# Response to Anonymous Referee #1

## 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.

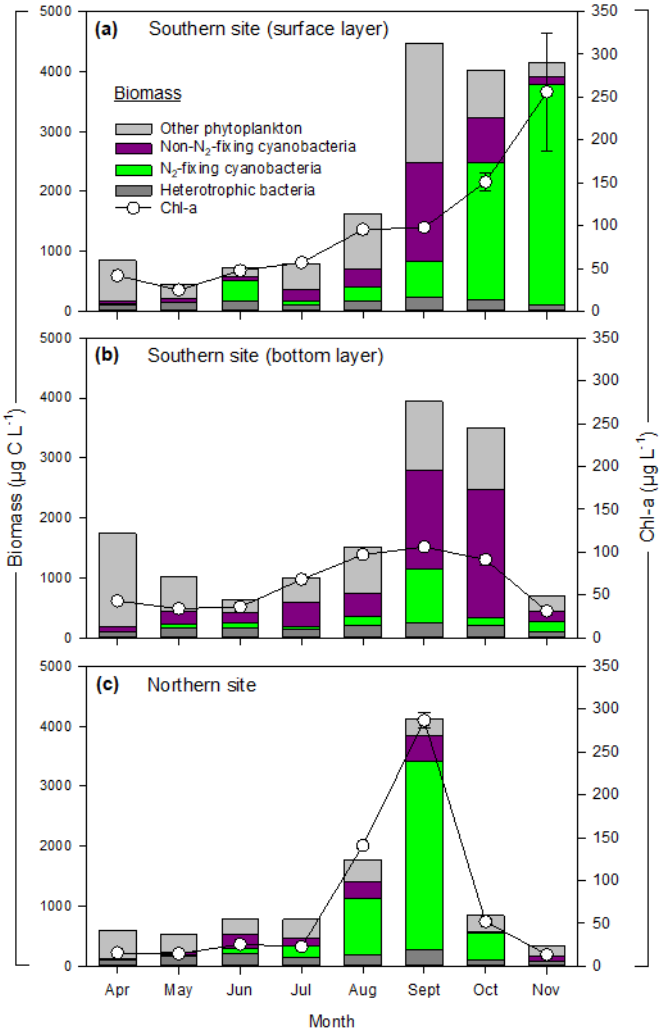


**Figure 2: Phytoplankton and bacteria biomass at southern (a, b) and northern (c) sites in the Curonian Lagoon during 2018. Chlorophyll-a concentrations are mean values and standard error (some error bars not visible) based on three replicates.**

**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).