Response (AC1) to Anonymous Referee #1 (RC1)

General comments

Reviewer comment (RC1). The manuscript has an interesting dataset where the authors combine *in situ* measurement with satellite imaging to estimate areal nitrogen fixation with the benefit of reducing bias due to patchiness of cyanobacteria blooms. I have however a few concerns and questions to the authors to address. I therefore suggest a revision before considering it for publication.

Reviewer comment (RC1). Something that was surprising to me was how come you didn't find any picocyanobacteria? In Zilius et al. 2020 I interpret it as you had about 20% of the community during summer? Also in Klawonn et al. 2016, colonial picocyanobacterial comprise ca. 5-10% of the cyanobacterial community in terms of carbon. It seems like you sampled on similar locations, maybe even at the same time, as in Zilius et al. 2020 so this needs an explanation. If it has to do with method differences, it needs to be explained or the statement of no picocyanobacterial removed and refer to previous study.

Answer: We acknowledge the reviewer for their positive comments. In this study, taxa referred as "colonial picocyanobacteria" by the reviewer were found with microscopy counting, and due to their relatively low contribution (generally <2% of total biomass) they were assigned to "non-N_2-fixing cyanobacteria", and thus not further discussed in the submitted manuscript (Fig. 2). In the revised version of our manuscript, we have added information related to cyanobacteria composition and their biomass: "*Non-filamentous colonial cyanobacteria, such as Aphanocapsa spp., Aphanothece spp., Merismopedia spp. and Cyanodictyon spp. exhibited low biomass (< 2% of total) except in June, when their contribution reached 12% at the northern site (Fig. 2). Picocyanobacteria were not detected during the study period at either site." (line 207-210*)

In Zilius et al. 2020, sequences were attributed to picocyanobacteria (not referring here as "colonial picocyanobacteria"). However, a volume of 50 to 70 ml was extracted for further sequencing and only few reads were assigned to picocyanobacteria. This means that picocyanobacteria were rare in this study and that they would not be detected by methods allowing quantification such as flow cytometry or epifluorescence microscopy. Both approaches are complementary and not contradictory since DNA methods can detect rare taxa but do not allow quantification yet.

Reviewer comment (RC1). I am also a bit concerned about the method you use for measuring N_2-fixation with injection of gas rather than pre-dissolved. I think this might cause an underestimation. Also the fact that you run 24 h incubations probably lead to underestimations of N_2-fixation per h since they do less in the night when its dark (1.8 times less; Klawonn et al. 2016). I think a potential underestimation should be discussed and rates presented as per day since this is what you measure.

Answer: Regarding the issue of hourly vs. daily rates of fixation, we agree with the reviewer's point that rates are likely to vary on a diel cycle (being lower at night). Therefore our diel incubations conducted under natural (outdoor) light conditions are more suitably expressed as daily rates than hourly rates since they are representative of both light and dark cycles. In the revised manuscript, we present daily values in figures and text.

With regards to methodology, we agree that there has been some debate about using the bubble method for N_2 fixation measurements (Mohr et al., 2010; Großkopf et al., 2012; White et al., 2020), but recent work (Wannicke et al., 2018) demonstrated that underestimation of rates is negligible (<1%) for incubations lasting 12–24 h. In the submitted version we have argued our choice for incubation duration: "*As the isotopic equilibration takes up to several hours (Mohr et al., 2010), we incubated the samples for 24 h, thus minimizing equilibration effects (Mulholland et al., 2012;*

Wannicke et al., 2018." (line 136-138). Eventually, our used technique avoids to have low labelling (percentage label should be between 5-10%) as the labelled seawater method often results in low quantities of $^{15}N_2$ gas in the water (e.g. Klawonn et al. (2015) had only 1% label in their experiment).

Reviewer comment (RC1). The theory of underestimation is further supported by that you have 1000-3000 µg cyanobacterial C per L and 120-200 nmol N2 fixation per h as compared to Klawonn 2016 where 100 µg cyanobacteria per L performed 80 nmol N_2 fixation per h. Why do you think you have so low rates as compared to your biomass? Can it be P limitation?

Answer: We agree with the reviewer's point that P limitation may play a role in limiting N fixation in the Curonian Lagoon. In a prior study, it was shown that P additions stimulated growth rates of N_2 fixing cyanobacteria from the Curonian Lagoon (Pilkaitytė and Razinkovas, 2007*)*. Likewise, addition of P stimulated diazotrophic community resulting in elevated N_2 fixation rates (Moisander et al. 2007). We may expect that dissolved P was limiting, which constrained N_2 fixation during summer . Thus, we suppose that DIP release from sediment and higher biomass of *Aphanizomenon* and diazotrophic activity frequently observed in the end of summer (Zilius et al. 2014, 2018) are not coincidence but rather consequence of increased P availability. We have modified the Discussion to address this point "*The proliferation of heterocystous cyanobacteria in the Curonian Lagoon is favoured by P (Pilkaitytė and Razinkovas, 2007), which is released from sediments, particularly when bloom conditions result in high water column respiration and transient (night-time) depletion of oxygen (Petkuviene et al., 2016; Zilius et al., 2014). Moisander et al. (2007) demonstrated that P can enhance diazotrophic activity of heterocystous cyanobacteria in microcosms. Release of dissolved P from sediments may in turn enhance rates N_2 fixation resulting in a positive feedback for cyanobacteria bloom development."* (line 299-304)

Reviewer comment (RC1). What effects do you think the fact that cyanobacteria only comprised about up to 36 or 86% of the phytoplankton fraction has for your correlations with chlorophyll and further areal estimates of N_2-fixation? I guess it must be very variable over the year how well your method can be applied? I think you should discuss this bias further.

Answer: With regard to our ability to model N_2 fixation on the basis of Ch-a, we specifically address this point in the discussion: "*We benefitted from prior work deriving Chl-a estimates from satellite images and their calibration to in situ measurements (Bresciani et al., 2014), but the success of the approach largely relied on the fact that heterocystous cyanobacteria dominated the summer–fall phytoplankton community of the lagoon, which provided a significant correlation between N_2 fixation and in situ Chl-a. However, it would be problematic to extrapolate this approach to periods outside of cyanobacteria dominance (e.g., spring diatom bloom) or to periods when other factors (e.g., low temperature in fall) constrain N_2 fixation*." (line 312-317)

References

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Pilkaitytė, R., and Razinkovas, A.: Seasonal changes in phytoplankton composition and nutrient limitation in a shallow Baltic lagoon. Boreal Environ. Res., 12, 551–559, 2007.

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Specific comments

Reviewer comment (RC1). Line 45-46, when they are dead I guess? Maybe clarify that this is when they are detritus on the bottom.

Answer: we assume that respiration of living cells rather detritus cause bottom hypoxia. During summer blooms, when plankton (mainly cyanobacteria and heterotrophic bacteria) respiration exceed diffusive oxygen supply to deeper layer, benthic community eventually depletes oxygen from adjacent bottom. We have clarified sentence and it reads now "*Large blooms of living cyanobacteria are associated with high oxygen demand in the water column, which results in transient (night-time) bottom hypoxia and enhances the release of dissolved inorganic phosphorus (DIP) from sediments (Petkuviene et al., 2016; Zilius et al., 2014).*" (line 45-47)

Reviewer comment (RC1). Line 52, any references to the patchiness? Maybe Rolff et al. 2007?

Answer: thanks for suggestion. We have included this reference.

Reviewer comment (RC1). Line 100, triplicates of samples or sampled from the same flask?

Answer: we collected water samples in triplicates, and in the laboratory each of them were filtered separately. Text modified: "*Triplicate water samples from each site or layer were filtered (Whatman GF/F, pore size 0.7 µm) for inorganic and organic nutrient analysis as previously described by Vybernaite-Lubiene et al. (2017).*" (line 100-101)

Reviewer comment (RC1). Line 109, maybe the Whatman and pore size should be on line 100 when first mentioned?

Answer: corrected accordingly.

Reviewer comment (RC1). Line 120, I think it would be good to provide heterocysts per number of vegetative cells as well since they change in density over the season (Svedén et al. 2015).

Answer: following the reviewer's suggestion, we added estimates of heterocyst frequency per cyanobacteria filament in the revised version of the manuscript, see updated Figure 6:

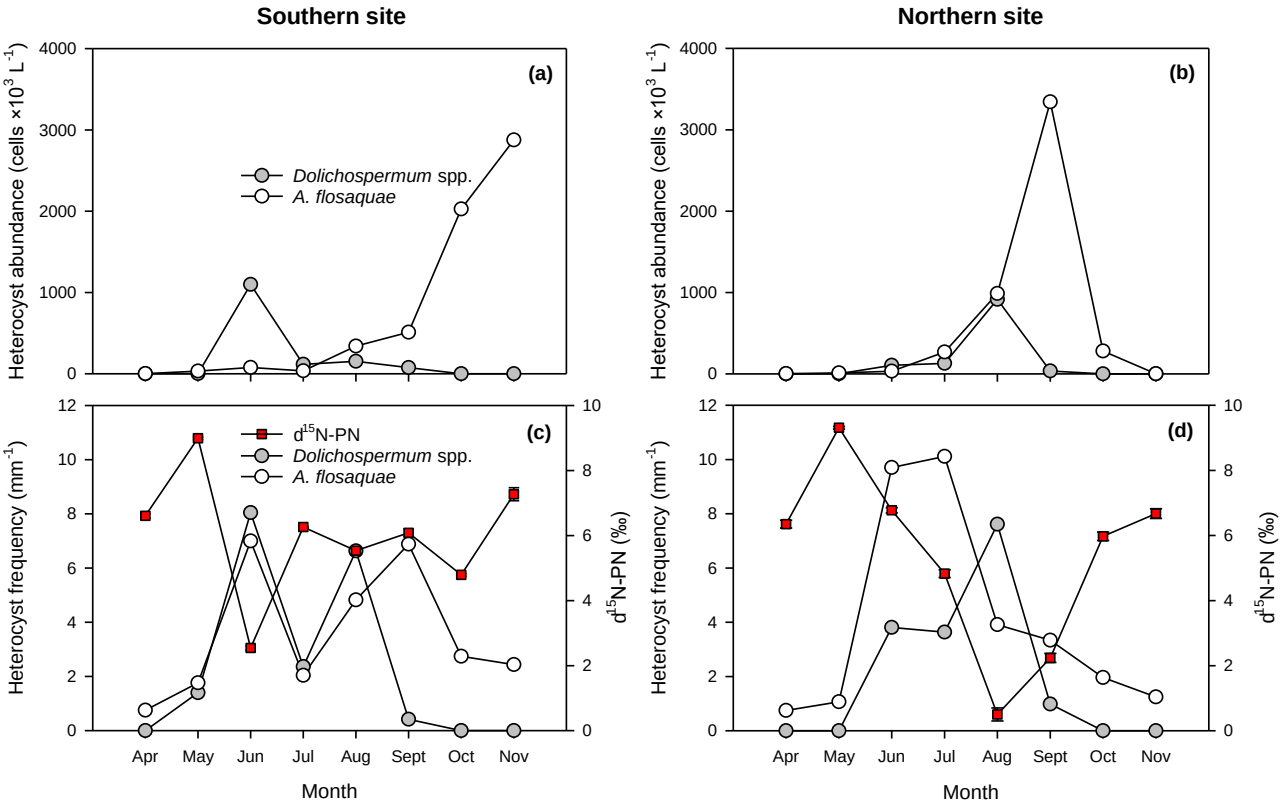

Fig. 6. Abundance of heterocysts of *Dolichospermum* spp. and *A. flosaquae* (a, b), heterocyst frequency per filament (c, d) and stable isotope composition of particulate nitrogen (δ^{15}N-PN) at southern and northern sites in the Curonian Lagoon during 2018. δ^{15}N-PN values are mean and standard error (some error bars not visible).

In the text, we have added following information:

„*The number of heterocysts (cell L^{-1}) and their frequency per millimeter of filament (mm^{-1}) was also determined.*“ (line 121-122)

„*Total heterocyst frequency per filament was higher at the beginning of summer (up to 15 mm^{-1}) at both sites, and gradually declined afterwards (Fig. 6c, d).*“ (line 249-250)

Reviewer comment (RC1). Line 128, are not many picocyanobacterial smaller than 3 um? Is this what is commonly used for picocyanobacterial? Did you use any certain settings on the flow cytometer to determine picocyanobacteria, for example a cyano-specific filter? Did you use Sybr? Were they in the same sample as the heterotrophic bacteria or on its own? I am asking this since I am surprised that you did not see any, while you did in Zilius et al. 2020.

Answer: we kindly note the use of the term “colonial picocyanobacteria” is misleading, since “colonial picocyanobacteria” do not belong to picoplankton owing to their colony size, and need to be enumerated by methods designed for nano- and microplankton. “Colonial picocyanobacteria” refer to cyanobacteria cells of 1-3 µm size embedded in mucilaginous colonies. The colonies are most commonly over 10 µm size and not very abundant (less than 1 colony mL^{-1}) in the Baltic Sea. As the colonies are large and mucilaginous inverted microscopy after sedimentation is the preferred method for detection and quantification. Following HELCOM recommendations, the biomass is estimated by converting in biovolume-carbon each cell from the colony. These colonies have typically small abundances <1 colony mL^{-1}. A larger volume of sample is required to detect such cyanobacterial taxa. In Klawonn et al (2016), free-living picocyanobacteria have not been counted, though they are present and abundant in Baltic Proper waters (B1 or BY31 stations). They counted the “colonial

picocyanobacteria" by inverted microscopy after sedimentation of 25 ml of Lugol-preserved samples. In this study, 10 to 25 ml of Lugol-preserved samples were counted by inverted microscopy and the cyanobacteria with cells <3 µm in colonies were assigned to the "non-N_2-fixing cyanobacteria" category. *Aphanocapsa* spp., *Aphanothece* spp., *Merismopedia* spp. and *Cyanodictyon* spp. were detected in low biomass (< 2% of total phytoplankton biomass) during the study period at either site, except in June when their contribution reached 12% at the northern site, as it is now specified in the text "*Non-filamentous colonial cyanobacteria, such as Aphanocapsa spp., Aphanothece spp., Merismopedia spp. and Cyanodictyon spp. exhibited low biomass (< 2% of total) except in June, when their contribution reached 12% at the northern site (Fig. 2). Picocyanobacteria were not detected during the study period at either site.*" (line 207-210)

Picocyanobacteria refer to free-living unicellular cyanobacteria with a size below 2 or 3 µm depending on the size definition chosen. And we used this original definition in the manuscript. They are free-living, belong to the picoplankton and are usually abundant (over 100 cells mL^{-1}) when present. Then can be detected and/or counted by flow cytometry or epifluorescence microscopy, methods designed to count picoplankton. By flow cytometry they are typically counted in volumes of 50-100 µL, as flow cytometry is designed to count small and abundant cells/particles.

In the present study, picocyanobacteria were counted with a flow cytometer following standard procedures. The preservation procedure, the running settings (flow rate, acquisition time, etc.) followed standard recommendations. The analyses for picocyanobacteria were performed independently from the analyses for heterotrophic bacteria. The BD Accuri C6 allows the detection of fluorescence from phycoerythrin (at 585/40 nm after excitation at 488 nm), phycocyanin (at 675/25 nm after excitation at 640 nm) and chlorophyll (>670 nm after excitation at 488 nm). During the analyses many cells showing chlorophyll fluorescence were detected but with no higher phycoerythrin or phycocyanin fluorescence over background level. Therefore, we concluded that no picocyanobacteria was detected in this study.

Reviewer comment (RC1). Line 133, does this mean that the flasks were top-filled without air during incubation? Did you shake/turn the flasks something to help with the mixing?

Answer: yes, the bottle was completely filled, and after injection of gas bubble was gently mixed. The missing information was added: "*The samples were filled into 500 ml transparent HDPE bottles and carefully sealed preventing formation of air bubbles. Each sample received 0.5 ml $^{15}N_2$ (98% $^{15}N_2$, Sigma-Aldrich) injected by syringe through a gas-tight septum, and then gently mixed for 10 min (Zilius et al., 2018).*" (line 134-136)

Reviewer comment (RC1). Line 137, in what way would pre-prepared isotopically enriched water be a risk of contamination? Contamination of what?

Answer: we mean that all ^{15}N label have been excreted into the surrounding waters within the incubation time is likely immediately reused and thus appears on the filters. During short-time incubations, this is in particularly relevant when proportion of excreted ^{15}N is relative close to quantities of dissolved $^{15}N_2$ gas, which can happen when using labelled seawater method water with low tracer percentage. To avoid any confusion this statement was removed from the text.

Reviewer comment (RC1). Line 139, I think its risk of underestimating rates when having flasks totally covered, 1% of light is still light and therefore it would have been more appropriate to have them covered instead. This would be good to mention in the results/discussion.

Answer: we disagree with this point. Measured PAR at 2 m depth was always < 5 µmol m^{-2} s^{-1} (June–November), which is well below 1% of surface water irradiance, see added information in revised version "*Surface water samples were incubated outdoors at ambient irradiance, while samples from 2.0–3.5 m were wrapped in aluminium foil as in situ irradiance was below 1% of surface*

PAR at these depths (< 5 µmol m^{-2} s^{-1} in the period of June–November)." (line 138-140). We appreciate that near-dark may not be quite the same as dark, but given the very low rates relative to surface (photic) values, we feel that this would not appreciably affect our findings. We feel that the more important methodological issue is that in these studies samples are almost always incubated at a fixed light intensity, whereas cyanobacteria mixing in the water column experience a dynamic light environment. This point is made in the Material and Methods: "*However, such fixed dark conditions is less representative to in situ conditions as cyanobacteria colonies can migrate upward to surface photic zone or use limited light for photosynthesis.*" (line 140-142) and in the Discussion: "*Our study, as well as prior work, is based on 24-h incubations, simulating conditions at a fixed depth, which may not be indicative of rates that could be sustained by diazotrophs circulating over a range of depth and light conditions*." (line 363-365)

Reviewer comment (RC1). Line 148-150, did you measure the final labelled concentration in the flask or is this only an estimate from calculations? In case you only estimated the added concentration this can be a bias for your later rate calculations.

Answer: unfortunately, $^{15}N_2$ concentration was not quantified in bottles. Therefore, it may lead underestimation of rates as suggested by White et al. 2020. Though we are aware of method use, there are number of studies still published without testing $^{15}N_2$ concentration in incubated bottles. This information was added in the revised version of manuscript: "*As we have used theoretical estimation of $^{15}N_2$ gas dissolution in bottles instead quantification with membrane inlet mass spectrometer, it can result in some underestimation of rates (White et al., 2020).*" (line 153-154)

References

White, A.E, Granger, J., Selden, C., Gradoville, M.R., Potts, L., Bourbonnais, A., Fulweiler, R.W., Knapp, A., Mohr, W., Moisander, P.H., Tobias, C.R., Caffin, M., Wilson, S.T., Benavides, M., Bonnet, S., Mulholland, M.R., Chang, X.B. 2020. A critical review of the $^{15}N_2$ tracer method to measure diazotrophic production in pelagic ecosystems. Limnology and Oceanography: Methods. doi:10.1002/lom3.10353

Reviewer comment (RC1). Line 153, how deep can you "see" with the satellites?

Answer: Optical remote sensing, i.e. the method based on passive radiometers operating in the visible and near-infrared wavelengths, is the only one which penetrates the surface of the waterbody (Robinson, 2010). The satellites can observe the water down to one optical depth, the portion of the water column where approximately 90% of the remote sensing observed signal originates (Gordon and McCluney, 1975; Werdell and Bailey, 2005). The optical depth is equivalent to the inverse of the diffuse attenuation coefficient (K_d) (Gordon and McCluney, 1975) and has also been shown to empirically relate to the Secchi disk depth (Lee et al., 2018). The range of $K_d(490)$ in Swedish coastal waters of the Baltic Sea during 2008 was 0.31–1.19 m^{-1} (Kratzer, Vinterhav, 2010). In Curonian Lagoon, estimated K_d value from daily buoy measurements was 2.7–5.7 m^{-1} during presence of cyanobacteria (2014-2015). This information was added in Material and Methods, see lines 159-163.

References

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Werdell, P.J., Bailey, S.W., 2005. An improved in-situ bio-optical data set for ocean color algorithm development and satellite data product validation. Remote Sens. Environ. 98 (1), 122–140.

Reviewer comment (RC1). Line 181, why linear regression and not correlations? Don't you expect both of them to be interdependent rather than one dependent?

Answer: while we consider that correlation coefficient represents the direction and strength of the relationship between chlorophyll and N_2 fixation rates, the regression coefficient determines the effect of chlorophyll a (independent variable) on the N_2 fixation (dependent variable), and determine the explained variation.

Reviewer comment (RC1). Line 189, please indicate in text and in figure legends when Chl is derived from *in situ* extractions and when from satellites.

Answer: thanks for the suggestion.

Reviewer comment (RC1). Line 193, who are the non N_2 fixing cyanobacteria if you did not have any picocyanobatceria? For example in November in Figure 2a?

Answer: the main non-N_2-fxing cyanobacteria are represented by *Planktotrix agardhii* and *Microcystis* spp. This information was also added in the text: "*The non-N_2-fixing cyanobacteria were dominated by Planktotrix agardhii and Microcystis spp.*" (line 203-204)

Reviewer comment (RC1). Lines 201-202, this surprises me, no picocyanobacterial at all? Is this common here? In Zilius et al. 2020 you had at least about 20% of the biomass?

Answer: see detail answer above.

Reviewer comment (RC1). Line 205, among how many samples? Do you mean micrograms on the y-axes, please use a proper "micro" symbol for this.

Answer: in the revised version, we have added information that chlorophyll a are presented as mean values and standard error (some error bars not visible) based on three replicates. Yes, on y-axis all units are in micrograms, according to the suggestions we applied proper style of "micro".

Reviewer comment (RC1). Line 209, please explain the clustered numbers (months?) in the figure. Maybe also add a legend title including what the symbols are (biomass?).

Answer: the information was added, and it is "*Phytoplankton biomass and community composition were generally similar between surface and bottom layers (April–September), except in October–November when the abundance of N_2-fixing cyanobacteria was greater in the surface layer (2500–3500 µg C L^{-1}) relative to the bottom layer (<100 µg C L^{-1}).*" (line 212-214). Also we have updated legend in Figure 2.

Reviewer comment (RC1). Line 228, among how many samples?

Answer: data are represented by mean values and standard error based on 3 replicates. Missing information was added to figure captions "***Figure 4****: Temporal patterns in temperature, dissolved organic carbon (a, b), dissolved and organic nitrogen (c, e), phosphorus (e, f), and DIN:DIP ratios (g, h) at southern (left panel) and northern (right panel) sites in the Curonian Lagoon during 2018 (error bars denote standard error based on 3 replicates; some not visible).*"

Reviewer comment (RC1). Figure 5, I think it is better to show N_2-fixation as per day since this is what you measured and since night-time is lower (See Klawonn et al. 2016 for night rates where they show the double rate at day time).

Answer: Thanks for the suggestion. In revised version, volumetric N_2-fixation rates are presented per day (µmol L^{-1} d^{-1}).

Reviewer comment (RC1). Figure 5b, is this low rates maybe related to lack of light?

Answer: yes, these rates were measured in the dark.

Reviewer comment (RC1). Line 236, how does number of heterocysts relates to total biomass/number of vegetative cells of cyanobacteria?

Answer: We have plotted heterocyst abundance versus number of vegetative cells of N_2-fixing cyanobacteria, which indeed provided nice results: "*The abundance of heterocysts varied seasonally depending on the number of vegetative cells of N_2-fixing cyanobacteria ($y=0.0251x+75.0$, $R^2=0.92$) with lowest values less than 2000 cells L^{-1} and peak values exceeding 2 million cells L^{-1} in late summer.*" (line 246-248)

Reviewer comment (RC1). Line 237, please abbreviate to *A. flosaque* throughout the manuscript except at first place mentioned.

Answer: done.

Reviewer comment (RC1). Figure 6, do you mean "per ml" with per mil? How come there is so many heterocysts in November in the southern station but almost no N_2 fixation nor cyanobacteria biomass at that time? In contrast the highest number of both N_2 fixation and heterocysts numbers correlates for the northern station. This needs to be discussed. Also, It would be good to also have heterocysts per filaments/vegetative cells here to see how it changes over the season (*Aphanizomenon* heterocyst density varies with season; Svedén et al. 2015).

Answer: "per mil" means delta units that are expressed in molecules per thousand, but for convenience we have change to "‰".

In revised version, we also provided heterocyst frequency, which better corresponded to N_2 fixation dynamic. The updated Figure 6 shows that patterns in heterocyst frequency was relatively low in November coinciding to decreased N_2 fixation rates. We assume that the October-November period represents the decline of the cyanobacteria bloom. In the submitted version we discussed that "*Results from this, and a prior study (Zilius et al. 2018), show that despite a high abundance of A. flosaquae at the end of fall, heterocyst frequency, and thus N_2 fixation rates declined substantially when water temperature dropped below 15 ^oC. Zakrisson et al. (2014) suggested that temperature controls the enzymatic activity of nitrogenase, which directly regulates the intensity of N_2 fixation in filaments*." (line 363-367)

Reviewer comment (RC1). Line 248, I think you have light limited N_2 fixation? 1% of surface light can still be 10-20 µm photons, which can be sufficient for carbon fixation.

Answer: we agree that N_2 fixation can be light limited in the turbid Curonian lagoon, but we note that the photic zone does not extend into the bottom layer (2–3.5 m depth), therefore we feel it was appropriate to incubate the bottom samples in the dark (see response to prior comment).

Reviewer comment (RC1). Lines 247-251, did you use correlations or regressions? If this is the regression models you later use this must be clear.

Answer: in present study, we have used linear regression, which later allowed to derived N_2 fixation estimates based on remote sensing Chl-a. We have reformulated the sentence to avoid confusion,

and now it reads "*N_2 fixation in the surface layer was significantly ($p < 0.001$) predicted by in situ Chl-a concentration ($R^2 = 0.91$), A. flosaquae biomass ($R^2 = 0.83$) and A. flosaquae heterocysts ($R^2 = 0.88$ all $p < 0.001$; Fig. 7). Whereas N_2 fixation in the bottom layer was weakly explained by in situ Chl-a ($R^2 = 0.52$, $p = 0.07$), but not A. flosaquae biomass or heterocysts. In situ chlorophyll-specific N_2 fixation derived from regression equations (Fig. 7a, b) was considerably lower in the deeper layer (0.002 ± 0.001 µmol N μg^{-1} Chl-a d^{-1}) relative to the surface layer (0.018 ± 0.002 µmol N μg^{-1} Chl-a d^{-1}).*" (line 258-262)

Reviewer comment (RC1). Fig. 7 Clarify that the Chl a from in situ extractions? Is this data from the whole year or only from the summer? For example if this is the whole year where is November value surface layer Chl of 250 µg L^{-1} but with no N_2-fixation.

Answer: we agree that some information is lacking. Not it reads "*Figure 7: Relationships between N_2 fixation and in situ measured Chlorophyll-a (a, b), Aphanizomenon flosaquae biomass (c, d) and their heterocysts (e, f) in surface (northern and southern sites) and bottom (southern site) layers of the Curonian Lagoon during April–September 2018."* (line 264-266)

Reviewer comment (RC1). Line 259-260, can you provide the equation you used for these estimates?

Answer: equation for this estimation is already showed in Fig. 7a, but to be clear we have added this equation in brackets: "*We used the relationship between N_2 fixation and Chl-a ($y = 0.018x – 0.459$; Fig. 7a) for the surface layer to derive estimates of N_2 fixation for the upper water column (0–2 m).*" (line 270-271)

Reviewer comment (RC1). Lines 260-261, how can you use this relationship when it was not significant, then there is no relationship?

Answer: The regression is marginally significant ($p = 0.08$) and has a reasonable R^2 value (0.52). Therefore, we felt that this model provided the best means for estimating bottom layer N_2 fixation. We also note that the bottom layer accounts for a relative small proportion of the lagoon's volume and an even smaller proportion of N_2 fixation (since surface rates are 7x higher). Therefore, our whole-lagoon estimates of N_2 fixation are not highly sensitive to assumptions about bottom water rates.

Reviewer comment (RC1). Line 263, values of what?

Answer: it was referred to estimated N_2 fixation rates. Now sentence reads "*The impact of the bloom on N_2 fixation can be visualized from the relatively low and uniform estimated rates throughout the lagoon during July, and the subsequent development of localized hotspots in the southern lagoon during August and September (Fig. 8).*" (line 275-276)

Reviewer comment (RC1). Line 282, if you have ten times more *Aphanizomenon* you maybe should also have higher N_2 fixation rates? This needs to be discussed and refer to data, for example Klawonn et al. 2016. Were they limited by something?

Answer: see our earlier answer.

Reviewer comment (RC1). Line 298-300, but cyanobacteria did not dominate all the time and never close to 100%? How does this affect the results? When they are less then 50% of the Chl community is this not overestimating the N_2-fixation rates? This needs to be discussed. The bottom layer had a lot of other organisms contributing to chl biomass.

Answer: see our earlier answer.

Reviewer comment (RC1). Lines 309 and below, can you also put these areal N_2-fixation estimates into perspective to other studies for the region? For example, Klawonn et al. 2016 and Olofsson et al. 2020 as well as references there in.

Answer: thanks for suggestion. We have put our estimates in the context of the Baltic region: "*These estimates reveal that summer N_2 fixation rates are slightly lower in the coastal site of SW Baltic (3.6 ± 2.6 µmol m^{-2} d^{-1}; Klawonn et al., 2016), but higher than those found in the Great Belt (~ 1 mmol m^{-2} d^{-1}; Bentzon-Tilia et al., 2015), Baltic Proper (0.4 ± 0.1 mmol m^{-2} d^{-1}; Klawonn et al., 2016), and Bothnian Sea (0.6 ± 0.2 mol m^{-2} d^{-1}; Olofsson et al. 2020b).*" (line 328-331)

Reviewer comment (RC1). Line 343, how can the heterocyst frequency be so high without cyanobacteria biomass being high?

Answer: Heterocyst abundance tracks cyanobacteria abundance, but in the revised version of manuscript, we also show that heterocyst frequency per filament decreases when biomass increases in November (see figure above).

Reviewer comment (RC1). You need to discuss problems with N_2 fixation from covered flasks and still standing flasks with gas injections somewhere in the discussion. "Caveats" with this study.

Answer: in the revised manuscript, we have stated that "*Our study, as well as prior work, is based on 24-h incubations, simulating conditions at a fixed depth, which may not be indicative of rates that could be sustained by diazotrophs circulating over a range of depth and light conditions.*" (line 359-361)

Technical corrections

Reviewer comment (RC1). Line 33, comma after algae.

Answer: added.

Reviewer comment (RC1). Line 39, I think you need to name this 2020a since it the first one appearing?

Answer: corrected.

Reviewer comment (RC1). Line 500, Please change to Riemann

Answer: done.

Response (AC2) to Anonymous Referee #1 (RC3)

General comments

Reviewer comment (RC3). I am pleased to see that the authors have improved the manuscript according to the comments below. Which includes being open about some caveats, comparison to other studies, some methods that were missing and additional data on heterocyst frequency. However, there is still some minor adjustments I would like to suggest before moving on. See specific comments below.

Answer: we acknowledge the reviewer for their positive comments, which are appreciated. Our responses to follow-up comments are provided below.

Reviewer comment (RC1). Something that was surprising to me was how come you didn't find any picocyanobacteria? In Zilius et al. 2020 I interpret it as you had about 20% of the community during summer? Also in Klawonn et al. 2016, colonial picocyanobacterial comprise ca. 5-10% of the cyanobacterial community in terms of carbon. It seems like you sampled on similar locations, maybe even at the same time, as in Zilius et al. 2020 so this needs an explanation. If it has to do with method differences, it needs to be explained or the statement of no picocyanobacterial removed and refer to previous study.

Answer: We acknowledge the reviewer for their positive comments. In this study, taxa referred as "colonial picocyanobacteria" by the reviewer were found with microscopy counting, and due to their relatively low contribution (generally <2% of total biomass) they were assigned to "non-N_2-fixing cyanobacteria", and thus not further discussed in the submitted manuscript (Fig. 2). In the revised version of our manuscript, we have added information related to cyanobacteria composition and their biomass: "*Non-filamentous colonial cyanobacteria, such as Aphanocapsa spp., Aphanothece spp., Merismopedia spp. and Cyanodictyon spp. exhibited low biomass (< 2% of total) except in June, when their contribution reached 12% at the northern site (Fig. 2). Picocyanobacteria were not detected during the study period at either site." (line 207-210*)

In Zilius et al. 2020, sequences were attributed to picocyanobacteria (not referring here as "colonial picocyanobacteria"). However, a volume of 50 to 70 ml was extracted for further sequencing and only few reads were assigned to picocyanobacteria. This means that picocyanobacteria were rare in this study and that they would not be detected by methods allowing quantification such as flow cytometry or epifluorescence microscopy. Both approaches are complementary and not contradictory since DNA methods can detect rare taxa but do not allow quantification yet.

Reviewer comment (RC3). Thank you for clarifying this in the revision of the manuscript and for looking further into this by also applying microscopy in addition to flow cytometry. Feel free to also include this extra information so that future readers do not confuse between the groups of colonial vs. free-living picocyanobacteria.

Answer: we have added following text: "*Though sequences were also attributed to diazotrophic picocyanobacteria (Synechococcus, Crocosphaera, Rippkaea, and Cyanothece), these were not detected with flow cytometry, suggesting low abundance."* (line 298-300).

Reviewer comment (RC1). I am also a bit concerned about the method you use for measuring N_2-fixation with injection of gas rather than pre-dissolved. I think this might cause an underestimation. Also the fact that you run 24 h incubations probably lead to underestimations of N_2-fixation per h since they do less in the night when its dark (1.8 times less; Klawonn et al. 2016). I think a potential underestimation should be discussed and rates presented as per day since this is what you measure.

Answer: Regarding the issue of hourly vs. daily rates of fixation, we agree with the reviewer's point that rates are likely to vary on a diel cycle (being lower at night). Therefore our diel incubations conducted under natural (outdoor) light conditions are more suitably expressed as daily rates than hourly rates since they are representative of both light and dark cycles. In the revised manuscript, we present daily values in figures and text.

With regards to methodology, we agree that there has been some debate about using the bubble method for N_2 fixation measurements (Mohr et al., 2010; Großkopf et al., 2012; White et al., 2020), but recent work (Wannicke et al., 2018) demonstrated that underestimation of rates is negligible (<1%) for incubations lasting 12–24 h. In the submitted version we have argued our choice for incubation duration: "*As the isotopic equilibration takes up to several hours (Mohr et al., 2010), we incubated the samples for 24 h, thus minimizing equilibration effects (Mulholland et al., 2012; Wannicke et al., 2018." (line 136-138).* Eventually, our used technique avoids to have low labelling (percentage label should be between 5-10%) as the labelled seawater method often results in low quantities of $^{15}N_2$ gas in the water (e.g. Klawonn et al. (2015) had only 1% label in their experiment).

Reviewer comment (RC3). I am not fully sure if the last sentence about the optimum labelling ranges is within the revision or only a reply to the comment. But if it is in the text, do you have a reference for the mentioned optimum labelling percentages? I do not see any problem with having 1 % labelling when using the dilution approach (which you refer to in Klawonn et al.) since you then have this labelling in the whole flask already from the beginning, and as long as you can trace it and measure the final concentration. In case there is no suitable reference for the optimum ranges, and you want to mention it in the manuscript, I would suggest that you rephrase it to only mention that this range of percentages works well when using the bubble method and not compare to Klawonn et al. as an example of where it has not worked well(?) since I do not know if you have proof of this, and its slightly different methods. Further, I think it is great that you changed to 24 h values since this is what you measured and it includes both day and night.

Answer: our reply with regard to ^{15}N labelling was provided only in the "Responses to Reviewer". Since we did not measure the percentage of labelling, this information will not be included in "Material and Methods".

Reviewer comment (RC1). The theory of underestimation is further supported by that you have 1000-3000 µg cyanobacterial C per L and 120-200 nmol N2 fixation per h as compared to Klawonn 2016 where 100 µg cyanobacteria per L performed 80 nmol N_2 fixation per h. Why do you think you have so low rates as compared to your biomass? Can it be P limitation?

Answer: We agree with the reviewer's point that P limitation may play a role in limiting N fixation in the Curonian Lagoon. In a prior study, it was shown that P additions stimulated growth rates of N_2 fixing cyanobacteria from the Curonian Lagoon (Pilkaitytė and Razinkovas, 2007). Likewise, addition of P stimulated diazotrophic community resulting in elevated N_2 fixation rates (Moisander et al. 2007). We may expect that dissolved P was limiting, which constrained N_2 fixation during summer. Thus, we suppose that DIP release from sediment and higher biomass of *Aphanizomenon* and diazotrophic activity frequently observed in the end of summer (Zilius et al. 2014, 2018) are not coincidence but rather consequence of increased P availability. We have modified the Discussion to address this point "*The proliferation of heterocystous cyanobacteria in the Curonian Lagoon is favoured by P (Pilkaitytė and Razinkovas, 2007), which is released from sediments, particularly when bloom conditions result in high water column respiration and transient (night-time) depletion of oxygen (Petkuviene et al., 2016; Zilius et al., 2014). Moisander et al. (2007) demonstrated that P can enhance diazotrophic activity of heterocystous cyanobacteria in microcosms. Release of dissolved P from sediments may in turn enhance rates N_2 fixation resulting in a positive feedback for cyanobacteria bloom development."* (line 299-304)

Reviewer comment (RC3). In addition to stating that they are favoured by it you should maybe also say that this might be why they are performing compare-wise lower N_2 fixation in comparison to other regions of the Baltic Sea and provide some reference to P concentrations in the lagoon during summer. Maybe they also have enough of other sources of N, such as ammonium, to support some of their needs?

Answer: thanks for the comment, which is opening a new question why so high biomass of cyanobacteria can be present in the Curonian Lagoon. We rephrased this section in the revised version of manuscript: "*Measured summer DIP concentration (0.3 µM) in the Curonian Lagoon was similar to that in other Baltic coastal sites (e.g. Klawonn et al., 2016), suggesting that higher biomass might be also supported by higher N availability. A recent study by Broman et al. (submitted) suggests that N_2 fixation in the lagoon satisfies only 13% of N demand for phytoplankton. Thus, other internal sources such as N release from sediment and mineralization in the water column are important to meeting algal N demands."* (line 316-320).

Specific comments

Reviewer comment (RC1). Line 45-46, when they are dead I guess? Maybe clarify that this is when they are detritus on the bottom.

Answer: we assume that respiration of living cells rather detritus cause bottom hypoxia. During summer blooms, when plankton (mainly cyanobacteria and heterotrophic bacteria) respiration exceed diffusive oxygen supply to deeper layer, benthic community eventually depletes oxygen from adjacent bottom. We have clarified sentence and it reads now "*Large blooms of living cyanobacteria are associated with high oxygen demand in the water column, which results in transient (night-time) bottom hypoxia and enhances the release of dissolved inorganic phosphorus (DIP) from sediments (Petkuviene et al., 2016; Zilius et al., 2014).*" (line 45-47)

Reviewer comment (RC3). Thank you for the explanation. Although this might be part of the problem, I think a majority of the oxygen on the bottom is consumed as they die and need to be degraded. I think you should add a sentence and a reference to this as well if you mentioned the above statement. See for example Conley et al. 2009 on Hypoxia in the Baltic Sea.

Answer: we agree that both respiration by algae and heterotrophic respiration of their decomposing remains contribute to water column oxygen demand. We have modified the text accordingly: "*Large blooms of living cyanobacteria are associated with high oxygen demand in the water column, which we attribute to heterotrophic respiration of algal biomass and respiration by the algae themselves. High oxygen demand results in transient (night-time) hypoxia and enhances the release of dissolved inorganic phosphorus (DIP) from sediments (Petkuviene et al., 2016; Zilius et al., 2014).*" (line 46-48).

Reviewer comment (RC1). Line 120, I think it would be good to provide heterocysts per number of vegetative cells as well since they change in density over the season (Svedén et al. 2015).

Answer: following the reviewer's suggestion, we added estimates of heterocyst frequency per cyanobacteria filament in the revised version of the manuscript, see updated Figure 6. In the text, we have added following information:

„*The number of heterocysts (cell L^{-1}) and their frequency per millimeter of filament (mm^{-1}) was also determined.*" (line 121-122)

„*Total heterocyst frequency per filament was higher at the beginning of summer (up to 15 mm^{-1}) at both sites, and gradually declined afterwards (Fig. 6c, d).*" (line 249-250)

Reviewer comment (RC3). Thank you for including heterocysts per filaments, I think this is more informative than just heterocysts per ml. However, I think you should be more specific. In the southern site the numbers were highest in June and August/September, so actually two peaks, and in the northern sites they peaked in June/July and August depending on the species, so not only early in the summer and declining as the sentence reads now.

Answer: thanks for comment. In the revised version we have specified temporal patterns in heterocyst abundance: "*Heterocyst frequency per filament showed distinctive temporal patterns between the studied sites depending on the species (Fig. 6c, d). At the southern site, two peaks up to 8.0 mm^{-1} in heterocyst frequency of both species was observed during June–September. Whereas heterocyst frequency at the northern site remained quite high through summer primarily contributed by A. flosaque (~10 mm^{-1}), later followed by Dolichospermum spp. (~8 mm^{-1}).*" (line 254-258).

Reviewer comment (RC1). Line 181, why linear regression and not correlations? Don't you expect both of them to be interdependent rather than one dependent?

Answer: while we consider that correlation coefficient represents the direction and strength of the relationship between chlorophyll and N_2 fixation rates, the regression coefficient determines the effect of chlorophyll a (independent variable) on the N_2 fixation (dependent variable), and determine the explained variation.

Reviewer comment (RC3). I guess this is fine as long as you state this as your tested hypothesis in the methods.

Answer: we appreciate that the two variables may be considered inter-dependent (i.e., blooms of diazotrophic cyanobacteria may result in higher rates of N_2 fixation, and increased rates of N_2 fixation may lead to expansion of cyanobacteria blooms). Our choice of x and y variables is dictated by the fact that Chl-a may be derived via remote sensing and thereby permit estimation of N_2 fixation over large spatial and temporal scales. From a modelling perspective, the converse is unlikely to be as useful (i.e., estimating Chl-a from N_2 fixation). The regressions models relating N_2 fixation to Chl-a simply serve to parameterize the needed variables (slope and intercept) and are not meant to test a hypothesis. Text modified as: "*Estimates of N_2 fixation were derived for each of the grid cells based on satellite-derived Chl-a and regression models relating N_2 fixation measurements to concurrent in situ measurements of Chl-a (regressions provided in Results). The linear regressions served to parameterize the model components (slope and intercept).*" (line 188-189).

Reviewer comment (RC1). Figure 6, do you mean "per ml" with per mil? How come there is so many heterocysts in November in the southern station but almost no N_2 fixation nor cyanobacteria biomass at that time? In contrast the highest number of both N_2 fixation and heterocysts numbers correlates for the northern station. This needs to be discussed. Also, It would be good to also have heterocysts per filaments/vegetative cells here to see how it changes over the season (*Aphanizomenon* heterocyst density varies with season; Svedén et al. 2015).

Answer: "per mil" means delta units that are expressed in molecules per thousand, but for convenience we have change to "‰".

In revised version, we also provided heterocyst frequency, which better corresponded to N_2 fixation dynamic. The updated Figure 6 shows that patterns in heterocyst frequency was relatively low in November coinciding to decreased N_2 fixation rates. We assume that the October-November period represents the decline of the cyanobacteria bloom. In the submitted version we discussed that "*Results from this, and a prior study (Zilius et al. 2018), show that despite a high abundance of A. flosaquae at the end of fall, heterocyst frequency, and thus N_2 fixation rates declined substantially*

when water temperature dropped below 15 °C. Zakrisson et al. (2014) suggested that temperature controls the enzymatic activity of nitrogenase, which directly regulates the intensity of N_2 fixation in filaments." (line 363-367)

Reviewer comment (RC3). I am still a bit perplexed about how chl a of cyanobacteria I November at the southern site is very low, the heterocysts per filaments is very low but the heterocysts per L is very high? I understand that N_2 fixation goes down with temperature, but how can it be so many heterocysts when there is no biomass?

Answer: we checked twice our dataset and manuscript, and found that the last bar, representing November in Figure 2a, has changed colour when converting from text file to PDF, which sometimes happens. The correct figure shows that Chl-a in surface layer was high as well as biomass of N_2-fixing cyanobacteria and absolute heterocystous number per litre.

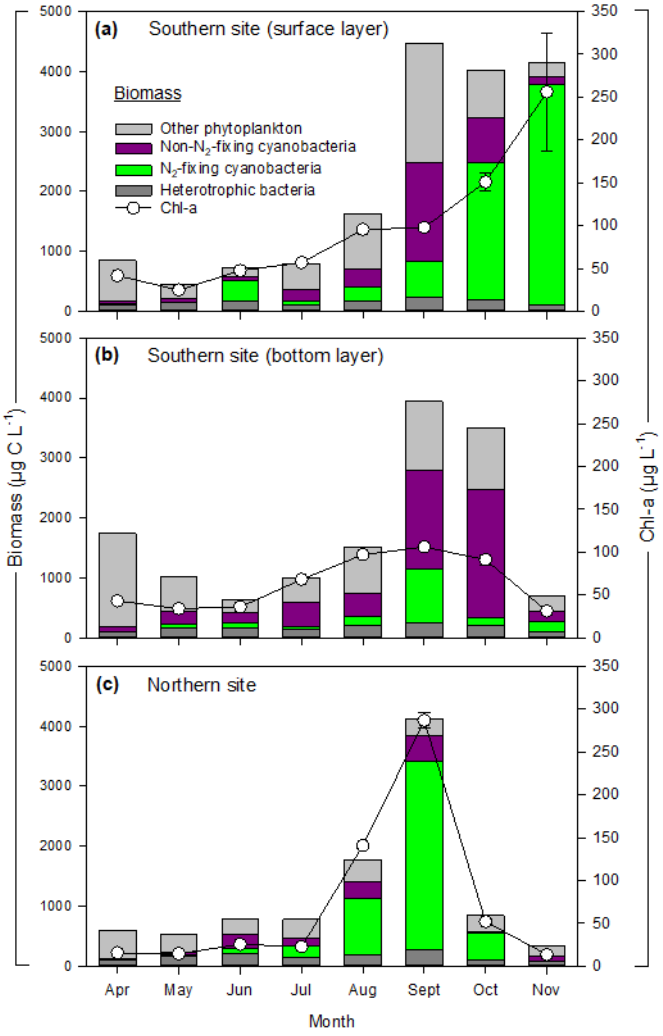

Figure 2: Phytoplankton and bacteria biomass at southern (a, b) and northern (c) sites in the Curonian Lagoon during 2018. Chlorophyll-a concentrations are mean values and standard error (some error bars not visible) based on three replicates.

Reviewer comment (RC1). Lines 260-261, how can you use this relationship when it was not significant, then there is no relationship?

Answer: The regression is marginally significant ($p = 0.08$) and has a reasonable R^2 value (0.52). Therefore, we felt that this model provided the best means for estimating bottom layer N_2 fixation. We also note that the bottom layer accounts for a relative small proportion of the lagoon's volume and an even smaller proportion of N_2 fixation (since surface rates are 7x higher). Therefore, our

whole-lagoon estimates of N_2 fixation are not highly sensitive to assumptions about bottom water rates.

Reviewer comment (RC3). Is this caveat explained also in the manuscript?

Answer: this caveat was added in the text "*We benefitted from prior work deriving Chl-a estimates from satellite images and their calibration to in situ measurements (Bresciani et al., 2014), but the success of the approach largely relied on the fact that heterocystous cyanobacteria dominated the summer–fall phytoplankton community of the lagoon, which provided a significant relationship between N_2 fixation and in situ Chl-a in surface layer. The regression model for estimating bottom layer N_2 fixation was marginally significant, and therefore we felt that the application of this model to deriving whole-water column rates was warranted. Whole-lagoon estimates were not highly sensitive to assumed rates in the bottom layer because this layer accounts for a relative small proportion of the lagoon's volume and because measured N_2 fixation rates in the bottom layer were 7 times lower than the surface."* (line 331-338).

Reviewer comment (RC1). Lines 309 and below, can you also put these areal N_2-fixation estimates into perspective to other studies for the region? For example, Klawonn et al. 2016 and Olofsson et al. 2020 as well as references there in.

Answer: thanks for suggestion. We have put our estimates in the context of the Baltic region: "*These estimates reveal that summer N_2 fixation rates are slightly lower in the coastal site of SW Baltic (3.6 ± 2.6 µmol m^{-2} d^{-1}; Klawonn et al., 2016), but higher than those found in the Great Belt (~ 1 mmol m^{-2} d^{-1}; Bentzon-Tilia et al., 2015), Baltic Proper (0.4 ± 0.1 mmol m^{-2} d^{-1}; Klawonn et al., 2016), and Bothnian Sea (0.6 ± 0.2 mol m^{-2} d^{-1}; Olofsson et al. 2020b).*" (line 328-331)

Reviewer comment (RC3). Please use the same units across all studies so its easier for the reader to compare. Maybe you need to formulate the sentence a bit clearer: "These estimates reveal that summer N_2 fixation rates are slightly lower in the Curonian Lagoon as compared to those measured at a coastal site of…".

Answer: we apologise for different units as it is typesetting mistake. The corrected version reads: "*These estimates reveal that summer N_2 fixation rates are slightly lower in the Curonian Lagoon as compared to those measured at a coastal site of SW Baltic (3.6 ± 2.6 mmol m^{-2} d^{-1}; Klawonn et al., 2016), but higher than those found in the Great Belt (~ 1 mmol m^{-2} d^{-1}; Bentzon-Tilia et al., 2015), Baltic Proper (0.4 ± 0.1 mmol m^{-2} d^{-1}; Klawonn et al., 2016), and Bothnian Sea (0.6 ± 0.2 mol m^{-2} d^{-1}; Olofsson et al. 2020b).*" (line 350-353).

Response (AC3) to Anonymous Referee #1 (RC4)

Reviewer comment (RC4). I am pleased to see that you have now met all my comments in a good way. I have no further comments on the manuscript except for that in the last comment you said the N_2 fixation rates for the Bothnian Sea in mol instead of what I think should be mmol?

Answer: acknowledge the reviewer for their positive comments. The reviewer is right for N_2 rates in the Bothnian Sea, which should be in mmol N m^{-2} d^{-1}. We have corrected this typesetting mistake.

Response (AC4) to Anonymous Referee #2 (RC5)

General comments

Reviewer comment (RC5). Overall, this is a very nicely written paper that integrates remote sensing data with empirical biogeochemical and biological data to estimate ecosystem-scale N fixation in an oligohaline coastal lagoon. Using remote sensing data to study processes such as N-fixation, given the good empirical relationship between N-fixation and chl-a in the late summer, is a very nice application of these data in coastal systems. As the authors point out, blooms of N-fixing cyanobacteria can significantly alter the N-budgets of enclosed coastal water bodies. Importantly, this can lead to these systems serving as 'sources' of N to the coastal ocean rather than serving as reactors for removing DIN via denitrification.

Answer: we acknowledge the reviewer for their positive comments, which are appreciated.

Reviewer comment (RC5). One concern I have is the spatial distribution of the water sampling locations. I agree that using the remote sensing approach appears to provide much more resolved estimates of N-fixation (this ms) than simply scaling up from the two sample locations (Line 310-312). Still, the entire southern half of the lagoon was not sampled. For example, it is noted at Line 78 in this ms that, "Longer water residence time in the southern lagoon provides favorable conditions for cyanobacteria bloom development (Bartoli et al., 2018)." Without actually measuring N-fixation rates vs chl-a concentrations at those southern areas (which could differ if the phyto community composition differs), there is still uncertainty about whether or not the remote-sensing based approach is yielding biased results in those southern reaches. This is particularly true because most of the high N-fixation rate hotspots in Figure 8 are further south than the 'southern' sampling location. It seems unlikely that this particular concern can be addressed using the same dataset but it is an important caveat that should be acknowledged. If the authors have evidence that the phytoplankton community in the southern part of the lagoon is the same as the community in the middle of the lagoon (i.e., the 'southern' sampling location) either from previous literature or their own unpublished work, then this would be an important pattern to note for readers.

Answer: we agree that whole lagoon sampling would be an ideal, but access to the southern region, which is located within Russian territorial waters, is problematic. Here, we improve on our ability to scale up these measurements by using remote sensing of Chl-a to infer spatial and temporal variation in N_2 fixation. Our whole-lagoon estimates are based on data collected at stations within the northern and central portions of the lagoon, as access to the southern region is problematic. Hydrodynamic modeling studies have shown that water renewal times in the central and southern portions of the lagoon are comparable (Umgiesser et al. 2016). Monitoring data suggest that Chl-a and phytoplankton community composition is similar in the central and southern regions (Bresciani et al. 2014; Semenova and Dmitrieva 2011). Therefore, we felt it was appropriate to derive whole-lagoon estimates of N fixation based on in situ measurements from these two sites.

References:

Bresciani, M., Adamo, M., De Carolis, G., Matta, E., Pasquariello, G., Vaičiūtė, D., and Giardino, C.: Monitoring blooms and surface accumulation of cyanobacteria in the Curonian

Lagoon by combining MERIS and ASAR data. Remote Sens. Environ., 146, 124–135, doi:10.1016/j.rse.2013.07.040, 2014.

Umgiesser, G., Zemlys, P., Erturk, A., Razinkova-Baziukas, A., Mežinė, J., and Ferrarin, Ch.: Seasonal renewal time variability in the Curonian Lagoon caused by atmospheric and hydrographical forcing. Ocean. Sci., 12, 391–402, doi:10.5194/os-12-391-2016, 2016.

Semenova A. S., and Dimitrieva O. A.: Spatial and temporal aspects of toxic effect of harmful algae on zooplankton in the Curonian Lagoon (the Baltic Sea) in New series 1(4) by Trudy AtlantNIRO. AtlantNIRO, Kaliningrad, RUS, 56–69, 2017.

Reviewer comment (RC5). The methods are very sparse for the TN riverine data collection. While not a central part of the analysis, these data are used to place the remote sensing results in an ecosystem context and are therefore important to the manuscript. In the text, reference is made to a previous paper rather than providing methods, but in the referenced paper (Zilius et al. 2018), the methods reported in that paper are limited to the following: "For the mass balance analysis, water samples were collected at the inflow (Nemunas River) and outflow (Klaipeda Strait) of the lagoon, and from an off-shore site in the Baltic Sea (55◦55âAš13.1"N and 21 ˇ ◦02âAš39.4"E), to estimate riverine inputs, lagoon export, and December 2014 to November 2015, except at the inflow site (Nemunas) where additional samples were obtained (at 1–2 week intervals) during the period of highest discharge (January–April)." (Zillius et al. 2018) It is important to see some additional details, even if they are only provided in the Supplemental file. Were samples collected monthly or at higher resolution at certain times of the year (as in Zilius et al. 2018)? Was there any effort to collect during average flow conditions? Where were the samples collected – mid stream in the river, or from the shore? Is the collection location the same location marine inputs, respectively (Fig. 1). Samples were collected monthly at each of the sites from referenced in the Zilius et al. 2018 paper?

Answer: we have provided some additional details of methodology to reduce reliance on the Zilius et al. 2018 paper: "*We also monitored total nitrogen (TN) concentrations in the Nemunas River (Fig. 1) to derive riverine N loads for comparison with atmospheric N inputs via N_2 fixation*. River samples were collected twice monthly during peak discharge (January-April) and monthly throughout the rest of the year (16 collections). *Water samples (2 L) were collected in triplicate, integrating the whole water column with repeated Ruttner bottle sampling at the surface (0.4 m depth) and bottom layers (3.0 m depth) as described in Vybernaite-Lubiene et al. (2018). Integrated water samples were transferred to opaque bottles, cooled with ice packs, and transported to the laboratory within the hour for subsequent analyses (see section 2.3 for details). Riverine N concentrations were used in combination with daily discharge measurements (provided by Lithuanian Hydrometeorological Service) to derive monthly N loads to the lagoon as previously described in Zilius et al. (2018).*" (line 98-105).

Specific comments

Reviewer comment (RC5). Figure 1. Please provide definitions for abbreviations (RUS, LT) and increase font size on some of the smaller figure elements such as the scale bar. There

is a grey rectangle just above the Nemunas River. Is that meant to be there and if so, what is it? Please show the river sampling location on the map.

Answer: thanks for suggestions. We have updated figure accordingly.

Reviewer comment (RC5). Line 114 – please provide a long-term estimate of d15N analytical precision for the UC Davis facility. They should have these numbers readily available. Otherwise, you could also report summary statistics on sample duplicates that were (presumably) interspersed with the submitted samples.

Answer: we have added missing information "*The long-term standard deviation is <0.3 ‰ for $\delta^{15}N$.*" (line 128-129)

Reviewer comment (RC5). Figure 3 – it is confusing to list *Anabaena* in the figure while referring to it as *Dolichospermum* in the text. There is a note in the figure legend that the two are the same but why not simply use *Dolichspermum* in the figure (or at least an abbreviation)?

Answer: thanks for suggestion. We have corrected figure accordingly (see below).

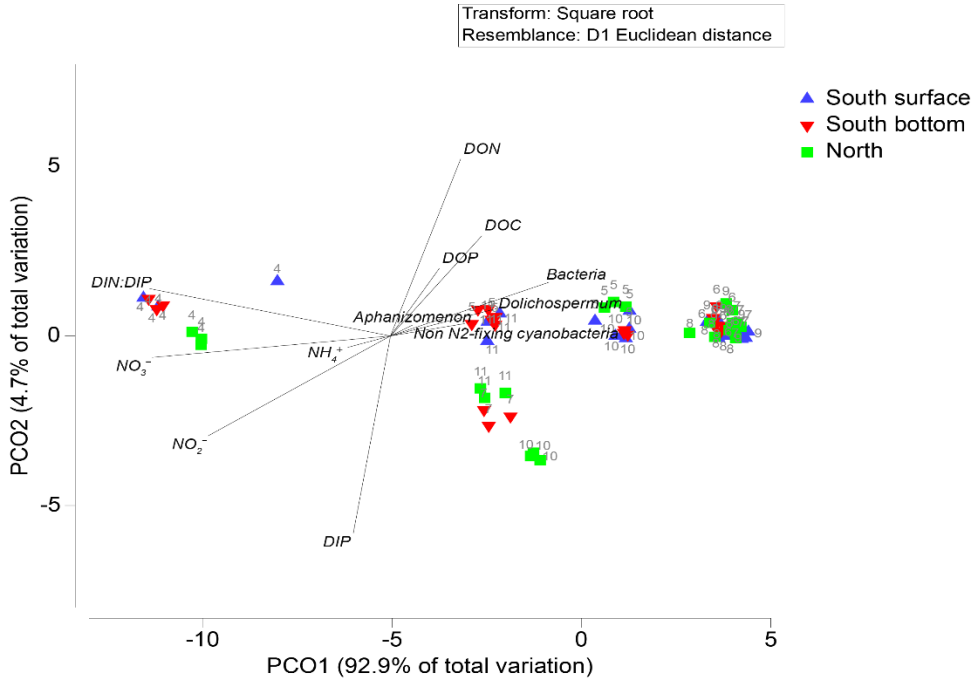

Figure 3: Principal coordinate biplots generated on Euclidean distances of normalized and forth-root transformed nutrient concentrations (DOC, NH_4^+, NO_2^-, NO_3^-, DON, DIP, DOP, and DIN:DIP). Overlaid vectors show individual chemical variables (those significantly correlating with either of the two primary axes, with Pearson correlations > 0.5) and plankton community biomass (*Aphanizomenon, Dolichospermum,* non-N_2-fixing cyanobacteria and heterotrophic bacteria).

Reviewer comment (RC5). Figure 4 – are the southern site values averaged between surface and bottom or are these only surface (or bottom) values? Can you please clarify in the figure legend?

Answer: revised caption is "*Figure 4: Temporal patterns in temperature, dissolved organic carbon (a, b), dissolved and organic nitrogen (c, e), phosphorus (e, f), and DIN:DIP ratios (g, h) at southern (surface layer; left panel) and northern (right panel) sites in the Curonian Lagoon during 2018 (error bars denote standard error based on 3 replicates; some not visible)*."

Reviewer comment (RC5). Line 346-360 – also see papers by Karlson et al. (2015), Woodland, Cook and others (2013, 2014) for evidence of diazotrophic N from cyanobacteria contributing to brackish food webs.

Answer: thanks for suggestion. We have included these references in manuscript.

Technical corrections

Reviewer comment (RC5). Line 182 – what do you mean by 'process' here? Is that word out of place or does it reference to a specific type of measurement taken in the surface and bottom waters? Can you please rephrase to make this more interpretable?

Answer: this is a redundant and has removed from the text.

Reviewer comment (RC5). Line 248 – add a comma after '0.88'

Answer: Done.

Reviewer comment (RC5). Line 249 – add a space between '=' and '0.07

Answer: Done.

Reviewer comment (RC5). Line 356-357 – replace 'their' with 'these blooms to have a' or something similar. The current phrasing is awkward.

Answer: this comment is not clear as indicated line reads "…abundance of *Microcystis* spp. and *Planktotrix agardhii*. Measured low $\delta^{15}N$ values (0.5 ± 0.2 ‰) in suspended living material suggest that fixed N can temporally support most of the nutritional needs for plankton (bacteria + phytoplankton) growth".

We may think that the reviewer had indicated line 365-367, "*Since intensifying blooms of cyanobacteria have already been observed in coastal areas of the Baltic Sea (Olofsson et al., 2020b), we may expect their stronger effect on ecosystem functioning in future*". We have rephrased this sentence, "*Since intensifying blooms of cyanobacteria have already been observed in coastal areas of the Baltic Sea (Olofsson et al., 2020a), we may expect these blooms to have a stronger effect on ecosystem functioning in future*".

Spatiotemporal patterns of N_2 fixation in coastal waters derived from rate measurements and remote sensing

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Abstract. Coastal lagoons are important sites for nitrogen (N) removal via sediment burial and denitrification. Blooms of heterocystous cyanobacteria may diminish N retention as dinitrogen (N_2) fixation offsets atmospheric losses via denitrification. We measured N_2 fixation in the Curonian Lagoon, Europe's largest coastal lagoon, to better understand the factors controlling N_2 fixation in the context of seasonal changes in phytoplankton community composition and external N inputs. Temporal patterns in N_2 fixation were primarily determined by the abundance of heterocystous cyanobacteria, mainly *Aphanizomenon flosaquae*, which became abundant after the decline in riverine nitrate inputs associated with snowmelt. Heterocystous cyanobacteria dominated the summer phytoplankton community resulting in strong correlations between chlorophyll-a (Chl-a) and N_2 fixation. We used regression models relating N_2 fixation to Chl-a, along with remote sensing-based estimates of Chl-a to derive lagoon-scale estimates of N_2 fixation. N_2 fixation by pelagic cyanobacteria was found to be a significant component of the lagoon's N budget based on comparisons to previously derived fluxes associated with riverine inputs, sediment-water exchange and losses via denitrification. To our knowledge, this is the first study to derive ecosystem-scale estimates of N_2 fixation by combining remote sensing of Chl-a with empirical models relating N_2 fixation rates to Chl-a.

1 Introduction

Biological dinitrogen (N_2) fixation plays an important role in the nitrogen (N) budget of aquatic ecosystems as it transforms gaseous N_2 into reactive forms, which are available for assimilation by microorganisms, algae, and plants (Gruber, 2004; Hayes et al., 2019). Coastal ecosystems contain diverse diazotrophic communities, comprised of unicellular cyanobacteria, colonial heterocystous cyanobacteria, and heterotrophic bacteria (Riemann et al., 2010; Bentzon-Tilia et al., 2014; Zilius et al., 2020). Unicellular cyanobacteria and heterotrophic diazotrophs dominate in tropical and oligotrophic marine systems (Zehr et al., 2003; Riemann et al., 2010; Farnelid et al., 2016), whereas colonial heterocystous cyanobacteria dominate N_2 fixation in temperate systems (Klawonn et al., 2016). The dominant colonial heterocystous cyanobacteria in the Baltic Sea and its coastal areas are *Aphanizomenon*, *Nodularia* and *Dolichospermum* (formerly *Anabaena*) (Olofsson et al., 2020ab). Their proliferation has the potential to alter N cycling and thereby influence N export to coastal waters.

Eutrophic lagoons, like those in the Baltic region (Asmala et al., 2017), undergo a shift from phosphorus (P) limitation in spring to N and silica limitation in summer and fall (Patuszak et al., 2005; Vybernaite-Lubiene et al., 2017). Such changes, which largely depend on the timing and magnitude of riverine nutrient inputs, create a temporal niche for diazotrophic cyanobacteria that are capable of overcoming N limitation, and thus often dominate the summer phytoplankton community (Paerl and Otten, 2013; Vybernaite-Lubiene et al., 2017). Large blooms of living cyanobacteria are associated with high oxygen demand in the water column, due ~~which~~ to autotrophic and heterotrophic. High oxygen demand results in transient (night-time) hypoxia and enhances the release of dissolved inorganic phosphorus (DIP) from sediments (Zilius et al., 2014; Petkuviene et al., 2016)~~results in transient (night time) bottom hypoxia and enhances the release of dissolved inorganic phosphorus (DIP) from sediments (Petkuviene et al., 2016; Zilius et al., 2014)~~. Thus, a positive feedback is established whereby surplus P favors N-limitation and further proliferation of cyanobacteria that are capable of fixing N_2. Although there has been progress in understanding the causes and expansion of cyanobacterial blooms in eutrophic coastal ecosystems (e.g.; Paerl et al., 2001; Paerl and Paul, 2012; Bartoli et al., 2018 and references therein), seasonal patterns and environmental controls of N_2 fixation associated with cyanobacterial blooms are not well understood. Particularly challenging is the estimation of N_2 fixation at larger scales, owing to the patchy distribution of cyanobacteria (Rolff et al., 2007).

In the present study, we analysed spatiotemporal patterns of pelagic N_2 fixation in relation to plankton community characteristics in the Curonian Lagoon. The lagoon is characterized by recurring~~massive~~ summer blooms of cyanobacteria, with chlorophyll a (Chl-a) concentrations as high as 400 µg L^{-1} (Bresciani et al., 2012; Vaičiūtė et al., 2021). Our prior work showed that the occurrence of cyanobacteria blooms had a large effect on N cycling and retention in this system (Zilius et al., 2018). Cyanobacteria blooms reduced annual N retention in the lagoon because summer N_2 fixation offsets winter denitrification. In addition, N contained in cyanobacteria biomass was more likely to be exported from the lagoon to the Baltic Sea, rather than buried in sediments, due to their positive buoyancy. A limitation of the prior study was that the patchy and dynamic nature of cyanobacteria blooms resulted in considerable uncertainty in estimating N_2 fixation at larger spatiotemporal

scales (e.g., for monthly, lagoon-scale N balances). In this follow-up study, we focus on seasonal changes in phytoplankton abundance and community composition and their utility in predicting N_2 fixation. We derive models relating N_2 fixation to heterocyst density, abundance of heterocystous cyanobacteria and Chl-a, and use remote sensing of Chl-a to derive estimates of N_2 fixation at larger spatiotemporal scales. We consider N_2 fixation in the context of other, previously measured inputs and losses of N from the lagoon.

2 Material and Methods

2.1 Study site

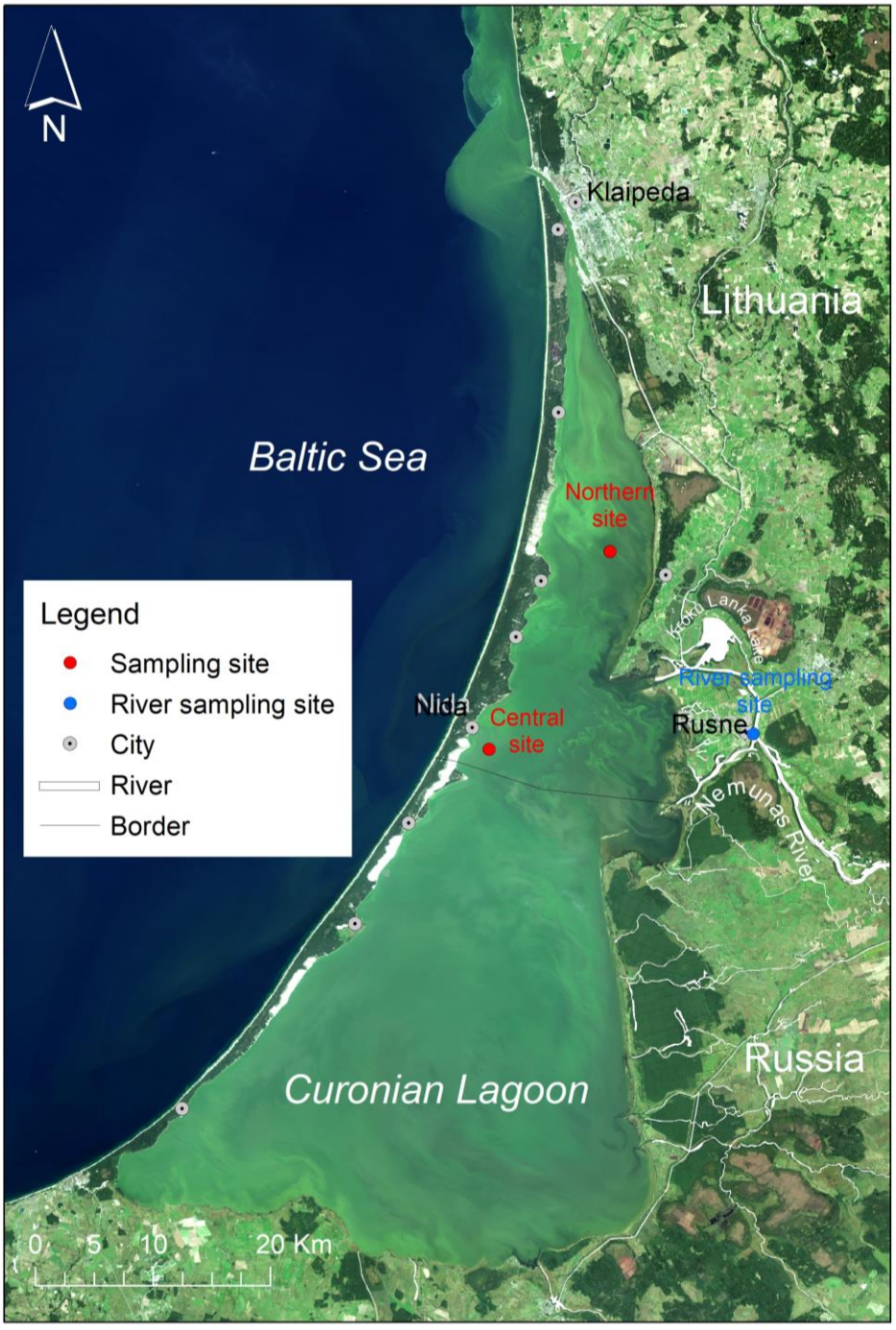
The Curonian Lagoon is located along the southeast coast of the Baltic Sea (Fig. 1). It is the largest coastal lagoon in Europe (area = 1584 km^2). The lagoon is a shallow waterbody (mean depth = 3.8 m) that discharges to the Baltic Sea through the narrow Klaipeda ~~s~~Strait and ~~occasionally~~ receives inputs from the Baltic during periods of wind-driven forcing (Zemlys et al., 2013). These events are typically of short duration and result in small increases in salinity (typically by 1–2, maximum = 7) in the northern portion of the lagoon. During sampling carried out for this study, salinity in the lagoon was below < 0.5, suggesting limited brackish water intrusions. There was little difference in temperature between surface and the bottom layers (< 2 ^oC) indicating well-mixed conditions within water column (Zilius et al., 2020).

The Nemunas River is the principal tributary (mean annual discharge = 16.4 km^3) and main source of nutrient inputs to the lagoon (Vybernaite-Lubiene et al., 2018; Zilius et al., 2018). The inflow of the Nemunas enters near the mid-point along the north-south axis of the lagoon. Hydrodynamic modelling studies suggest that the bulk of riverine inputs travel north toward the Klaipeda Strait resulting in shorter water residence time in the northern lagoon (Umgiesser et al., 2016). Longer water residence time in the central and southern lagoon provides favorable conditions for cyanobacteria bloom development (Bartoli et al., 2018). Patterns of phytoplankton seasonal succession are similar throughout the lagoon, transitioning from diatom and chlorophyte dominance in spring to cyanobacteria-dominated blooms in summer and fall (Semenova and Dmitrieva, 2011; Zilius et al., 2018). Spatially-extensive blooms of heterocystous cyanobacteria (*Aphanizomenon* and *Dolichospermum*) are occasionally observed, particularly during low-wind conditions (Bartoli et al., 2018; Vaičiūtė et al., 2021).

2.2 Sample collection

We measured pelagic N_2 fixation and characterized bacterioplankton and phytoplankton communities at stations located in northern and south-central regions of the lagoon during April–November 2018 (Fig.1). Water samples were collected monthly at two depth layers (0–1.5 m, and 2.0–3.5 m) in the deeper, central site~~area~~ (~~southern site;~~ mean depth = 3.5 m), and at one depth layer (0–1.5 m) in the shallow northern site (mean depth = 1.5 m). Water samples were transferred to 1) sterilized amber borosilicate bottles (0.5 L) for bacteria counting, 2) to opaque HPDE bottles (2 L) for nutrient analyses, and 3) 20 L jars for N_2 fixation measurements. All samples were transported on ice (except for N_2 fixation experiment) within half an hour after

collection for subsequent laboratory processing and analyses. During each sampling, water temperature, salinity and dissolved oxygen were measured *in situ* at the surface (0.5 m depth) and bottom (0.5 m above the sediment) using a YSI 460 multiple probe (Xylem). Vertical profiles of photosynthetically active radiation (PAR) were measured with a LI-192 underwater quantum sensor (LI-COR®). We also monitored total nitrogen (TN) concentrations in the Nemunas River (Fig. 1) to ~~compare~~ derive riverine N loads for comparison with atmospheric N inputs via N_2 fixation ~~(as described in Zilius et al., 2018)~~. River samples were collected twice monthly during peak discharge (January–April) and monthly throughout the rest of the year (16 collections). Water samples (2 L) were collected in triplicate, integrating the whole water column with repeated Ruttner bottle sampling at the surface (0.4 m depth) and bottom layers (3.0 m depth) as described in Vybernaite-Lubiene et al. (2018). Integrated water samples were transferred to opaque bottles (2 L), cooled with ice packs, and transported to the laboratory within the hour for subsequent analyses (see section 2.3 for details). Riverine N concentrations were used in combination with daily discharge measurements (provided by Lithuanian Hydrometeorological Service) to derive monthly N loads to the lagoon as previously described in Zilius et al. (2018).


N
Klaipeda
Lithuania
Baltic Sea
Northern site
Legend
Sampling site
River sampling site
City
River
Border
Nida
Central site
River sampling site
Rusne
Nemunas River
Russia
Curonian Lagoon
0 5 10 20 Km

105

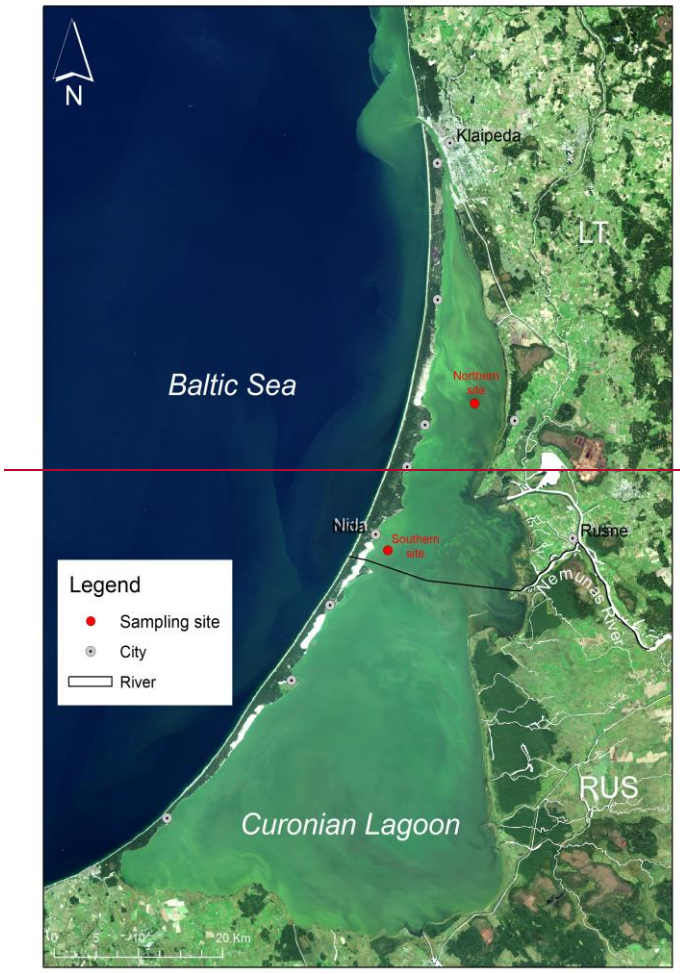


Figure 1: Satellite image by OLI/Landsat-8 (18/09/2014) showing summer blooms in the Curonian Lagoon with the sampling sites (red circles) representing the northern and south-central regions, and monitoring site at the Nemunas River (blue circle). LT = Lithuania, RUS = Russia, and black line indicates a border between two countries.

2.3 Chemical analysis

Triplicate w~~W~~ater sample~~s~~ from each site and layer were filtered (Whatman GF/F, pore size 0.7 µm)~~filtered~~ in triplicate for inorganic and organic nutrient analysis as previously described by Vybernaite-Lubiene et al. (2017). Dissolved inorganic nutrients (NH_4^+, NO_2^-, NO_3^-, and DIP) were determined colorimetrically using a continuous flow analyser (San^{++}, Skalar) following the methods described in Grasshoff et al. (1983). Total dissolved nitrogen (TDN) was analyzed by the high temperature (680 ºC) combustion, catalytic oxidation/NDIR method using a Shimadzu TOC 5000 analyzer with a TN module. Dissolved organic nitrogen (DON) was calculated as difference between TDN and DIN ($NH_4^+ + NO_2^- + NO_3^-$). Dissolved organic carbon (DOC) was determined with a Shimadzu TOC 5000 analyzer using an acetanilide dilution series as a standard

(Cauwet, 1999). Total dissolved phosphorus (TDP) was determined after digestion and oxidation of the organic P forms with alkaline peroxodisulphate acid digestion (Koroleff, 1983). Dissolved organic phosphorus (DOP) was calculated as difference between TDP and DIP. Water samples for Chl-a were filtered through ~~Whatman~~ GF/F filters ~~(nominal pore size 0.7 µm)~~. Pigments were extracted with 90% acetone (24 h at 4 °C) and measured by spectrophotometry (Jeffrey and Humphrey, 1975; Parsons et al., 1984). Particulate matter was collected on pre-ashed (4 h at 550 ºC) Advantec GF75 filters (nominal pore size 0.3 µm) for particulate nitrogen (PN, river water) and isotopic signature (δ^{15}N-PN, lagoon) analysis. Prior to analysis, filters were dried at 60 ºC to constant weight and later analysed with an Elementar Vario EL Cube (Elementar Analysen systeme GmbH) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS, Sercon Ltd) at the Stable Isotope Facility of UC Davis, USA. The long-term standard deviation is <0.3 ‰ for δ^{15}N. TN was calculated as a sum of dissolved and particulate fractions.

2.4 Phytoplankton and bacteria counts

Samples for phytoplankton counting were immediately preserved with acetic Lugol's solution and examined at magnifications of ×200 and ×400, using a LEICA DMI 3000 inverted microscope. Phytoplankton community composition was determined using the Utermöhl method (Utermöhl, 1958) according to HELCOM recommendations (HELCOM, 2017). Phytoplankton biomass (mg L^{-1}) was calculated according to the methodology described in Olenina et al. (2006) and converted into carbon units (µg C ~~cell~~ L^{-1}) following Menden-Deuer and Lessard (2000). The number of heterocysts ~~was also determined~~ (cells L^{-1}) and their frequency per millimeter of filament (mm^{-1}) was also determined.

The abundance of heterotrophic bacteria and picocyanobacteria was determined in filtered samples (50 µm size mesh), which were preserved in 0.25% glutaraldehyde (final concentration) and stored at −80 °C until analysis (Marie et al., 2005). Samples for determination of heterotrophic bacteria biomass were stained with SYBR Green I (Invitrogen) to a final concentration of 1:10000 (Marie et al., 2005), diluted with Milli-Q water, and analysed with a flow cytometer (BD Accuri™C6, DB Biosciences) at a medium flow rate (35 µL min^{-1}) for 2 min. Microspheres of 1 µm (Fluoresbrite plain YG, Polysciences) were added to samples as an internal standard. Samples for picocyanobacteria (≤ 3 µm) were analysed at a flow rate of 66 µL min^{-1} with an acquisition time of 2 min. Microspheres of 3 µm (Fluoresbrite plain YG, Polysciences) were used as an internal standard. A factor of 20 fg C per cell was used to convert bacteria counts to carbon biomass (µg C L^{-1}; Lee and Fuhrman, 1987).

2.5 Nitrogen fixation

Monthly measurements of N_2 fixation in the water column were performed using the $^{15}N_2$ technique described in Montoya et al. (1996). Rates were measured in two depth layers (0–1.5 m, and 2.0–3.5 m) at the deeper, ~~southern~~ central site, and for the whole water column (0–1.5 m) at the shallow northern site. The samples were filled ~~(avoiding air bubbles)~~ into 500 mL~~l~~ transparent HDPE bottles and carefully sealed preventing formation of air bubbles ~~bottles and carefully sealed preventing~~

~~formation of air bubbles~~. Each sample received 0.5 mL~~l~~ $^{15}N_2$ (98% $^{15}N_2$, Sigma-Aldrich) injected by syringe through a gas-tight septum, and then gently mixed for 10 min (Zilius et al., 2018). As the isotopic equilibration takes up to several hours (Mohr et al., 2010), we incubated the samples for 24 h, thus minimizing equilibration effects which can lead to underestimation of rates (Mulholland et al., 2012; Wannicke et al., 2018) ~~but prevents potential contamination through the production of isotopically enriched water~~. Surface water samples were incubated outdoors at ambient irradiance, while samples from 2.0–3.5 m were wrapped in aluminium foil as *in situ* irradiance was below 1% of surface PAR at these depths (< 5 µmol m^{-2} s^{-1} in the period of June–November). However, such fixed dark conditions is less representative to *in situ* conditions as cyanobacteria colonies can migrate upward to surface photic zone or use limited light for photosynthesis. After incubation, suspended material was collected on pre-ashed (4 h at 450 ºC) Advantec GF75 filters (nominal pore size 0.3 µm). This nominal pore size filter was used instead the conventional (0.7 µm) as it allows for quantitative collection of smaller cells comprising the active diazotrophic community (Bombar et al., 2018). All samples were stored frozen until analysis with a continuous-flow ~~isotope ratio mass spectrometer~~IRMS (~~Thermo Finnigan,~~ Delta S, Thermo-Finnigan~~Bremen~~) at the Leibniz Institute for Baltic Sea Research Warnemünde (IOW). Volumetric rates of N_2 fixation were calculated following Eq. 1 (Montoya et al., 1996):

$$N_2 \text{ fixation rate } (\mu\text{mol N } L^{-1} d^{-1}) = \frac{V}{2}\overline{PN} \approx \frac{V}{2} \times \left(\frac{[PN]_0 - [PN]_f}{2}\right) \quad (1)$$

Where V (the specific rate of N_2 uptake) is derived from Eq. 2:

$$V = \frac{1}{\Delta t} \times \frac{\left(A_{PN_f} - A_{PN_0}\right)}{\left(A_{N_2} - A_{PN}\right)} \quad (2)$$

A_{PN} is the ^{15}N atom % enrichment of the PN pool at the beginning (t_0) and end (t_f) of an incubation, A_{N2} is the ^{15}N atom % enrichment of the dissolved N_2 gas in the incubated water, and PN is the concentration of PN at the beginning (t_0) and end (t_f) of the incubation. As we have used theoretical estimation of $^{15}N_2$ gas dissolution in bottles instead quantification with membrane inlet mass spectrometer, it can result in some underestimation of rates (White et al., 2020). Volumetric N_2 fixation rates were converted to areal rates (mmol N m^{-2} d^{-1}) taking into account the depth of the water column and the thickness of each depth layer (see above).

2.6 Remote sensing

Prior studies have developed and validated techniques for remote sensing of Chl-a in the Curonian Lagoon (Bresciani et al., 2014; INFORM, 2016; Riddick et al., 2019). The satellites can observe the water down to one optical depth, the portion of the water column where approximately 90% of the remote sensing observed signal originates (Gordon and McCluney, 1975; Werdell and Bailey, 2005). The optical depth of the Curonian Lagoon is typically less than 0.5 m (Vaiciute, unpublished). We obtained satellite images for 6 dates spanning the period when phytoplankton communities were dominated by heterocystous cyanobacteria and water temperatures exceeded 15 ºC (July–September). Chlorophyll-a concentrations were obtained from optical satellite images using an on-board Multispectral Instrument (Sentinel-2: September 7 and 20) and an on-board Ocean and Land Colour Instrument (Sentinel-3: July 4 and 24, August 8 and 23). Satellite images were resampled to a nominal pixel

size of 300 m resulting in a grid matrix of ~17,000 cells comprising the area of the lagoon. Cells adjacent to the shoreline were excluded from analyses due to potential interference from aquatic vegetation and benthic algae. Atmospheric correction was carried out using the Second Simulation of the Satellite Signal in the Solar Spectrum-Vector code (6SV; Vermote et al., 1997) previously used in other satellite applications for the Curonian Lagoon (Bresciani et al., 2014). The parametrization of the 6SV code was performed using the Maritime model. Values of Aerosol Optical Thickness (AOT) were obtained from AERONET sites and using MODIS-derived AOT values available from the NASA Giovanni application (https://giovanni.gsfc.nasa.gov/giovanni/). Chl-a concentrations were derived after the application of a semi-empirical band-ratio model that uses reflectance in the red and near-infrared spectral regions (Gitelson et al., 2007; De Santi et al., 2019).

Equation 3 used for Sentinel-2 data:

$$Chl-a, mg\ m^{-3} = (76.36 \pm 2.29) \times \frac{Ref_{705}}{Ref_{665}} - (51.57 \pm 0.26), \quad (3)$$

Equation 4 used for Sentinel-3 data:

$$Chl-a, mg\ m^{-3} = (52.19 \pm 1.81) \times \frac{Ref_{708}}{Ref_{665}} - (32.07 \pm 0.57), \quad (4)$$

where Ref_x indicates the reflectance of the band with central wavelength x.

Prior work validating satellite-derived Chl-a against *in situ* observations in the Curonian Lagoon showed good agreement for both Sentinel-2 (R^2 = 0.91, root-mean-square error (RMSE) = 18.6 mg m^{-3}) and Sentinel-3 R^2 = 0.95, RMSE = 7.4 mg m^{-3}) images (INFORM, 2016; Riddick et al., 2019). Estimates of N_2 fixation were derived for each of the grid cells based on satellite-derived Chl-a and regression models relating ~~measured~~ N_2 fixation measurements to concurrent *in situ* measurements of Chl-a (regressions provided in Results).

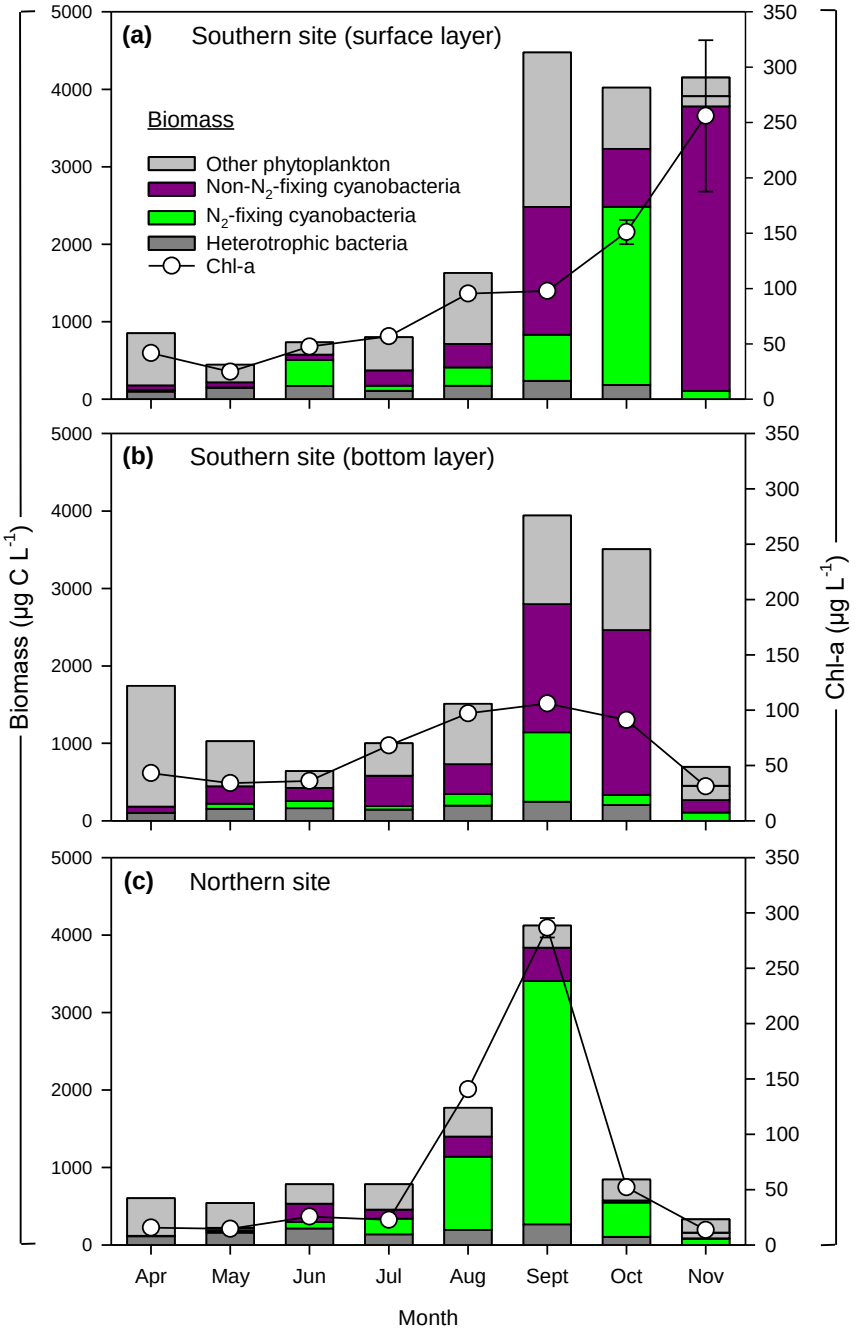
2.7 Statistical analysis

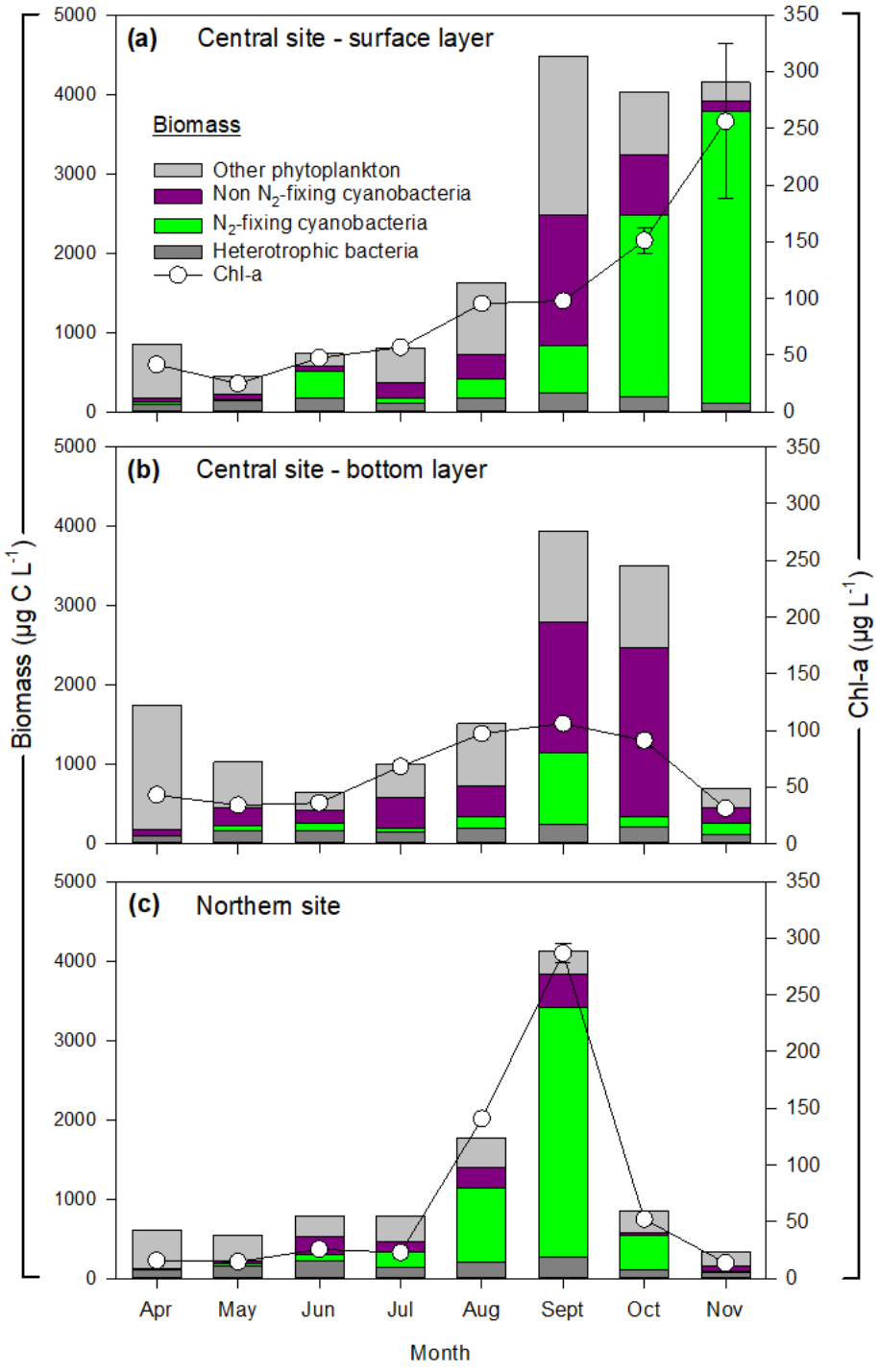
Principal coordinates analysis (PCoA) was performed to visualize spatiotemporal patterns in plankton community variables (*Aphanizomenon*, *Dolichospermum*, non N_2-fixing cyanobacteria, and heterotrophic bacteria biomass) and their relationship to nutrient concentrations. This analysis was performed using Primer 7 software (v.7, Primer-E Ltd.~~, v.7~~; ~~Plymouth, United Kingdom;~~ Clarke and Gorley, 2015) on Euclidean distances of normalized and forth-root transformed variables. The linear regression was used to predict N_2 fixation rates based on *in situ* Chl-a concentration. In addition, variance analysis (two-way ANOVA) was used to test differences in Chl-a concentration between ~~process~~ surface and bottom layers. The assumptions, data normality, and homogeneity of variance, were checked using Shapiro–Wilk test and Cochran's test, respectively. For significant factors, post hoc pairwise comparisons were performed using the Student-Newman-Keuls (SNK) test. The significance level was set at $\alpha = 0.05$. Analyses were performed in SigmaPlot 14.0 software.

3 Results

3.1 Phytoplankton and bacteria communities

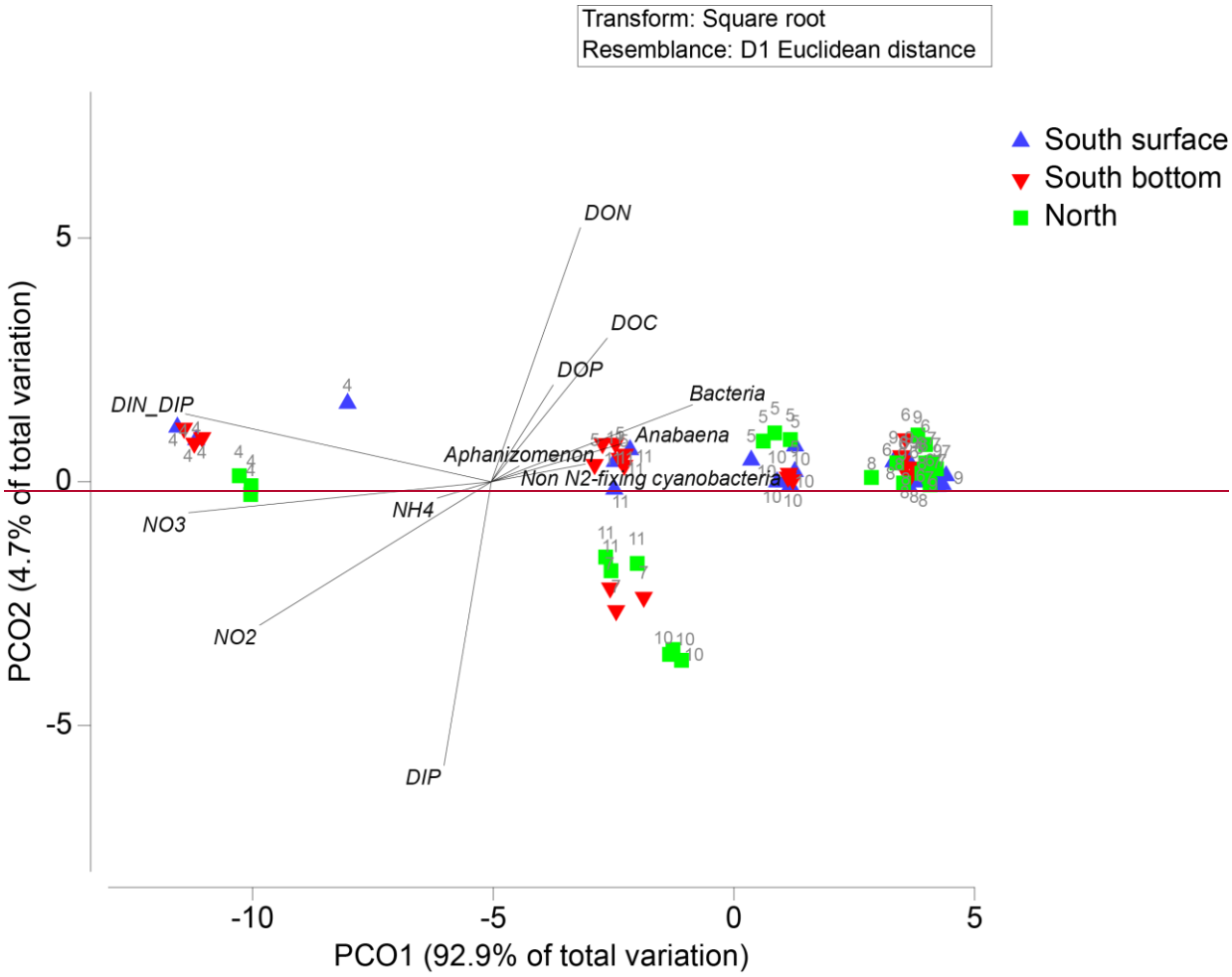
Seasonal patterns in phytoplankton biomass and community composition followed expected trends based on prior work at this site (Fig. 2). *In situ s*~~S~~urface Chl-a during April–July ranged from 25 to 57 µg L^{-1} at the ~~southern~~ central site and from 14 to 26 µg L^{-1} at the northern site. Higher Chl-a was observed in late summer with values ranging from 52 to 286 µg L^{-1} at the northern site and from 96 to 256 µg L^{-1} at the ~~southern~~ central site. Phytoplankton biomass showed corresponding changes, increasing from ~1000 to 4000 µg C L^{-1} during spring to late summer. Diatoms dominated the spring phytoplankton community (April–May) accounting for up to 94% of total biomass. During June–July, diatoms were replaced by non- and N_2-fixing cyanobacteria; the later accounted for up to 36% of total phytoplankton biomass. The non N_2-fixing cyanobacteria were dominated by *Planktotrix agardhii* and *Microcystis* spp. Between August and November, N_2-fixing cyanobacteria dominated the community (86% of total biomass). The main N_2-fixing cyanobacteria were *Dolichospermum* spp. and *Aphanizomenon flosaquae*. Heterotrophic bacteria accounted for ~30% of the total plankton biomass (bacteria + phytoplankton) during the early successional (diatom-dominated) phase. Non filamentous colonial cyanobacteria, such as *Aphanocapsa* spp., *Aphanothece* spp., *Merismopedia* spp. and *Cyanodictyon* spp. exhibited low biomass (< 2% of total) except in June, when their contribution reached 12% at the northern site (Fig. 2c). Picocyanobacteria were not detected during the study period at either site. Heterotrophic bacteria biomass was higher during the cyanobacterial bloom (June–October), increasing from ~80 to 250 µg C L^{-1}, however, their relative contribution to total plankton biomass decreased to ~17%. Phytoplankton biomass and community composition were generally similar between surface and bottom layers (April–September), except in October–November when the abundance of N_2-fixing cyanobacteria was greater in the surface layer (2500–3500 µg C L^{-1}) relative to the bottom layer (< 100 µg C L^{-1}).~~Picocyanobacteria were not detected during the study period at either site.~~


(a) Southern site (surface layer)
Biomass
Other phytoplankton
Non-N2-fixing cyanobacteria
N2-fixing cyanobacteria
Heterotrophic bacteria
Chl-a
(b) Southern site (bottom layer)
(c) Northern site
Biomass (µg C L-1)
Chl-a (µg L-1)
0
1000
2000
3000
4000
5000
50
100
150
200
250
300
350
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Month


(a) Central site - surface layer
Biomass
Other phytoplankton
Non N2-fixing cyanobacteria
N2-fixing cyanobacteria
Heterotrophic bacteria
Chl-a
(b) Central site - bottom layer
(c) Northern site
Biomass (µg C L-1)
Chl-a (µg L-1)
0
1000
2000
3000
4000
5000
50
100
150
200
250
300
350
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Month

230

Figure 2: Phytoplankton and heterotrophic bacteria biomass at ~~southern~~ central (a, b) and northern (c) sites in the Curonian Lagoon during 2018. Chlorophyll-a concentrations are mean values and standard error (some error bars not visible) based on three replicates.


Transform: Square root
Resemblance: D1 Euclidean distance
South surface
South bottom
North
DON
DOC
DOP
Bacteria
Anabaena
Aphanizomenon
Non N2-fixing cyanobacteria
DIN_DIP
NH4
NO3
NO2
DIP
PCO2 (4.7% of total variation)
PCO1 (92.9% of total variation)
5
0
-5
-10
-5
0
5

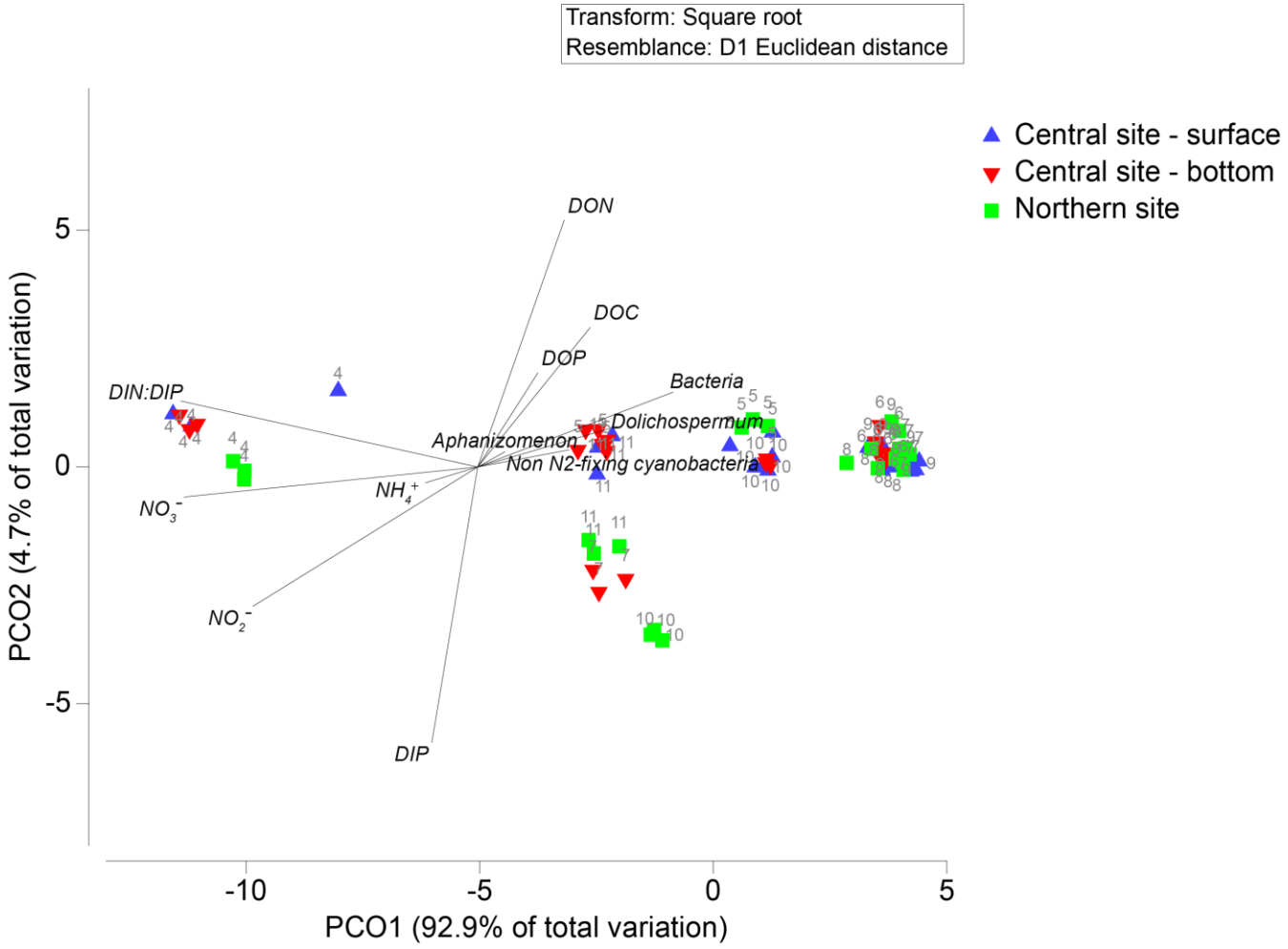
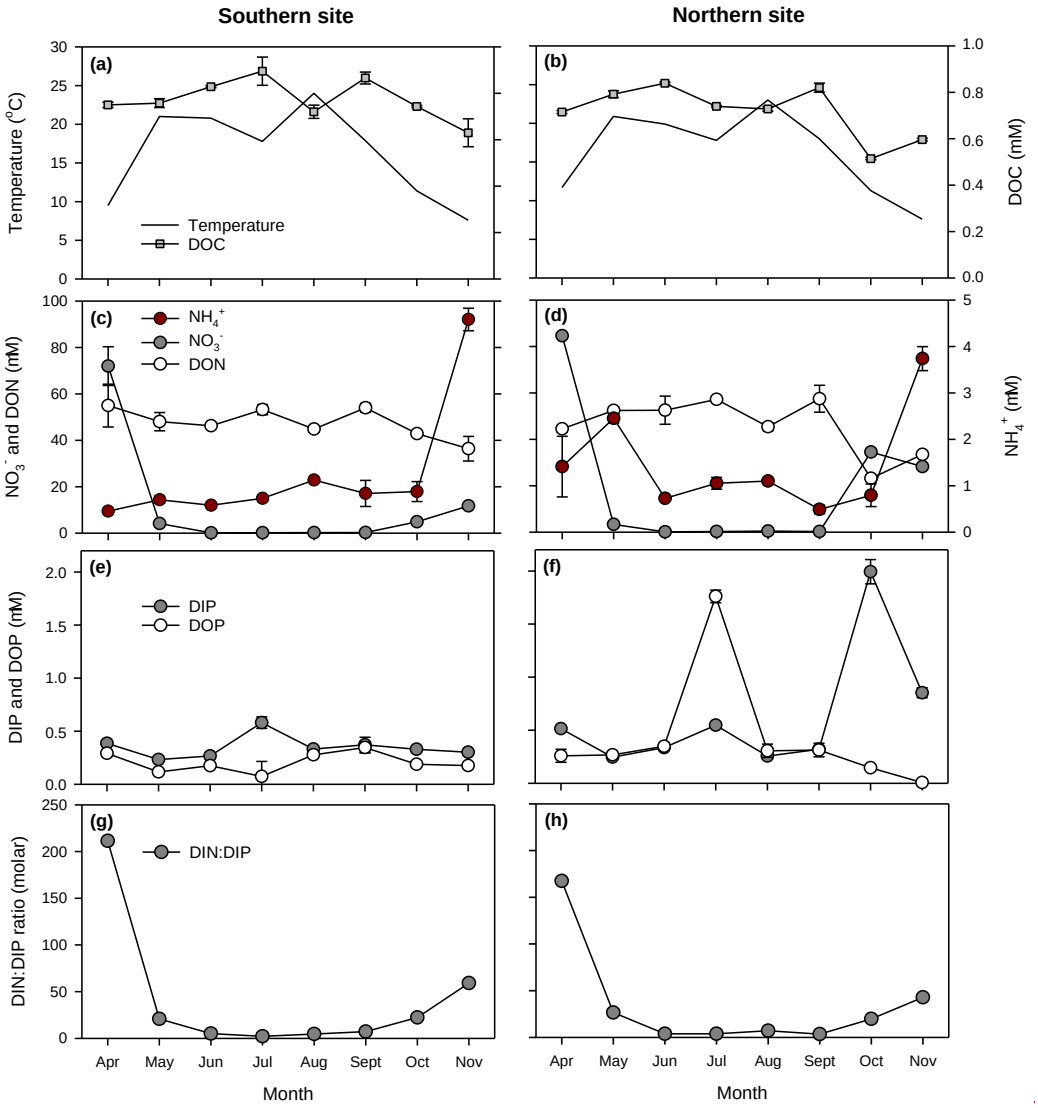


Figure 3: Principal coordinate biplots generated on Euclidean distances of normalized and forth-root transformed nutrient concentrations (DOC, NH_4^+, NO_2^-, NO_3^-, DON, DIP, DOP, and DIN:DIP). Overlaid vectors show individual chemical variables (those significantly correlating with either of the two primary axes, with Pearson correlations > 0.5) and plankton community biomass (*Aphanizomenon, Dolichospermum*, non -N_2-fixing cyanobacteria and heterotrophic bacteria).

Principal coordinates analysis revealed spatiotemporal differences in nutrient concentrations and plankton community characteristics (Fig. 3). The first principal coordinate axis explained 93% of the total variation by differentiating April samples on the basis of high NO_3^- (~80 µM) and high DIN:DIP (~200 molar) relative to samples collected in May–November (NO_3^- mean = 2.5 µM; DIN:DIP mean = 14 molar; Fig. 3, 4). Biomass of *A~~ph~~. flosaquae*, *Dolichospermum* spp., non N_2-fixing cyanobacteria and heterotrophic bacteria were positively associated with axis 1 indicating their dominance during low N and low DIN:DIP conditions. The second principal coordinate axis accounted for 5% of variation and separated samples collected in July from the bottom layer (~~southern~~ central site) and during October–November at the northern site. These samples were

characterized by higher DIP (up to 2 µM) and lower DON (< 40 µM) relative to samples collected at other sites and dates (DIP < 0.5 µM; DON 40–60 µM), but did not reveal differences in community characteristics. Overall, NO_3^- was the dominant fraction of dissolved N in spring, whereas DON was the main fraction in summer and fall. Seasonal variation in DIP and DOP was small (< 2 µM) in comparison to N, such that changes in the relative availability of N vs. P were largely determined by N concentrations.


Southern site
Northern site
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Temperature (°C)
DOC (mM)
Temperature
DOC
NO3- and DON (mM)
NH4+ (mM)
NH4+
NO3-
DON
DIP and DOP (mM)
DIP
DOP
DIN:DIP ratio (molar)
DIN:DIP
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Month

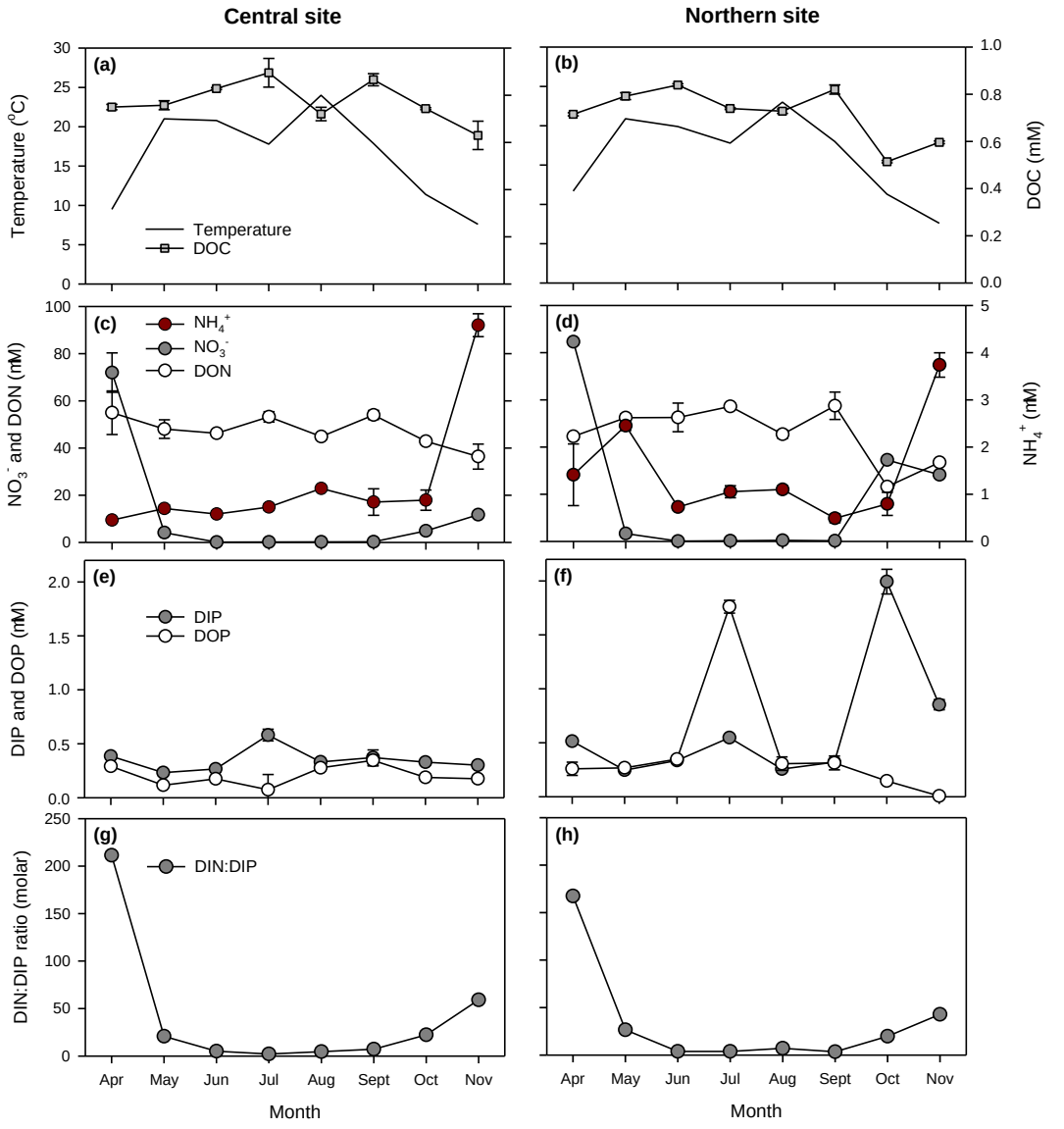
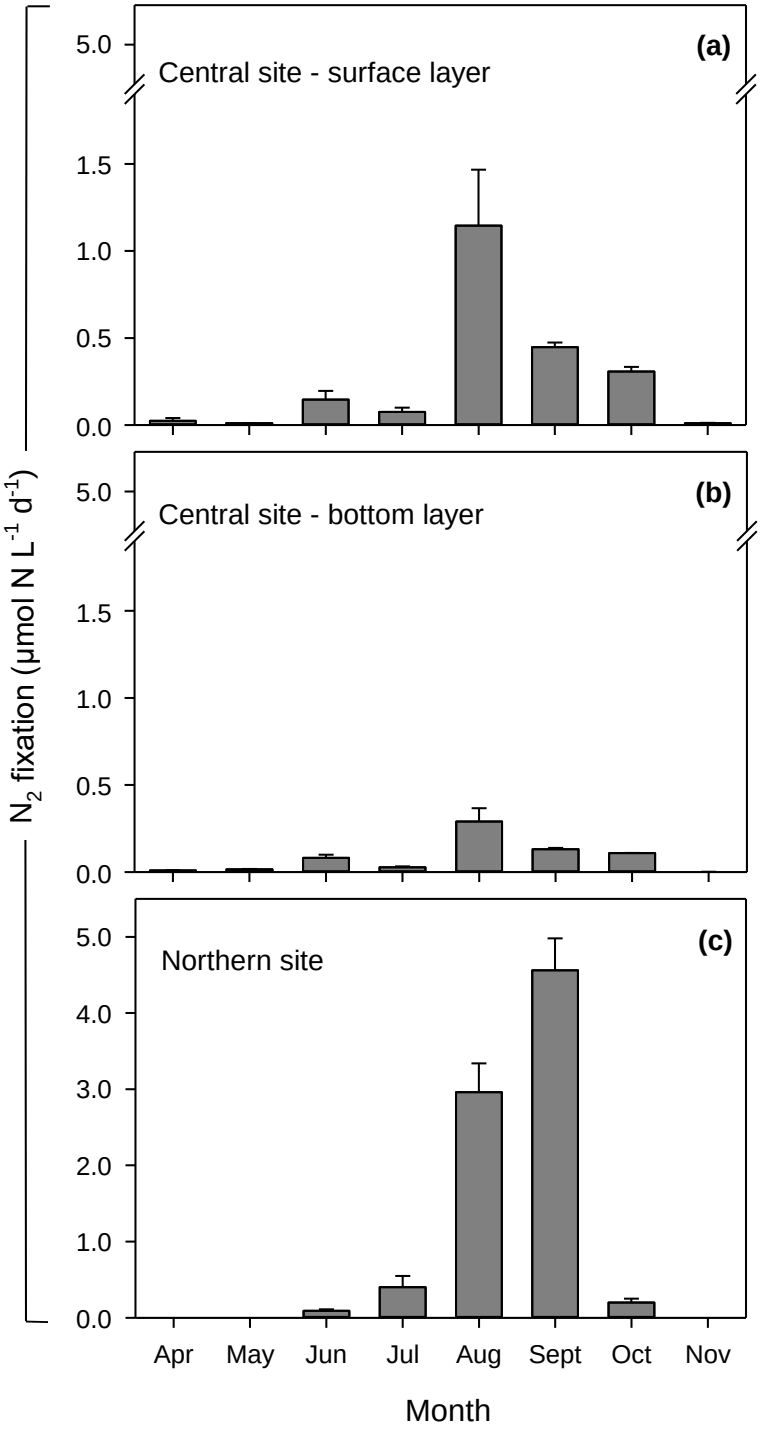


Figure 4: Temporal patterns in temperature, dissolved organic carbon (a, b), dissolved inorganic and organic nitrogen (c, e), phosphorus (e, f), and DIN:DIP ratios (g, h) at ~~southern~~ central (surface layer; left panel) and northern (right panel) sites in the Curonian Lagoon during 2018 (error bars denote standard error based on 3 replicates; some not visible).


(a)
Central site - surface layer
(b)
Central site - bottom layer
(c)
Northern site
5.0
1.5
1.0
0.5
0.0
4.0
3.0
2.0
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Month
N2 fixation (µmol N L-1 d-1)

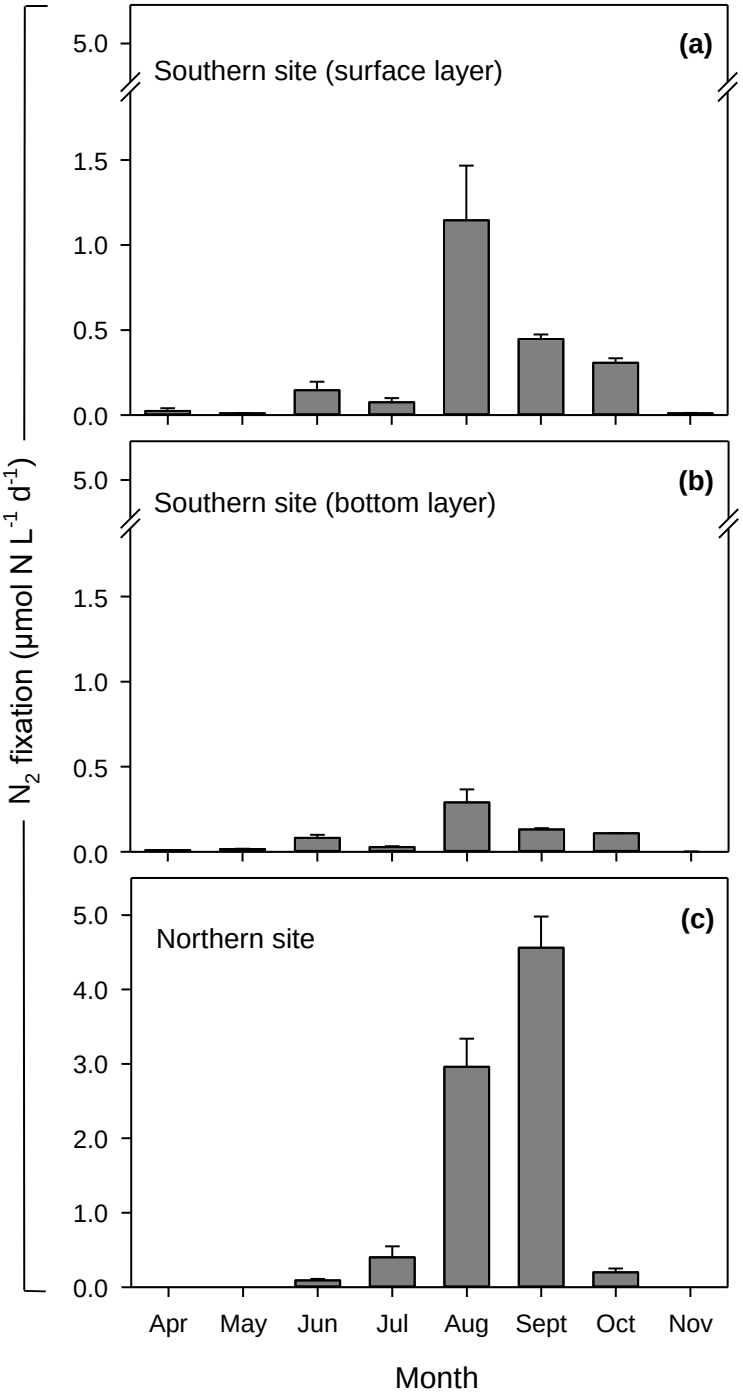
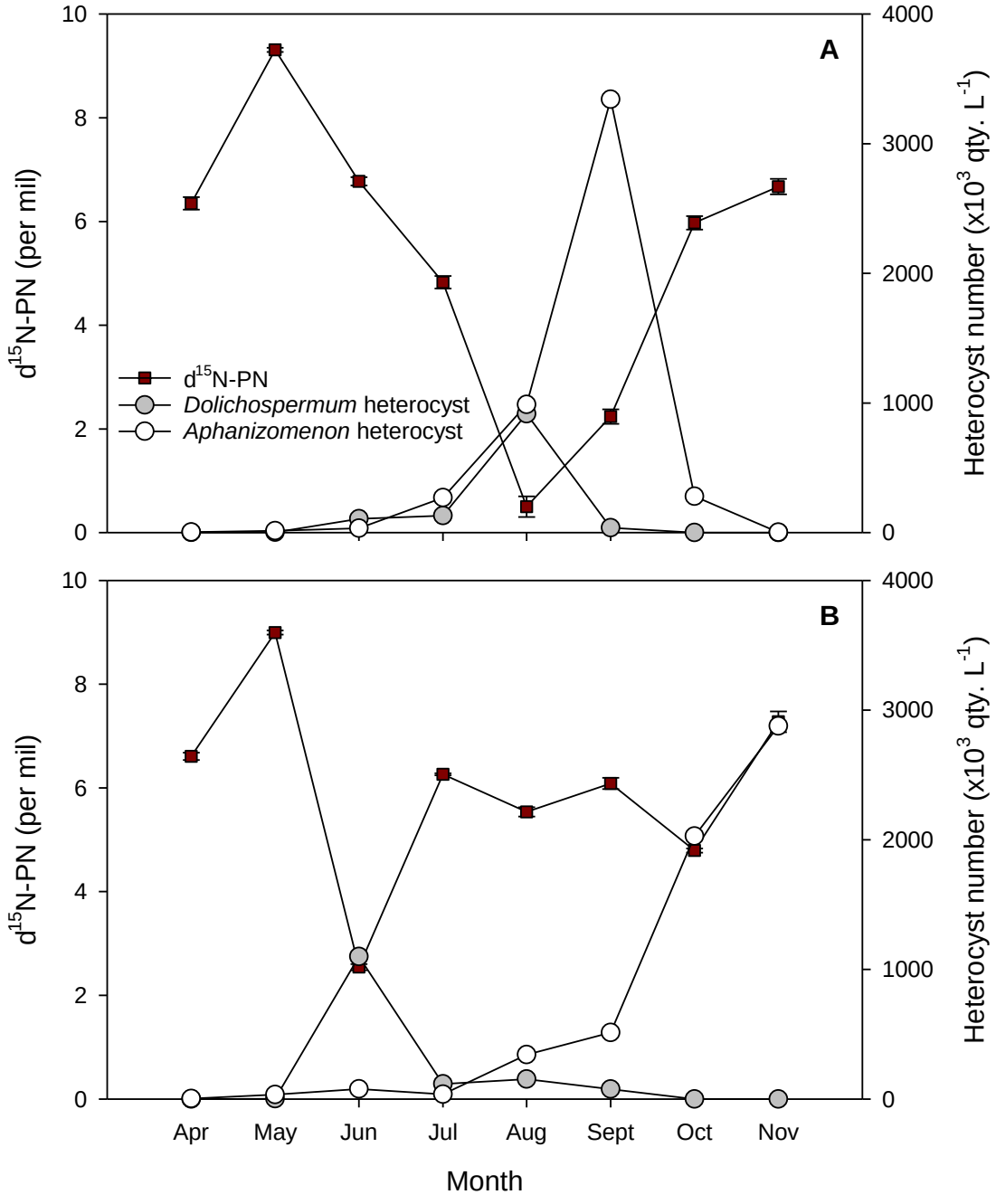


Figure 5: Rates of N_2 fixation at central~~northern~~ (a, b) and ~~southern~~ northern (~~b,~~ c) sites in the Curonian Lagoon in 2018 (data are mean values and standard error based on 3 replicates).

3.2 N_2 fixation

Rates of N_2 fixation varied by over two orders of magnitude (< 0.1 to > 5~~200~~ µ~~n~~mol N L^{-1} d~~h~~$^{-1}$) with the highest rates measured at the northern site in August and September (3.0~~123~~ ± 0.4~~16~~ and 4.6~~190~~ ± 0.4~~17~~ µ~~n~~mol N L^{-1} d~~h~~$^{-1}$, respectively; Fig. 5). A comparison of N_2 fixation in the surface and bottom layers at the ~~southern~~ central site showed that rates were consistently lower (<0.4~~15~~ µ~~n~~mol N L^{-1} d~~h~~$^{-1}$) in the deeper layer. The abundance of heterocysts varied seasonally depending on the number of vegetative cells of N_2-fixing cyanobacteria ($y = 0.0251x + 75.0$, $R^2 = 0.92$) with lowest values less than 2000 cells L^{-1} and peak values exceeding 2 million cells L^{-1} in late summer (Fig. 6 a, b). Heterocysts of *A~~ph~~. flosaquae* accounted for > 95% of total heterocysts as contributions from *Dolichospermum* spp. were small by comparison (< 150,000 cells L^{-1}). The heterocyst frequency per filament showed distinctive temporal patterns between sites depending on the species~~was higher at the beginning of summer (up to 15 mm^{-1}) at both sites, and gradually declined afterwards (Fig.~~ (Fig. 6c, d). At the central site, two peaks up to 8.0 mm^{-1} in heterocyst frequency of both species was observed during June–September. Whereas heterocyst frequency at the northern site remained quite high through summer primarily contributed by *A. flosaquae* (~10 mm^{-1}), later followed by *Dolichospermum* spp. (~8 mm^{-1}). The N isotopic signature of PN declined with increases in heterocyst frequency~~abundance~~ and rates of N_2 fixation. Prior to the cyanobacteria bloom, $\delta^{15}N$ values were 6–9 ‰ and declined to less than 1 ‰ by August before rebounding in October–November (Fig. 6c, d). Smaller declines in $\delta^{15}N$ values were observed at the southern site.


A
B
d15N-PN (per mil)
Heterocyst number (x10^3 qty. L^-1)
d15N-PN
Dolichospermum heterocyst
Aphanizomenon heterocyst
0
2
4
6
8
10
0
1000
2000
3000
4000
Apr
May
Jun
Jul
Aug
Sept
Oct
Nov
Month

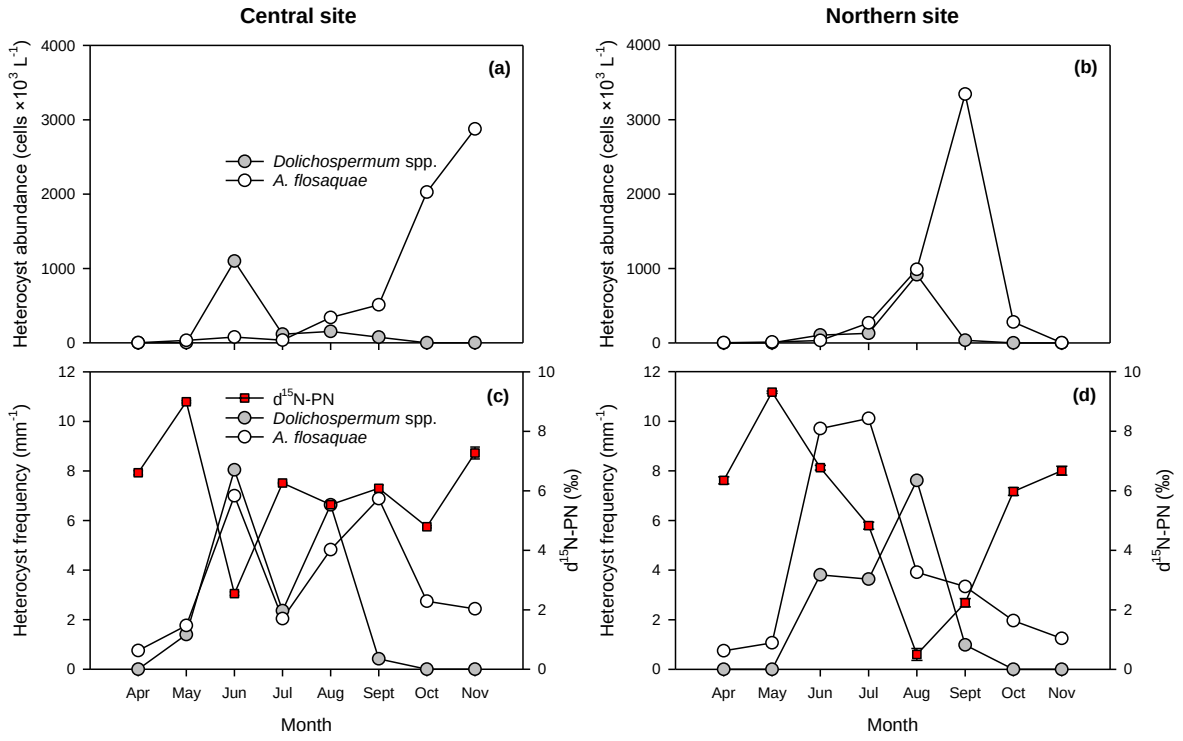
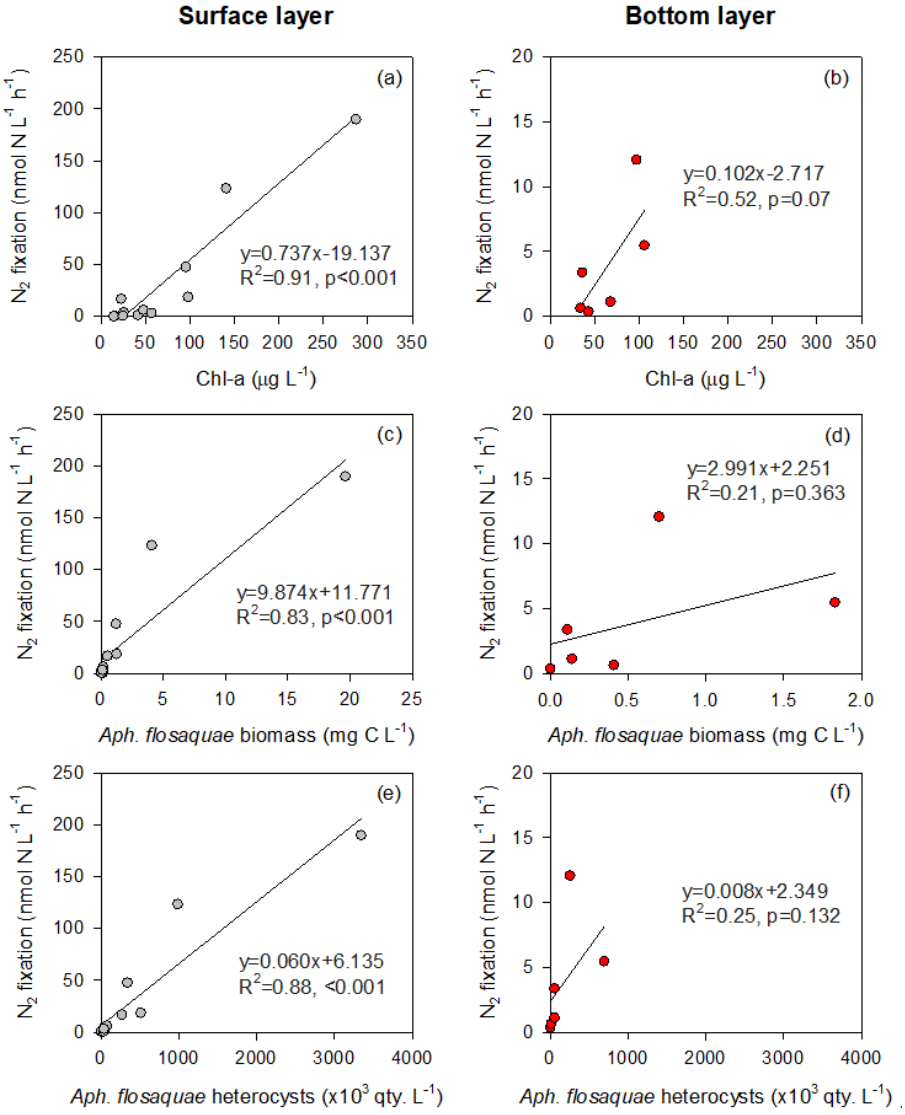


Figure 6: Abundance of heterocysts of *Dolichospermum* spp. and *A~~ph~~. flosaquae* (a, b), heterocyst frequency per filament length and stable isotope composition of particulate nitrogen (δ^{15}N-PN) (c, d) at ~~southern~~ central (surface, left panel~~s~~) and northern sites (right panel~~s~~) in the Curonian Lagoon during 2018. δ^{15}N-PN values are mean and standard error based on 3 replicates (some error bars not visible).

N_2 fixation in the surface layer was significantly ($p < 0.001$) predicted by *in situ* Chl-a concentration ($R^2 = 0.91$), *A~~ph~~. flosaquae* biomass ($R^2 = 0.83$) and *A~~ph~~. flosaquae* heterocysts ($R^2 = 0.88$, all $p < 0.001$; Fig. 7). Whereas N_2 fixation in the bottom layer was weakly explained ~~correlated~~ by *in situ* Chl-a ($R^2 = 0.52$, $p = 0.08$), but not *A~~ph~~. flosaquae* biomass or heterocysts. *In situ* c~~C~~hlorophyll-specific N_2 fixation ~~derived from regression equations (Fig. 7a, b)~~ derived from regression equations (Fig. 7a, b) was considerably lower in the deeper layer (0.002 ± 0.001 µ~~0.102 ± 0.049 n~~mol N µg^{-1} Chl-a d~~h~~$^{-1}$) relative to the surface layer (0.018 ± 0.002 µ~~0.737 ± 0.076 n~~mol N µg^{-1} Chl-a d~~h~~$^{-1}$).


Surface layer
Bottom layer
(a)
(b)
(c)
(d)
(e)
(f)
N_2 fixation (nmol N L^{-1} h^{-1})
Chl-a (μg L^{-1})
y=0.737x-19.137
R^2=0.91, p<0.001
y=0.102x-2.717
R^2=0.52, p=0.07
Aph. flosaquae biomass (mg C L^{-1})
y=9.874x+11.771
R^2=0.83, p<0.001
y=2.991x+2.251
R^2=0.21, p=0.363
Aph. flosaquae heterocysts (x10^3 qty. L^{-1})
y=0.060x+6.135
R^2=0.88, <0.001
y=0.008x+2.349
R^2=0.25, p=0.132

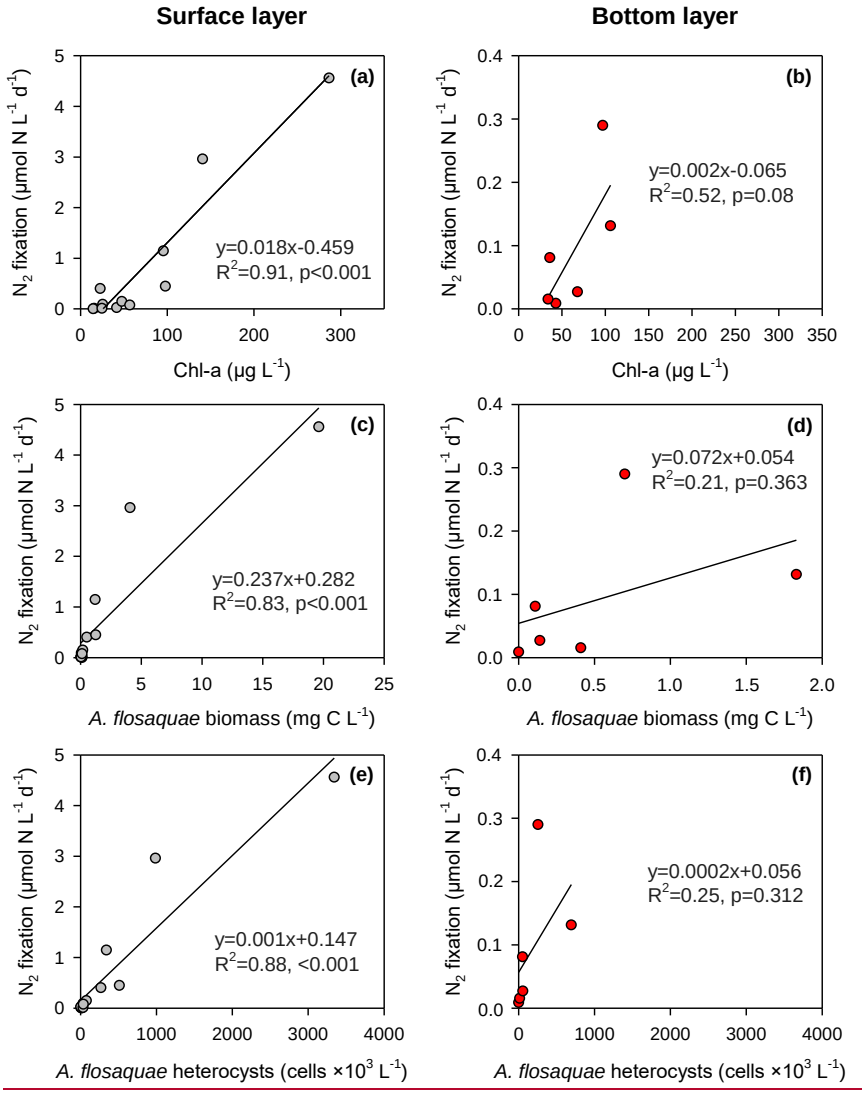


Figure 7: Relationships between N_2 fixation and *in situ* measured Chlorophyll-a (a, b), *A. flosaquae* biomass (c, d) and their heterocysts (e, f) in surface (northern and ~~southern~~ central sites, left panel) and bottom (southern site, right panel) layers of the Curonian Lagoon during April–September 2018.

Remote sensing-based estimates of lagoon-wide Chl-a increased from 65.4 ± 0.9 µg L^{-1} (July 4) to 88.7 ± 0.2 µg L^{-1} (August 8), and thereafter remained relatively stable throughout August and September (means = 84 to 89 µg L^{-1}). Satellite-derived Chl-a values for each grid cell were used along with the regressions relating N_2 fixation to Chl-a ~~(Fig. 7)~~ to derive estimates of N_2 fixation for each cell. We used the relationship between N_2 fixation and *in situ* Chl-a (y = 0.018x – 0.459; Fig. 7a) for the surface layer to derive estimates of N_2 fixation for the upper water column (0–2 m). For deeper areas, we used the relationship between N_2 fixation and *in situ* Chl-a for the bottom layer. We assumed that surface Chl-a (from remote sensing)

was representative of Chl-a in the deeper layer as we did not find significant differences between the two layers sampled at the ~~southern~~ central site (SNK test, $p < 0.05$). The impact of the bloom on N_2 fixation can be visualized from the relatively low and uniform rates~~values~~ throughout the lagoon during July, and the subsequent development of localized hotspots in the southern lagoon during August and September (Fig. 8). Lagoon-wide average values of N_2 fixation increased from 1.5~~1559~~ (July 4), to 2.5~~467~~ (August 8) and thereafter ~~ranged from 2324 to 2352~~remained ~2.3 m~~μ~~mol m^{-2} d^{-1} through the end of September.

4 Discussion and Conclusion

We characterized seasonal variation in phytoplankton communities in relation to nutrient conditions to better understand the mechanisms regulating pelagic N_2 fixation in the Curonian Lagoon. Findings based on this study and our prior work (Zilius et al., 2018) suggest that the decline in riverine NO_3^- inputs following spring snowmelt, and the subsequent depletion of DIN in the lagoon, provides favourable conditions for an active diazotrophic community during summer and fall. Stoichiometric ratios of dissolved inorganic nutrients are frequently used to identify potential limiting elements and their role in driving community succession (Ptacnik et al., 2010; Perez et al., 2011). The occurrence of elevated NO_3^- concentrations and high DIN:DIP after spring runoff was followed by an extended period (8 months) of persistent low N availability, creating a temporal niche for heterocystous cyanobacteria (Supplement, Fig. S1). In a related study, we used molecular techniques to document the diversity of diazotrophs of the Curonian Lagoon and found that the community shifted from N_2-fixing heterotrophic bacteria in spring to photosynthetic heterocystous cyanobacteria in summer–fall (Zilius et al., 2020). Though sequences were also attributed to diazotrophic picocyanobacteria (*Synechococcus*, *Crocosphaera*, *Rippkaea*, and *Cyanothece*), these were not detected with flow cytometry, suggesting low abundance. Here, we show that spatial and temporal variation in rates of N_2 fixation were primarily determined by the abundance of heterocystous cyanobacteria. The maximum abundance of heterocysts occurred during the bloom of *A~~ph~~. flosaquae* and coincided with the peak in N_2 fixation rates and the decline in δ^{15}N-PN to values similar to atmospheric N. The heterocyst frequency per filament of *A. flosaquae* declined with increasing biomass, possibly indicating N_2 fixation, which requires lower number of heterocysts. Heterocyst formation is triggered by inorganic N depletion (e.g. Kumar et al., 2010) and in the Curonian Lagoon we observed the peak in heterocyst frequency (up to 15 per filament)~~abundance~~ in early summer as DIN was depleted to < 1 μM. Similar to other sites in the Baltic, the peak in heterocyst frequency was found when *A. flosaquae* biomass was still low (Walve and Larsson, 2007; Zakrisson and Larsson, 2014; Klawonn et al., 2016). Our estimates of *A~~ph~~. flosaquae* abundance were an order of magnitude higher than those previously reported for temperate and boreal estuarine systems (Bentzon-Tilia et al., 2015; Klawonn et al., 2016; Olofsson et al., 2020a~~b~~). T~~All t~~hese findings support the idea that *A~~ph~~. flosaquae* is the principal contributor to N_2 fixation in the brackish Baltic Sea and its adjacent coastal areas. The proliferation of heterocystous cyanobacteria in the Curonian Lagoon is favoured by P (Pilkaitytė and Razinkovas, 2007), which is released from sediments, particularly when bloom conditions result in high water column respiration and transient (night-time) depletion of oxygen (Zilius et al., 2014; Petkuviene et al., 2016). ~~These results support~~

~~prior work showing that Aph. flosaquae is the principal contributor to N2 fixation in the brackish Baltic Sea and its adjacent coastal areas (Klawonn et al., 2016; Olofsson et al., 2020a).~~ Moisander et al. (2007) demonstrated that P can enhance diazotrophic activity of heterocystous cyanobacteria in microcosms. Release of DIP from sediments may in turn enhance rates N_2 fixation resulting in a positive feedback for cyanobacteria bloom development. Measured summer DIP concentration (0.3 µM) in the Curonian Lagoon was similar to that in other Baltic coastal sites (e.g. Klawonn et al., 2016), suggesting that higher biomass might be also supported by higher N availability. A recent study by Broman et al. (submitted) suggests that N_2 fixation in the lagoon satisfies only 13% of N demand for phytoplankton. Thus, other internal sources such as N release from sediment

and mineralization in the water column are important to meeting algal N demands.

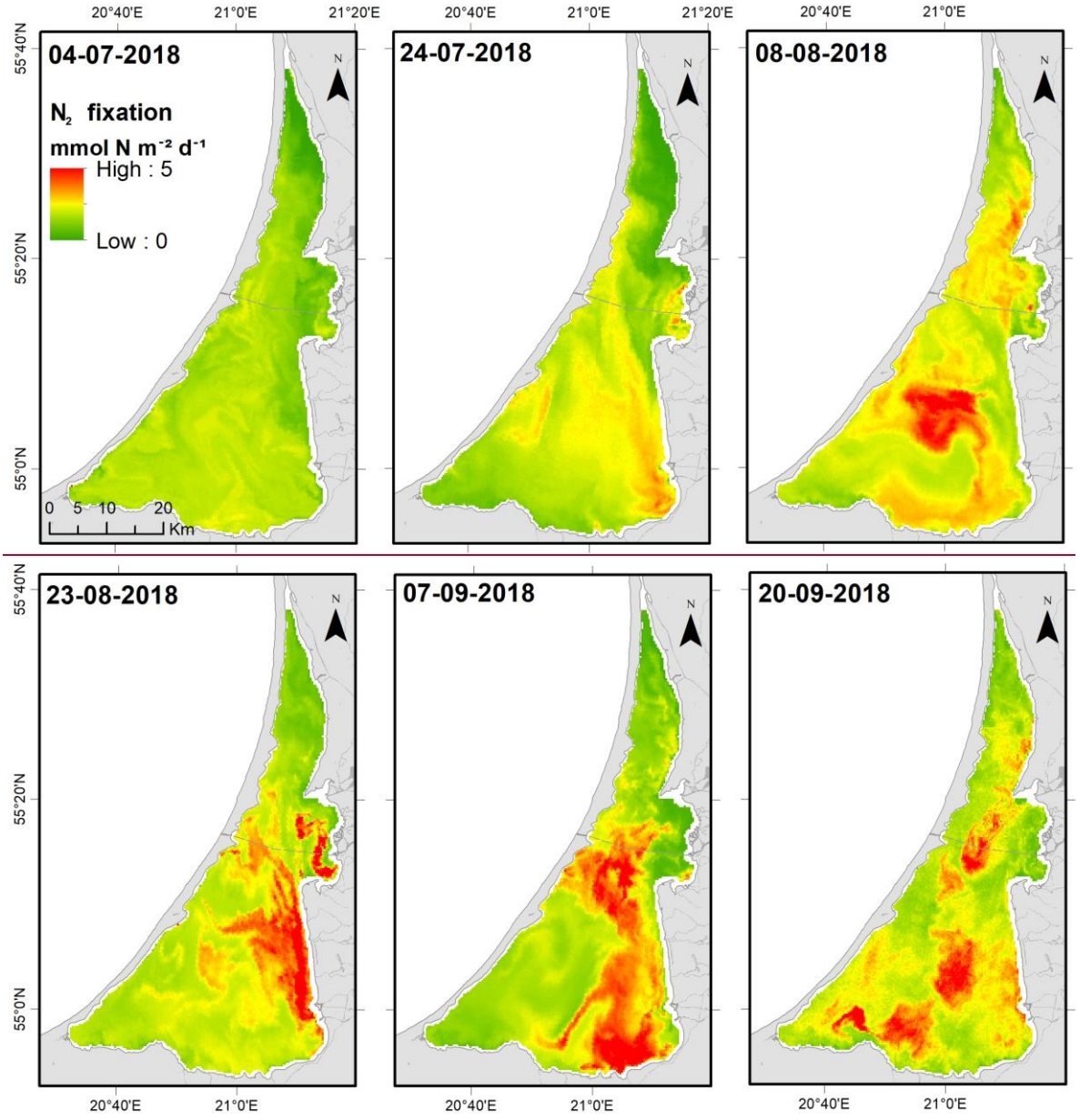


Figure 8: Estimates of pelagic N_2 fixation in the Curonian Lagoon derived from remote sensing of Chlorophyll a.

The patchy distribution of cyanobacteria poses a significant challenge to reliably extrapolating results from site-specific measurements to the ecosystem scale (Zilius et al., 2014; 2018). Surface accumulation of positively-buoyant cyanobacteria and subsequent wind dispersion adds a dynamic component to biogeochemical processes in eutrophic lakes and estuarine

systems (e.g. Gao et al., 2014; Zilius et al., 2014; Klawonn et al., 2015). Our previous work describing N fluxes in the Curonian Lagoon relied on a simple extrapolation of N_2 fixation rates measured at the two stations also used in this study. Here, we improve on our ability to scale up these measurements by using remote sensing of Chl-a to infer spatial and temporal variation in N_2 fixation. Our whole-lagoon estimates are based on data collected at stations within the northern and central portions of the lagoon, as access to the southern region is problematic. Hydrodynamic modeling studies have shown that water renewal times in the central and southern portions of the lagoon are comparable (Umgiesser et al., 2016). Monitoring data suggest that Chl-a and phytoplankton community composition is similar in the central and southern regions (Semenova and Dmitrieva, 2011; Bresciani et al. 2014; Vaičiūtė et al., 2021). Therefore, we felt it was appropriate to derive whole-lagoon estimates of N fixation based on *in situ* measurements from these two sites. We benefitted from prior work deriving Chl-a estimates from satellite images and their calibration to *in situ* measurements (Bresciani et al., 2014), but the success of the approach largely relied on the fact that heterocystous cyanobacteria dominated the summer–fall phytoplankton community of the lagoon, which provided a significant ~~correlation~~ relationship between N_2 fixation and *in situ* Chl-a in surface layer. The regression model for estimating bottom layer N_2 fixation was marginally significant, and therefore we felt that the application of this model to deriving whole-water column rates was justified. Whole-lagoon estimates were not highly sensitive to assumed rates in the bottom layer because this layer accounts for a relative small proportion of the lagoon's volume and because measured N_2 fixation rates in the bottom layer were 7 times lower than the surface. However, it would be problematic to extrapolate this approach to periods outside of cyanobacteria dominance (e.g., spring diatom bloom) or to periods when other factors (e.g., low temperature in fall) constrain N_2 fixation. The transferability of this approach to other systems would likely depend on this facet; in systems where heterocystous cyanobacteria account for a small and variable fraction of Chl-a, the utility of Chl-a as a predictor of N_2 fixation may be limited. Prior studies have used remote sensing to infer N_2 fixation, though by less direct means. For example, Hood et al. (2002) used SeaWiFS-derived estimates of *Trichodesmium* Chl-a, and modelled relationships between N_2 fixation and underwater irradiance to infer N_2 fixation in the tropical Atlantic Ocean. Coles et al. (2004) used remote sensing of Chl-a to estimate phytoplankton production in the North Atlantic and infer rates of N_2 fixation needed to support production. Other studies have related taxa-specific N_2 fixation to *in situ* measurements of Chl-a or algal biomass, including recent work in the Baltic Sea (Olofsson et al., 2020b). To our knowledge, ours is the first study to derive ecosystem-scale estimates by combining remote sensing of Chl-a with empirical models relating rates of N_2 fixation to Chl-a.

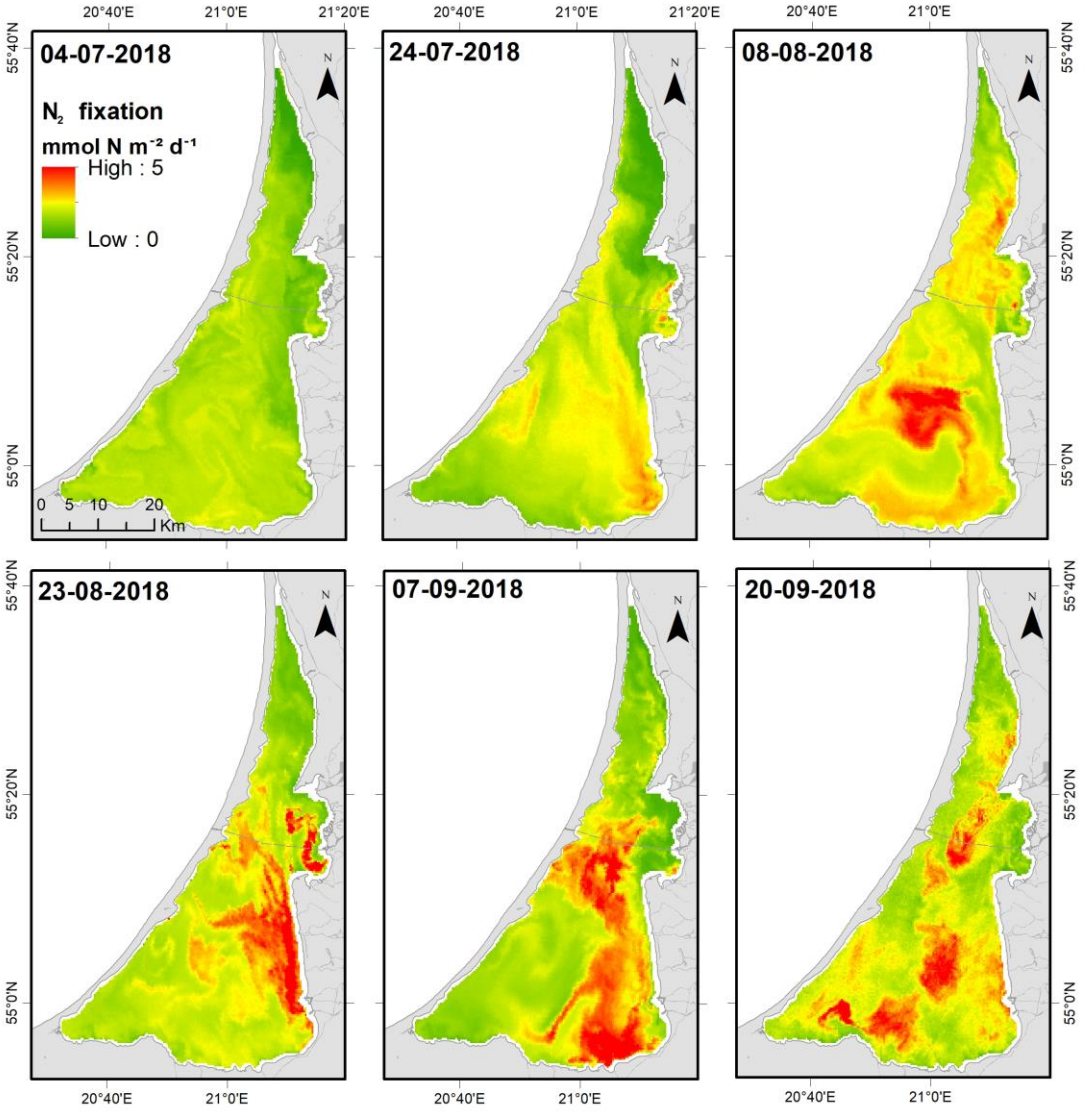


Figure 8: Estimates of pelagic N_2 fixation in the Curonian Lagoon derived from remote sensing of Chlorophyll-a.

Our remote sensing-based estimates of N_2 fixation for the Curonian Llagoon ranged from 11.5559 to 2.52467 mμmmol N m^{-2} d^{-1} (mean = 2.1140 ± 0.1147 mμmol N m^{-2} d^{-1}) during July–September. These estimates reveal that summer N_2 fixation rates are slightly lower in the Curonian Lagoon as compared to those measured at a coastal site of SW Baltic (3.6 ± 2.6 mmol N m^{-2} d^{-1}; Klawonn et al., 2016), but higher than those found in the Great Belt (~ 1 mmol N m^{-2} d^{-1}; Bentzon-Tilia et al., 2015), Baltic Proper (0.4 ± 0.1 mmol N m^{-2} d^{-1}; Klawonn et al., 2016), and Bothnian Sea (0.6 ± 0.2 mmol N m^{-2} d^{-1}; Olofsson et al., 2020b). By comparison, *in situ* estimates scaled using our prior method (based on the proportion of lagoon area represented by the two stations) yielded estimates ranging from 0.360 to 3.6590 mμmol N m^{-2} d^{-1} (mean = 1.930 ± 0.9870 mμmol N m^{-2} d^{-1}) for corresponding dates. The two approaches yielded similar mean values, though with lower variability among those

based on remote sensing. We attribute this to the shifting spatial distribution of cyanobacteria in the lagoon, which results in greater variability in site-specific measurements relative to the lagoon-scale assessments captured by remote sensing. These new estimates confirm our prior findings regarding the importance of N_2 fixation to the N balance of the lagoon. During periods of low river discharge, rates of N_2 fixation were twofold higher compared to monthly TN loads from the Nemunas River (June–October 2018 range = 0.83~~0~~ to 1.1~~2~~~~0~~ m~~μ~~mol N m^{-2} d^{-1}) (Supplement, Fig. S1). N_2 fixation during summer and fall largely offset annual average denitrification (3.2~~1~~~~90~~ m~~μ~~mol N m^{-2} d^{-1}) and was equivalent to half of the measured sediment-water TDN exchange (3.8~~7~~~~90~~ m~~μ~~mol N m^{-2} d^{-1}; Zilius et al., 2018). Our prior work also showed enhanced PN export to the Baltic Sea during periods when the lagoon was dominated by cyanobacteria. Positive buoyancy allows *A~~ph~~. flosaquae* and other cyanobacteria to remain suspended in the water column, which favours export in lagoon outflow, rather than retention via sedimentation (Bukaveckas et al., 2019). Overall, these findings suggest that the occurrence of heterocystous cyanobacteria blooms has substantially diminished the potential for the lagoon to attenuate N fluxes to the Baltic Sea. As blooms of N_2-fixing cyanobacteria are common among the three large Baltic lagoons (Curonian, Oder, and Vistula), their effect in diminishing lagoon N retention may be regionally important (Bangel et al., 2004; Dmitrieva and Semenova, 2012). Our approach using remote sensing combined with local, empirical models relating N_2 fixation to Chl-a may provide a useful means for assessing the role of cyanobacteria blooms in the context of N budgets for the Baltic Sea (e.g. Savchuk, 2018).

Our research has allowed us to better understand the environmental conditions that favour the occurrence of heterocystous cyanobacteria blooms and their contributions to the N budget of the lagoon. Important questions remain regarding the factors that regulate rates of N_2 fixation and the fate of atmospherically-derived N. Underwater irradiance is likely an important factor influencing biomass-specific N_2 fixation given its energetic costs. The Curonian Lagoon is a relatively turbid system in which the photic zone typically occupies less than 30% of the water column (Zilius et al., 2014). Our no-light incubations simulating the deeper layer of the south-central~~ern~~ lagoon showed that N_2 fixation was occurring, but at biomass-specific rates that were 7-fold lower in comparison to the surface layer. Low N_2 fixation rates during dark incubations were also observed in cyanobacteria filaments collected from other coastal sites of the Baltic Sea (Svedén et al., 2015). Though heterocystous cyanobacteria continue to fix N_2 in the dark, it remains unknown for how long due to the high energetic costs. Previous measurements of N_2 and carbon fixation in *Aphanizomenon* from the Baltic Sea suggest that respiration of stored cell products may provide energy for N_2 fixation under low light conditions (Svedén et al., 2015). Our study, as well as prior work, is based on 24-h incubations, simulating conditions at a fixed depth, which may not be indicative of rates that could be sustained by diazotrophs circulating over a range of depth and light conditions. Positive buoyancy and periodic mixing toward the surface may allow cyanobacteria to capture sufficient light energy to sustain N_2 fixation (Stal and Walsby, 2000). In addition to light availability, water temperature is likely an important constraint on seasonal patterns of N_2 fixation in temperate systems. Results from this, and a prior study (Zilius et al., 2018), show that despite a high abundance of *A~~ph~~. flosaquae* ~~heterocyst~~ at the end of fall, heterocysts frequency, and thus N_2 fixation rates declined substantially when water temperature dropped below

15 °C. Zakrisson et al. (2014) suggested that temperature controls the enzymatic activity of nitrogenase, which directly regulates the intensity of N_2 fixation in filaments.

Recent work has shown that N fixed by diazotrophs is subsequently distributed to the planktonic food web (Woodland et al., 214; Karlson et al., 2015~~Adam et al., 2016~~), which likely involves a variety of mechanisms including grazing (Woodland et al., 213), leakage of NH_4^+ and DON (Ohlendieck et al., 2007; Adam et al., 2016), and remineralization of N following algal senescence (Eglite et al., 2018). The relative importance of these pathways is not well known, though our data for the Curonian Lagoon suggests that heterotrophic bacteria, non-N fixing cyanobacteria and a diverse group of grazers benefit from the activities of heterocystous cyanobacteria. The biomass of heterotrophic bacteria increased during the bloom of heterocystous cyanobacteria to levels (250 µg C L^{-1}) that were appreciably higher than other coastal (Gulf of Finland and Archipelago Sea = 30–55 µg C L^{-1}) or open areas of the Baltic Sea (Bothnian Sea ~80 µg C L^{-1}; Baltic Proper = 16–44 µg C L^{-1}; Heinänen, 1991). It is likely that heterocystous cyanobacteria release dissolved organic matter which stimulates the growth of heterotrophic bacteria (Bertos-Fortis et al., 2016; Hoikkala et al., 2016; Berg et al., 2018; Berner et al., 2018). There is also evidence that non-N_2-fixing cyanobacteria benefitted from the bloom of heterocystous cyanobacteria as indicated by higher abundance of *Microcystis* spp. and *Planktotrix agardhii*. Measured low $\delta^{15}N$ values (0.5 ± 0.2 ‰) in suspended living material suggest that fixed N can temporally support most of the nutritional needs for plankton (bacteria + phytoplankton) growth. Lastly, our prior work using stable isotopes tracked atmospherically-derived N from cyanobacteria to a diverse group of consumers and suggested that 50–80% of secondary production was supported by cyanobacteria during bloom events (Lesutiene et al., 2014).

In conclusion, our study contributes to a better understanding of the activity of coastal diazotrophs and their seasonal dynamics in eutrophic estuarine systems. The use of remote sensing allowed us to estimate N_2 fixation rates at the ecosystem scale and to show that these rates are high and relatively stable despite the dynamic and patchy distribution of cyanobacteria. The propensity for cyanobacteria to form dense, localized aggregates may influence the efficiency with which by-products of their carbon and N_2 fixation are disseminated by creating biogeochemical hotspots (Klawonn et al., 2015, 2019). Since intensifying blooms of cyanobacteria have already been observed in coastal areas of the Baltic Sea (Olofsson et al., 2020a~~b~~), we may expect these blooms to have a~~their~~ stronger effect on ecosystem functioning in future. Therefore, further work combining remote sensing and *in situ* studies may provide greater insights as to the fate of atmospherically-derived N and its implications for ecosystem energetics.

Authors contribution

MZ, PAB and DV conceived the ideas and designed methodology. MZ, IVL, SB and TB led the field survey and experimental activities. IV-L, DV, DO, EG, IL, SBr, AA and AZ assisted with analysis and data collection and analysis. AZ assisted with statistical analyses. MZ and SB secured funding for the investigation. MV provided use of specialized facilities, MZ and PAB wrote the first draft of the paper, and all co-authors contributed to writing review and editing. ~~We kindly thank the Editor and anonymous reviewer for their constructive comments.~~

Conflict of interest

The authors declare that they have no conflict of interest.

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