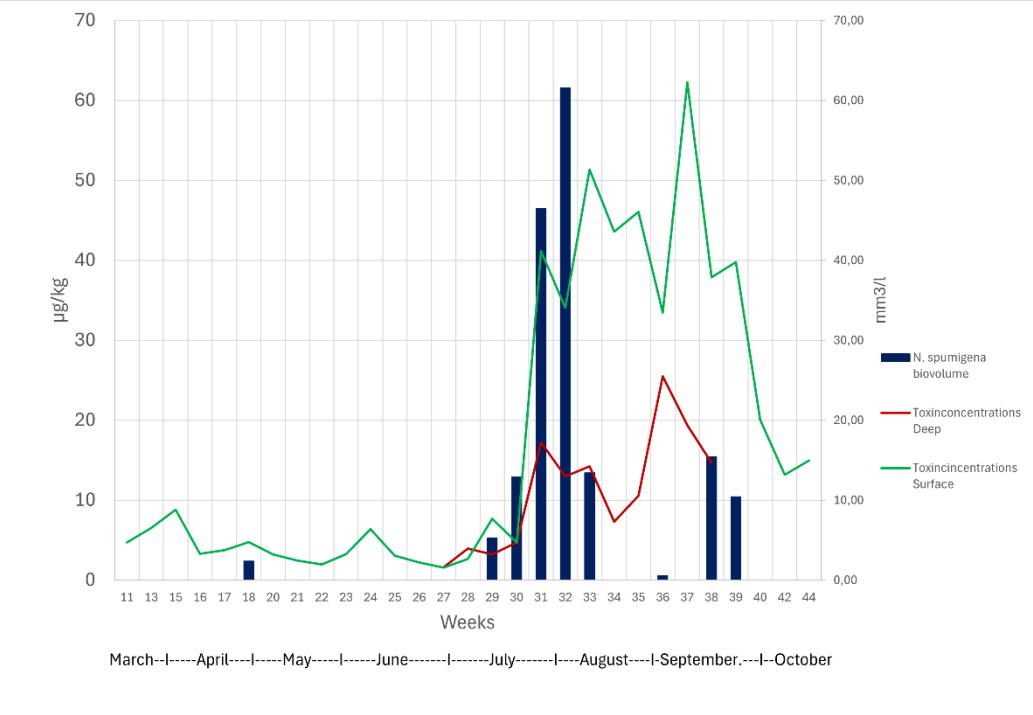


*Figure 2. Results from the LC-MS analysis of cyanotoxins in mussel tissue, for Deep and Surface samples, in µg/kg, as well as N. spumigena biovolume in $mm^3/l$.*

LC-MS analysis was conducted on the same toxin extractions as the ELISA analysis, but with very different results. The levels of mussel tissue toxins from the surface were low from week 11 to 28, where *Nodularia spumigena* populations also remained near non-existent or low (Fig. 2). Starting with a sharp increase to 41µg/kg during week 31, toxin levels fluctuated but remained high until week 39, when they started gradually decreasing, reaching similar levels as week 11-28 by week 46. The deep samples show an overall lower toxin concentration compared to the surface ones. Similarly to surface toxins, there is an increase following week 30, compared to the measurements from the previous weeks. It is notable that the highest concentration of surface toxins occurs during week 37, several weeks after the first peak of *N. spumigena* biovolume decreased to below detection, and one week before the second peak.

## 3.2 Cyanotoxin concentrations in biomass in relation to temperature, nutrients, and cyanobacterial biovolume

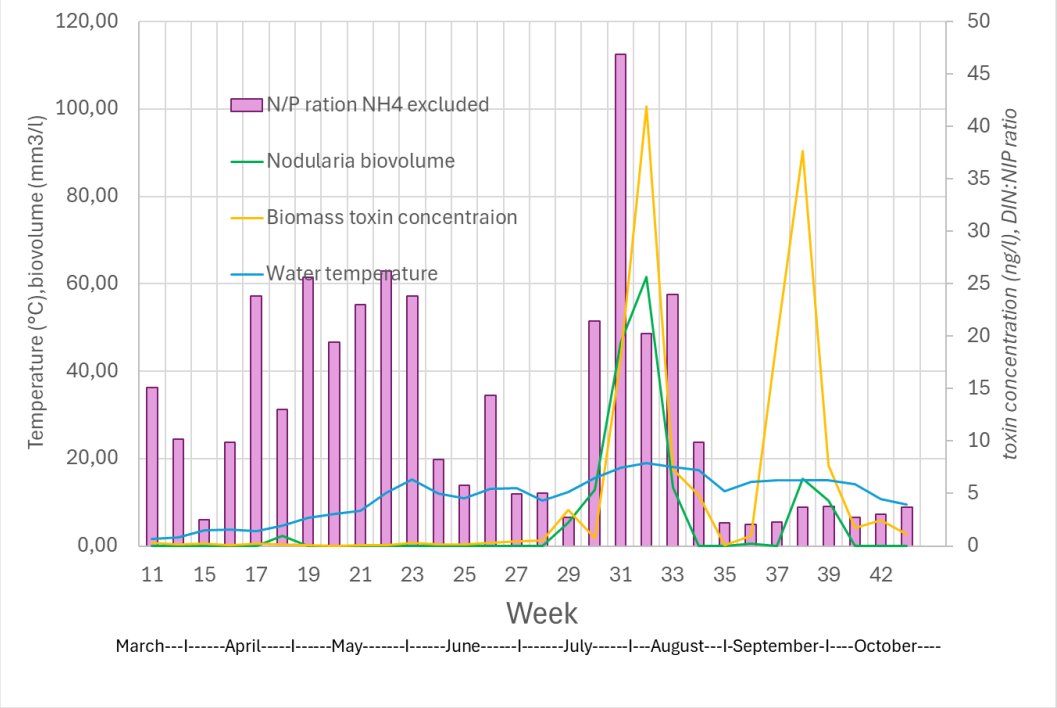


*Figure 3. Relation of biomass toxin concentration (ng/l) to N. spumigena biovolume mm3/l, with regards to the environmental factors water temperature and DIN:DIP ratio (excluding NH4).*

Until June, (figure 3) dissolved inorganic N:P ratio (moles of NOx-N: moles of PO4-P; visualized by the pink bars) was generally over 16, meaning that phosphorus was limiting according to Redfield's ratio (Redfield at al. 1963). Simultaneously *N. spumigena* biovolume and biomass toxicity per litre was low or under detection limit. From week 24 to 30, the nutrient balance shifts, with nitrogen becoming the limiting factor, which benefits diazotrophs like *N. spumigena,* who as previously stated start increasing rapidly around week 30. It is also during week 30 that another shift in the DIN:DIP ratio occurs, and values over 16 once more indicate a DIP limitation. This increase in DIN:DIP ratio aligns very well with the first *N. spumigena* biovolume peak: both of them decrease drastically by week 34-35. The second peak in biomass (week 38) occurs during a DIN limitation. Figure 3 also shows a somewhat similar trend between cyanotoxins from biomass and *N. spumigena* biovolume, with a clear increase in both around weeks 32 and 38. However, while biomass toxins are at a quite

similar level during both peaks, the difference in biovolume is quite large. Fluctuations in water temperatures have a somewhat similar, but much less drastic trend: with a dip in temperature during week 35, in between the two toxin- and biomass peaks where temperatures were generally higher.

The inorganic nitrogen from $NH_4^+$ was excluded due to surprisingly high values yielded, from which it was concluded that some microbial processes may have interfered with the water, and/or that cell ruptures during filtering may have led to $NH_4^+$ being released.

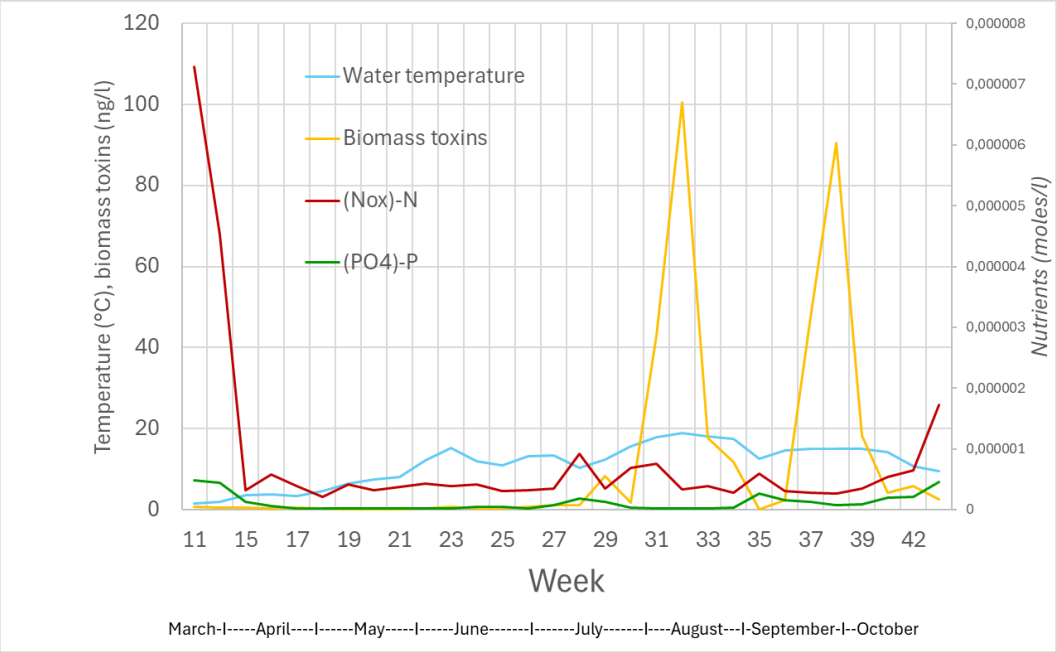


*Figure 4. The environmental factors inorganic phosphorus availability (moles/l) (from $PO_4^-$), total nitrogen from $NO_2^-$ and $NO_3^-$ (moles/l) and water temperature, in relation to total toxins in biomass samples from surface waters (ng/l).*

*Table 2. Differences between the two peaks in N. spumigena biovolume with regards to measured phosphorus, nitrogen concentration, and water temperature.*

| **Environmental factors** | **Peak 1 (week 31-33)** | **Peak 2 (week 38-39)** |
|---|---|---|
| Available phosphate (mmoles/l) | 0.02 | 0.09 |
| Available DIN from $NO_2^-$ and $NO_3^-$ (mmoles/l) | 0.49 | 0.30 |
| Average temperature (C°) | 18.3 | 15.1 |

Table 2 outlines differing environmental factors during the two peaks in *N. spumigena* biovolume during week 31-35 and week 38-39. During the second peak, phosphate was more abundant, nitrate less available, and the temperature slightly lower, compared to the first.

## 3.3 Nodularin concentrations in relation *to N. spumigena* biovolume

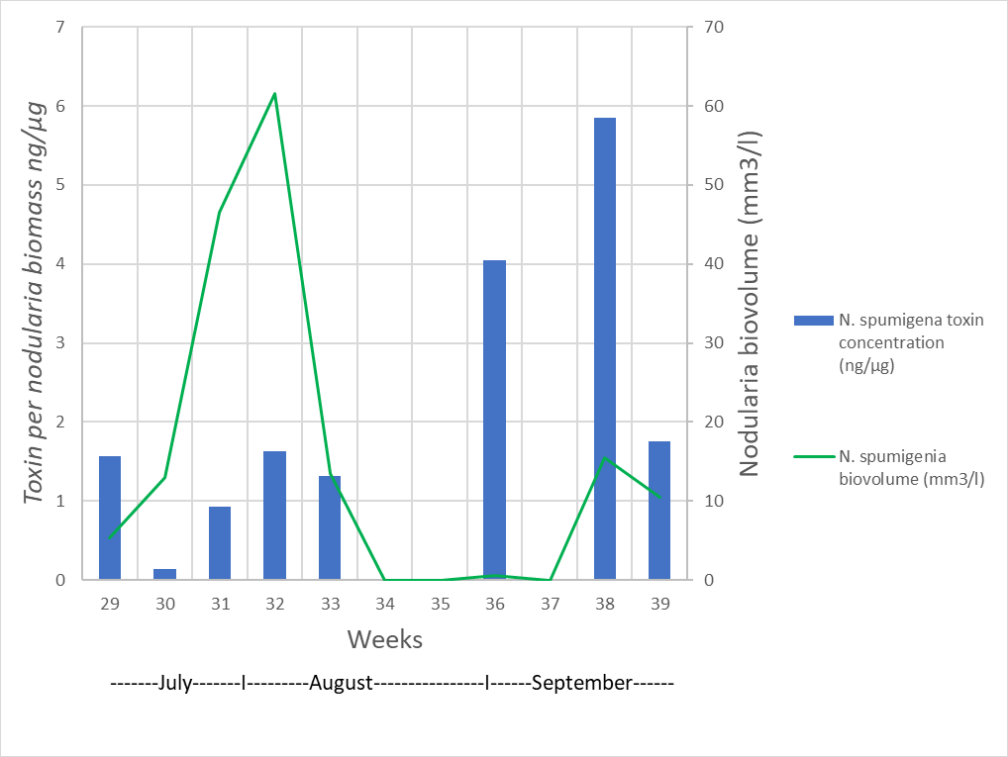


*Figure 5. Concentration of toxins per weight of N. spumigena (ng/µg), along with N. spumigena biovolume ($mm^3$/l).*

The concentration of nodularin per biovolume of *N. spumigena* was relatively low during the first peak (week 29 to 34), but during week 36 and 38, the toxin levels were higher. The empty bars for toxin concentration during week 34, 35 and 37 are due to that the recorded *N. spumigena* volume was 0. However, there were toxins found in the phytoplankton biomass at each of these time points. During week 35 and 36, these levels were very low (0,1 and 2,3 ng/l, respectively).

# 4. Discussion

The results of the LC-MS analysis of mussel tissue is visualized in a time plot in figure 2. Since no microcystins were recorded, it was concluded that while there was presence of *Dolichospermum* during the months sampled they have not produced toxins to any extent large enough to detect. Instead, all toxins quantified are nodularins, probably produced by the cyanobacteria *Nodularia spumigena.* Hereafter, when biovolume of cyanobacteria is considered, only *N. spumigena* is taken into account since that is the most common found toxic cyanobacteria responsible for high nodularin concentrations in the Baltic Sea. The LC-MS results differ greatly from the previous ELISA results from the same mussel tissue toxin extractions, and have been deemed to be a much more accurate representation of reality for reasons soon discussed.

The first peak of toxins in mussel tissue occurred during week 30 (late July). It was also during this week that the biovolume of *N. spumigena* started to increase rapidly, though an increase from levels earlier in the year (week 11-27) could be seen already two weeks before, during week 28. The peak in toxins in mussel tissue coincides with the presence of *N. spumigena*. The trends in both *N. spumigena* biovolume and mussel tissue toxicity after week 30 however, differ greatly. Despite a decline in the *N. spumigena* population by week 34, with no detection and 0.57 $mm^3/l$ biovolume being recorded until week 37, toxin levels remain high in the mussel tissue, even increasing during weeks 33, 35 and 37. This highlights that while the presence of significant concentrations of *N. spumigena* is necessary for toxins to accumulate in mussel tissue, it is not a straightforward, linear relationship. Mussel tissue toxicity continued rising after the *N. spumigena* population crashed, likely due to nodularins being left in the water, and/or bound in zooplankton.

It is notable that at no time point was any tested mussel tissue completely negative for nodularins. The lowest value was detected during week 16 (mid-April) of 3.3µg/kg. Since there was no *N. spumigena* recorded in the water samples at this time, it is expected that the toxin concentration are at their lowest during this time. It also suggests a sort of threshold value where the mussels are not able to rid themselves of nodularins below the value of around 4µg/kg. This value was found by taking the average of toxin levels between the weeks 11 and 28, which were chosen due to *N. spumigena* biovolume being near detection limit at this time. Later in the season they clearly show being able to purify themselves of nodularins, decreasing in toxic concentration from  to 62 µg/kg to 7 µg/kg in 9 weeks (from week 37 to 46), an ability that has been well established in previous studies (Burmester et al., 2012,  Camacho-Muñoz et al., 2021). With basis in the

LC-MS analysis data, 4 µg/kg can be established as a crude baseline of toxicity for mussels farmed in Dalarö/Stockholm archipelago during the year 2024. The surface and deep samples show quite different toxin concentrations, with the deep samples having a significantly lower concentration of toxin. As previously discussed, this is likely due to cyanobacteria congregating near the surface, leading to much lower levels of toxins at 10 metres deep. It is notable that the deep samples appear to be following the same trend as the surface ones, with the toxin concentrations rising between weeks 31 and 38.

One of the factors that appear to have a strong effect on the amount of toxins ending up in the mussel tissue was temperature. During the weeks 29 to 43, there were prolonged periods of water temperature higher than 15°C. While the water temperature was above this limit during week 23, this does not appear to be enough to stimulate the growth of *N. spumigena,* in turn leading to no significant toxin accumulation. The seasonality of mussel tissue nodularin toxicity sampled at 3 m can thus be established as low, steady concentrations of around 4 µg/kg during most of the year, with a strong peak during the end of summer and early autumn, following the rapid expansion of *N. spumigena.* A similar trend in nodularin concentration can be found in Olofsson et. al's 2025 study of the same mussel farm, but with lower levels: a max value of 22 µg/kg versus this study's findings of 62µg/kg. Despite fluctuations in the *N. spumigena* abundance, the toxin levels remained high in the mussel tissue, as the mussels continue accumulating toxins remaining in the water, only dropping when the population of *N. spumigena* decreases permanently for the season, and the toxins left in the body subsequently dissipated, around 3-4 weeks later. A similar seasonality can be established for mussels farmed on a deeper level, but with a less dramatic increase, and overall lower levels of toxins.

The seasonality and overall toxin levels of the mussel tissues can help guide safe harvesting practices. The Australian issued maximum limit of nodularins in shellfish intended for human consumption of 51 µg/kg was exceeded on two occasions in the surface samples: during week 33 and 37. It is important to note however, that the limit is only exceeded marginally during week 33: by 0.33µg/kg. Even so, the toxin levels exceeding, and even being close to the limit of safe consumption should be treated with respect. It is hard to establish with certainty if 2024 is representative of a "normal year", but comparisons can be made with Olofsson et. al's 2025 study, which found similar nodularin trends, but of a lower concentration. While more data is needed to fully establish "normal toxin levels", this suggests that there is little risk for 2024 having been abnormally low in toxin concentrations, which could give a false indication of low toxin rates. Due to climate change induced increasing sea temperature, the notion of a normal

year is also becoming more and more diffuse, since toxin production is likely to increase in the future (Neumann 2013). However, the base line of 4µg/kg established is well below the threshold value. It can be inferred that if harvest of shallowly (3m) farmed mussels is conducted during the colder months of the year (October-May), human consumption is safe. It would be beneficial to determine a more precise window of time based on how fast the mussels are able to rid themselves of toxins, for how long nodularins stay in the water body and aquatic organisms after rapid decreases of *N. spumigena* biovolume, but for this more data is needed, and it is beyond the scope of this thesis. For mussels farmed at deeper levels of 10m, harvest was safe throughout the whole time that mussels were sampled.

Figure 3 illustrates discrepancies between *N. spumigena* biovolume and biomass toxins. Most notably, the second, much smaller peak of *N. spumigena* biovolume around week 36 produces an almost equal amount of toxins as the first. The factors behind this occurrence were examined through the lens of nutrient limitations, overall nutrient levels and temperature. The sharp decrease in the first *N. spumigena* population peak may be explained by phosphate limitation. In tandem with the population crash, the DIN:DIP ratio rapidly decreases to low values around 4. This was, along with fluctuations in temperature, likely to be the reason for the sharp biovolume decrease: there was not enough phosphate left to support a soaring population. However, the measurements for phosphorus were only conducted on its inorganic form, phosphate, not on organic phosphorus. With the possibility of *N. spumigena* using organic phosphorus for growth, this would also need to be measured in order to give a more complete picture. The second, smaller peak in *N. spumigena* biovolume was not limited by DIP, but instead DIN. While diazotrophs can fix their own nitrogen, this is a more energy consuming process than taking up available nutrients from the water body, which may have contributed to the smaller population peak.

Another environmental factor to consider in order to explain the rapid decreases in population is possible large scale water movements. It is possible that sharp decrease in population around week 33 is not purely attributed to nutrient availability and temperature decreases, but that weather events causing currents may have dispersed the cyanobacteria away from the test site. It was not within the scope of this thesis to study water currents and weather events, but this may be an important aspect to examine and control for in future research.

The reason for the large biomass toxin concentration differences during the two peaks becomes apparent in figure 5, that visualizes not just the overall toxin concentration from biomass found in a set amount of water, but the toxin

concentration specifically of *N. spumigena* biomass. While the first concentration measurement is dependent on the totalbiovolume of *N. spumigena* present in the water sample, the second focuses solely on toxin concentration per cyanobacterial biomass. In the later peak, the concentration of nodularins per biovolume of *N. spumigena* is much higher than the first. This would explain why the lower overall biomass of *N. spumigena* in the second peak was able to produce a similar amount of toxin as the first. On average, every individual *N. spumigena* produced more toxin than the individuals in the first peak.

The reasons behind the relatively high toxin concentration per biovolume of *N. spumigena* are perplexing, and not fully examined in this thesis.
The environmental differences between the two peaks of *N. spumigena* populations are visualized in figure 4 and can be summarized, with regards to the second, less abundant peak as: a lower temperature, a higher phosphate but a lower $NO_x$-availability. There are several studies that report *N. spumigena* shifting into a higher gear of toxin production when nutritionally stressed (Stolte et. al 2002), but this is with regards to phosphate limitations. Since the less abundant peak had both a lower N:P ratio, and overall more available phosphate, this is at odds with the literature. A lower temperature has in some cases been found to increase toxin production (Silveira and Odebrecht 2019, Hameed et al. 2017), but those findings cannot directly be applied to these circumstances of around 15 °C. This warrants questions about how much weight can be put into the recorded toxin concentrations per biovolume. While not impossible, more replicas of water samples would be needed. With just one sample collected per sampling time point, there is quite a risk of this not being fully representative of the environment at that time.

A question mark that remains is the high peak of nodularins in mussel tissue during week 37, despite the *N. spumigena* population being absent at this time point. In itself a rising toxin concentration is not unexpected: mussels accumulate toxins in their tissues and nodularins being able to remain in the water body and other organisms for sometime even after a *N. spumigena* population crash. What stands out with week 37 is that it had been 4 weeks since any *significant N. spumigena* biomass, and the overall trend in mussel tissue toxin was downwards: suggesting that the rate with which the mussels were purifying themselves was larger than the rate that they were taking up any remaining nodularins. The way in which the mussels purify themselves is largely through excretion of faeces and pseudofaeces, very little is attributed to limited metabolic breakdown or transformation of toxins (Camacho-Muñoz et al 2021). The sudden reversal of this trend, despite no significant *N. spumigena* population around, was therefore unexpected.

The reason is likely that the water sample collected, later analysed to produce biovolume data, was not representative of the whole water body, and that was in fact a significant *N. spumigena* population at this time. During the previous week, there was a very small *N. spumigena* population recorded, with the highest toxicity per biovolume of the whole season, which may also have had an effect. A combination of the two is possible. These are possible explanations, but cannot be said with that much certainty. Once again, the fact that only one water sample was collected per sampling point limits how much weight can be put into conclusions based on *N. spumigena* biovolume and concentrations of toxins from cyanobacterial biomass.

The results from the ELISA analysis (figure 1) were not in accordance with expectations. Firstly, the toxin concentration spikes several times when there were very low levels of cyanotoxins in the water samples, like in week 9, 11 and 19. Toxin levels from cyanobacterial biomass were still very low during these periods, and did not have any kind of notable increase until week 32 (shown in figure 3). While it is possible to miss a spike of cyanotoxin production when water sampling, if the samples are far in between, this is not deemed likely in this case as the discrepancy is too large, and it does not make sense seasonally. The weeks with the biggest peaks are in the colder seasons at the beginning of the year, where cyanobacteria levels are very low and do not bloom.

Secondly, there was a very high fluctuation in the toxin levels, the most extreme case being a decrease of 87% between week 11 and 12. While mussels have been reported being able to purify themselves of cyanotoxins (Burmester et al., 2012, Camacho-Muñoz et al., 2021), a rate this dramatic is not expected. The fluctuations do not follow any sort of trend seen in cyanobacterial abundance or toxin levels found in cyanobacterial biomass. Thirdly, there are not the expected instances of negative or low in toxicity mussels in the colder months, where cyanobacterial abundance is very low. If the mussels do indeed have such an effective way of ridding themselves of toxins as figure 1 suggests, it is surprising and unlikely that they do not exhibit stable, lower toxin levels during early and late weeks, where cyanobacterial biovolume is very low.

Lastly, the relationship, or in a way, the lack thereof, between the deep and the surface samples are surprising. Since many cyanobacteria have developed buoyancy, it is well established that they concentrate mostly in the surface water. Thus, toxin concentrations are expected to be higher near the surface, with deep samples having lower levels, but following a similar pattern as the surface samples. In figure 1, deep toxin concentrations do not follow a similar trend as the

surface samples, and more importantly: they are on four occasions higher than the surface toxin levels. If this was the only unexpected result it would warrant further investigation, but together with the first three reasons it is deemed highly probable that something has interfered with the analysis and that the results do not represent reality. To establish this, the ELISA results are contrasted with the LC-MS analysis, which was conducted with the same methanol extractions.

These unrealistic ELISA results have several probable causes. One is the risk of more than 1% of methanol being left in the extractions, which would interfere with the ELISA analysis. Care was taken to avoid this by letting the extraction samples evaporate for about 5 days until the residue was of a dry, sticky, tar-like consistency. This was taken as a sign that all methanol had evaporated, however there is a small possibility of methanol remnants large enough to disturb the analysis. Another possible problem regarding methanol is in the extraction process itself. Methanol is a very potent polar solvent, and in hindsight there is risk that it extracted a wide variety of compounds, not limited to just cyanotoxins. Other compounds like amino acids, vitamins, fatty acids, peptides and more may have been dissolved and resulted in a disturbance of the ELISA analysis. This is deemed as the most likely source of error. A final source of error can be found in the sampling process. At the farm, some sample locations were at times nearing depletion of mussel individuals. To combat this, the mussel farmers would refill the locations, taking mussels from other places around the farm. This introduces possible error, as the samples taken are no longer representative of that location.

This does not mean that an ELISA analysis is unsuitable for further analysis of mussel tissue microcystin and nodularin in absolute terms. There are many protocols for extracting toxins from mussel tissue that could prove effective. The issue faced is often that of time limitations, with many other protocols requiring substantially more time. In order to examine if the error lies in too much methanol being left in the samples, the methanol could be changed to another solvent and/or that could have been combined with $N_2$ evaporations to ensure complete methanol evaporation. The limitation is once again time, and thus, cost.

# 5. Conclusions

With the LC-MS results, insights into the seasonality of microcystins and nodularin concentrations in mussel tissue could be determined. No microcystin was detected at any time of the year, while a concentration of around 4 µg/kg of Nodularins was deemed a baseline toxicity. The mussels, while able to purify themselves of substantially higher levels of toxins, were not able to decrease the concentration much below the baseline. During sustained periods of water temperatures above around 15°C, rapidly increasing *N. spumigena* populations and subsequent nodularin production led to higher levels in the toxin in the mussel tissue. These levels dropped once *N. spumigena* populations had dropped near absence, and the toxins contained in the water body as well as other organisms had dissipated, a process taking about 4 weeks. This depuration period can give mussel farmers a guideline for safe harvesting.

The mussels from the surface samples (3 m) were at two occasions above the threshold value for safe human consumption. However, we show that there was only a risk for harmful levels of nodularins during the relatively warmer months. If harvested between October and May, toxin levels were consistently low enough to be deemed safe with regards to nodularins. Mussels from the deep samples (10m) were not above the available regulatory limits during any point in the year. With warming temperatures due to climate change it is possible that the window of safe harvesting shrinks. There is no risk however, that the Baltic Sea in the near future would have such warm temperatures that *N. spumigena* would produce toxins year round, so while the safe harvesting window may shrink, it will not disappear. These results are only regarding nodularin and microcystins and *N. spumigena*, and can therefore not with certainty say that mussels farmed in the Baltic are safe for human consumption. Further analysis of other phytoplankton toxins must be assessed before such a statement with such weight can be made.

The relationship between *N. spumigena* population size and nodularin production is not linear. Analysis using biovolume of *N. spumigena* suggested great differences between the concentration of nodularins per biovolume of *N. spumigena* between the two main peaks in populations recorded. Possible explanations for this discrepancy lies in differences in environmental factors like temperature and nutrient availability, causing mild stress which can lead to increased toxin production. These explanations remain possible, theoretical ones, as the lack of water sample replicates prevents certainty. The results should be taken with extra caution, since increased toxin production following nitrogen stress is at odds with current literature.

Analysing mussel tissue toxins using ELISA analysis, in combination with a methanol toxin extraction yielded unreliable results. This was likely due to the methanol dissolving other compounds that interfered with the assay, and/or methanol still remaining in the extractions, also causing interference. While measures like solvent change and different extraction methods in theory could enable high quality results using ELISA analysis, in practice it is deemed that these measures are not viable due to their time consumption, the lack of which is the biggest advantage of ELISA analysis. In contrast, LC-MS analysis of mussel tissue toxins yielded realistic results. Another advantage of LC-MS in the method's classification as well as quantification, giving insight into the different rates of toxin types.

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# Annex 1 LC-MS analysis

**Quantitative Method**

The compounds were analysed using liquid chromatography tandem mass spectrometry at Lund University, Sweden. Samples were extracted at SLU, Ultuna.
10 µl sample was injected and an ACQUITY HSS T3 UPLC column (100Å, 1.8 µm, 2.1 mm x 100 mm) was used for separation. The analysis was performed on a 6400 Series Triple Quadrupole LC/MS system (Agilent Technologies). The mobile phases were MilliQ water and acetonitrile (A), both with 0.1% formic acid, and the flow was 0.45 mL/min. The gradient was held at 1% B for 1 min, and was then linearly increased to 70% B within 9, then 90% B at 9.10 min and held there for 1 min, before decreasing back to 1% B within 10 s and left at 1% B for another 2 min.

Analytical method details are presented in Table S1. Blank controls and recovery standards were analysed with every analytical batch. The limit of detection (LOD) was 0.1 ng/ml and was calculated from water blanks and defined as three times the standard deviation of the concentration corresponding to the peak at the same retention time as the individual compounds.

*Table S1. Analytical details for quantification of eight compounds in water samples, including retention time (RT), quantitative and qualitative ion transition and collision energies (CE in eV).*

| **Compound** | **RT (min)** | **Transition** | **CE (eV)** |
|---|---|---|---|
| NOD | 6.4 | 825 -> 135 (quantitative) | 70 |
| | | 825 -> 103 (qualitative) | 137 |
| Cylsper | 2.4 | 416 -> 194 (quantitative) | 37 |
| | | 416 -> 336 (qualitative) | 21 |
| mc-Asp-RR | 6.1 | 513->135 (quantitative) | 36 |
| | | 513->103 (qualitative) | 79 |

| | | | |
|---|---|---|---|
| mc-RR | 6.3 | 520->135 (quantitative) | 34 |
| | | 520->127 (qualitative) | 46 |
| mc-Asp-LR | 6.1 | 981.5->135 (quantitative) | 70 |
| | | 981.5->213 (qualitative) | 58 |
| mc-LR | 7.0 | 995.5->135 (quantitative) | 67 |
| | | 995.5->213 (qualitative) | 77 |
| mc-HilR | 7.2 | 1009.5->135 (quantitative) | 64 |
| | | 1009.5->213 (qualitative) | 76 |
| mc-WR | 7.2 | 1068.5->135 (quantitative) | 70 |
| | | 1068.5->213 (qualitative) | 60 |
| mc-YR | 6.9 | 1045.5->135 (quantitative) | 72 |
| | | 1045.5->213 (qualitative) | 58 |

**Names:**
Nodularin, Cylindrospermopsin, Microcystin-RR, Microcystin-LR, Microcystin-YR, Microcystin-WR, Microcystin-HilR, Desmethyl-Microcystin LR and Desmethyl-Microcystin RR

# Annex 2 Phytoplankton identification and quantification

Qualitative and quantitative analysis of phytoplankton were done using an inverted microscope (Delta Optical, Poland) equipped with phase contrast and 100×, 200× and 400× magnification. Lugol's samples were settled in 2-3, 10 or 25 ml sediment chambers for minimum 3 or 24 hours depending on phytoplankton density. Next, the material was analysed using Utermöhl's method, according to the recommendations made by the Helsinki Commission (HELCOM 2021). Units were considered to be individual cells, cenobia, colonies and 100 μm trichome sections (ind./l). The volume and biomass of cells were calculated on the basis of geometric formulas according to the shapes of particular cells, and using size classes in accordance with the guidelines set out by Olenina et al. (2006), updated appendix available at ICES website (www.1.). Phytoplankton was identified at the lowest possible taxonomic level using appropriate literature and keys for marine and freshwater environments (e.g. Komárek and Anagnostidis 1999, 2005; Komárek 2013; Pliński and Hindák 2010; Pliński and Owsianny 2011, Pliński and Witkowski 2009, 2011). The preparation of a list of currently binding taxa was based on the World Register of Marine Species (www.2.) and AlgaeBase (www.3.).


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# Annex 3 DIN analysis

## Determination of $PO_4$-P, $NH_4$-N, and $NO_2^-$+$(NO_3)^-$-N from water samples

Around 12-15 ml of water was filtered through a 0.22 µm PVDF filter after water collection and frozen at -20°C until the determination of $PO_4$-P, $NH_4$-N, and $NO_2$+$(NO_3)$-N.

The measurement of $NH_4$-N was done according to **Grasshof et al. (1999)**, for the measurement of $PO_4$-P **the SS-EN ISO 15681-2:2018** was used, and for the determination of $NO_2$+$(NO_3)$-N the method described in **SS-EN ISO 13395:1996** was applied. All three methods were modified for **Alpkem SFA.**

The measurement range for $NH_4$-N was 0.5 – 2500 µg/l, for $PO_4$-P 0.5 – 500 µg/l and for $NO_2$+$(NO_3)$-N 0.3 – 1600 µg/l.

| Measured Parameter | Method | Principle | Sample Type | Measurement Uncertainty *(expanded, coverage factor k=2)* | Measurement Range |
|---|---|---|---|---|---|
| **Ammonium Nitrogen** | Grasshof et al. (1999), modified for Alpkem SFA | SFA, determination of formed indophenol blue | 1:1, 1:3 | <3 µg/L: 0.5 µg/L<br>3–30 µg/L: 1.7 µg/L<br>>30 µg/L: 5% | 0.5–2500 µg/L |
| **Phosphate Phosphorus** | SS-EN ISO 15681-2:2018, modified for Alpkem SFA | SFA, determination of formed antimony-phospho-molybdenum blue (reduction with ascorbic acid) | 1:1, 1:3 | <2 µg/L: 0.5 µg/L<br>2–25 µg/L: 1.0 µg/L<br>>25 µg/L: 5% | 0.5–500 µg/L |
| **Nitrite + Nitrate Nitrogen** | SS-EN ISO 13395:1996, modified for Alpkem SFA | SFA, reduction of nitrate to nitrite in Cd/Cu-reductor. Determination of nitrite after formation of an azo dye. | 1:1, 1:3 | <2 µg/L: 0.3 µg/L<br>2–20 µg/L: 1.3 µg/L<br>>20 µg/L: 4% | 0.3–1600 µg/L |

The reported measurement uncertainty is an expanded measurement uncertainty with a coverage factor k=2, which approximately corresponds to a 95% confidence

interval, calculated according to the "Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories," SP Report 2015:74 (translation of the NORDTEST report TR 537), together with the MUKit software.

(*) The reported measurement uncertainty is an expanded measurement uncertainty with a coverage factor k=2, which approximately corresponds to a 95% confidence interval, calculated according to the Eurachem/CITAC Guide, *Quantifying Uncertainty in Analytical Measurement* (2nd Edition, 2000).

Sample types:

- 1:1 = freshwater
- 1:3 = brackish and seawater
- 10:4 = sludge/sediment

Measurement range = analyzable concentration range without dilution. Any impact on samples during sampling and transport is beyond the Institute for Ecology, Environment and Botany of Stockholm University’s control.

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