The study contains an interesting data set with UCYN-A including both genes and potential N2 fixing transcripts in the southern Baltic Proper. However, the study was conducted a bit off season as compared to expected and the manuscript lacks an argumentation to why this study is needed? Was it late in the season on purpose and if so why?

Authors' response (AR): We thank the reviewer for considering our manuscript interesting, for a thorough and helpful review of our manuscript, and would like to say that we really appreciate the time the reviewer invested for helping us improving the manuscript.

We conducted the study off season because exactly because it rarely has been done and we were hoping to come with this to an understanding of N_2 fixation over the course of the year. Most studies are conducted in and around the bloom seasons, but fall/ winter studies are rarely presented. We, however, find it important to do so, and hope this study can contribute to a better assessment of N_2 fixation over the course of the year, and possibly inform models by doing so. In order to clarify this point, we provided an explanation in L.89 of the revised version of the manuscript:

'To now explore the impact and importance of salinity for diazotrophy in the Baltic Sea and in order to complement existing datasets on N2 fixation from high-productivity seasons, we carried out a survey…'

I think the combination of genes and transcripts is interesting, but I wonder if the transcripts can be somehow quantitative. Maybe you can present also transcripts per gene copies so its normalized to abundance? I think this particular data is what is novel with this study and should be lifted in the manuscript and aims. Especially UCYN-A has not been widely studied in the Baltic Sea and not this late in the season.

AR: We used a qPCR (quantitative real time PCR) for both gene copies and transcript copies, which is a fully quantitative method measured against a standard dilution series with known copy numbers. To present transcripts per gene copies is a good idea and a valid point, we now added a figure showing the transcriptional activity in that way.

I am a little bit confused by the use of the bubble method here for N2 fixation as I thought this was not used any more due to the risk of underestimation (e.g., White 2012) and recent studies in the Baltic Sea has used dilution method (e.g., Klawonn et al. 2016). I think this should be discussed in the manuscript and not lifted as an advantage as it is now. Can it be that rates were underestimated?

AR: True, and some of the authors were directly involved in developing the pre-dissolution method, which has later been improved by Klawonn et al. The initial purpose of the study was indeed to compare our data, however, to older datasets to reconstruct annual cycles in a Baltic Sea regional ocean model, therefore, we aimed at keeping the data comparable to older studies. Rates, especially those at the lower end of N_2 fixation according to Grosskopf et al might be underestimated, therefore, the estimate here is conservative and not fully quantitative. On the other hand, Klawonn et al, 2015 report a potential impact of trace metal contamination leading to an overestimation when using the

dissolution method, our aim was to then rather remain conservative and not risk overestimation. We added the following explanation to L. 155 ff of the manuscript:

'We used the bubble addition method (Montoya et al., 1996) to ensure comparability to previous studies from the Baltic Sea, although we are aware that more recent studies (Klawonn et al., 2016) used the dilution method. The latter study used the dissolution method, which is prone to trace metal contamination and might lead to an overestimation of N_2 fixation (Klawonn et al., 2015; Benavides et al, 2016), which we wanted to avoid. Our rates, however, seem to be within the range of all earlier studies, despite they represent a conservative assessment and possibly an underestimation (White et al., 2012, Grosskopf et al, 2014)'

References:

Benavides, M., et al. (2016), Basin-wide N2 fixation in the deep waters of the Mediterranean Sea, Global Biogeochem. Cycles, 30, 952– 961, doi:10.1002/2015GB005326.

Klawonn, I., G. LaviK, and P. Böning (2015), Simple approach for the preparation of 15N2-enriched water for nitrogen fixation assessments: Evaluation, application and recommendations, Front. Microbiol., doi:10.3389/fmicb.2015.00769.

The statistics needs some work in the paper, better describe the PCA in the text and why you have two graphs presenting what seems like contradicting results. As it is now it is difficult to tell apart what is correlated to what since all arrows has the same colors and point in different directions in A and B. Maybe it is better to run an NMDS analysis for stations and taxonomic groups correlated with environmental factors (as arrows on top), and only one panel? With significant arrows in one color and non-significant in another.

AR: The PCA is now described in more detail. The colors don't have any information; however, the directions and lengths of the arrows do. The directions tell us which components are positively (same directions) or negatively (opposite directions) correlated. The length tells us the "contribution" to the explained variance. Thus, the longer the arrows are the more weight the explanation of the variance we have. Lastly, we show the panels A and B of the figure because a PCA plot is a three-dimensional analysis with x number of components. In A, we show PCA1 and 2. In B we show another perspective using PCA3. The text has been modified to make it better understandable.

I suggest that the manuscript need major revision before being considered for publication.

Detailed comments

Lines 7-8, change to “decreased salinity due to altered precipitation related to climate change”?

AR: Done.

Line 8, clarify N2 by including nitrogen (N2) fixing?

AR: Done.

Line 17, Nodularia spumigena?

AR: Yes, corrected.

Lines 18 and 19, gene copies? Cells? Filaments?

AR: Yes, this should be gene copies, which has been revised.

Line 20, are you sure they are N_2 fixers or should you say potential before?

AR: The N_2 fixers were detected using specific key functional marker genes for either nifH or anf/vnfD which were amplified using PCR. Based on this in combination with a bioinformatic (including individual BLAST search on NCBI and alignment with related nitrogenase sequences) and phylogenetic analysis they were identified as N_2 fixers. Therefore, genetically those microbes are N_2 fixers, and this is typically used as a basis to call them N_2 fixers. If we now of course think about N_2 fixers being only organisms which are actively fixing N_2 we would have to add ‘potential’. It is therefore rather a linguistic but interesting problem. We would therefore be tempted to leave it like it is.

Line 20, I think statistical testing here is redundant. Either say what test or just skip it and start the sentence from “salinity was identified”.

AR: Done.

Line 22, similarly significant? Either it is significant or not?

AR: Done.

Line 38, change to severely affects?

AR: Done.

Line 44, is this how the reference should look like?

AR: No, we reformatted the reference.

Line 46, remove the comma after both?

AR: Done.

Line 66, Anabaena/Dolichospermum if often referred to as spp. since its not only one species. You should state Klawonn et al. 2016 or similar study here when saying these three carry out the N2 fixation.

AR: Yes, this has been adjusted, Klawonn et al has been added, here.

Line 68, maybe also mention rates from newer studies such as Klawonn et al. 2016.

AR: Done.

Line 69, I don't understand this huge deviation between what the fix and consume, the 35% in Ploug et al. would not explain that big difference in rates.

AR: Thanks for this comment, indeed there seems to have been a calculation error which has been corrected, now. The rates are now 115-295 nmol N L^{-1} d^{-1} fixed and 33.33 – 85.74 nmol N L^{-1} d^{-1} leaked. Roughly 30% leaked out which is more in line with Klawonn et al. 2016, a dataset that is now also presented along with our data in the text. Further, a deviation could result from different assessments used. We were referring to Larsson et al. (2001), where N_2 fixation rates were estimated based on the increase in total nitrogen in the upper 20 m of the water column, the incorporation of N into biomass is, however, based on C:N ratio. Thus, the estimates presented by Ploug et al might indeed be more accurate. We adjusted the respective text, it now reads:

'… contributed massively to N_2 fixation with rates of up to 115 – 295 nmol N L^{-1} d^{-1} (Larsson et al., 2001). At the same time, cyanobacteria consume only a fraction of the N they fix, with estimates in the range of 33.33 – 85.72 nmol N L^{-1} d^{-1} leaking out (Janson et al., 1994; Larsson et al., 2001; Wasmund, 1997) roughly in line with estimates given in Klawonn et al. (2016) based on isotope measurements and cell-specific N_2 fixation experiments of *Aphanizomenon sp* and *N. spumigena*, which demonstrated a release of a substantial fraction of up to 35% of fixed N available for other primary producers (Ploug et al., 2010, 2011).'

Line 72, missing period.

AR: Added.

Line 81, what unit?

AR: This refers to salinity, which is unitless now, and also was given without a unit in the paper referred to.

Ln 82, how does it affect the nitrogenase activity?

AR: We can only guess that there might be a physiological optimum for the nitrogenase depending on the salinity in the medium or the environment. It might also have to do with the impact of salinity on gas solubility. However, there is no clear explanation provided.

Line 86, abbreviate to N. spumigena after first mentioned. I think you should broaden the range, Aphanizomenon is extremely common in the Baltic Proper with salinities around 5-6, with higher densities than the Bothnian Sea.

AR: Done. The information on the salinity range has been revised.

Line 87, change “speak for” to indicate?

AR: Changed.

Line 89, include Southern Baltic Sea?

AR: Done.

Line 90, why did you choose a low productive season?

AR: Please see the above comment, we added a text to explain this to the manuscript.

Line 91, what do you mean by controls?

AR: We were referring to any environmental parameter, or factor, which could have a potential impact on N_2 fixation. This has now been clarified in the text:

‘…to explore how various environmental parameters impact on Baltic Sea N_2 fixation, and…’

Lines 89-92, I think you should revise this aims section as you need to argue for why you choose the low productive season and also that you actually look at genes and transcripts which is really cool. That salinity affects the diazotrophic community composition is already known, but it is novel that you here look at other diazotrophic taxa than the heterocystous ones, I think this should somehow be told here too.

AR: Thanks for this encouraging comment, we emphasized that more now, the section now reads:

'To now explore the impact and importance of salinity for diazotrophy in the Baltic Sea and in order to complement existing datasets on N_2 fixation from high-productivity seasons, we carried out a survey including chemical profiling, and rate measurements in the low productive fall season. The focus of this study is a molecular genetic assessment of the diazotrophic community diversity and genetic activity including heterocyst and unicellular cyanobacteria, heterotroph diazotrophs and diazotrophs carrying alternative nitrogenase genes.'.

Line 97, change on to using and remove "to the Baltic Sea"?

AR: Done.

Line 102, both ammonium, nitrate, and nitrite?

AR: This has been clarified, it was only nitrate and nitrite.

Line 110, change to fixed?

AR: Done.

Lines 112-113, revise so that it is clear that the 0.5-1 L was filtered onto the membrane filters and change filter to plural. On all stations and depths?

AR: Done. We added a table with all stations and depths, and indicated, which samples were taken, and where.

Line 115, it is not clear to me how these samples were collected, all from the 0.5-1 L filtered samples?

AR: Yes, this refers to the sentence before. We changed the sentence to clarify this to:

'For nucleic acid extraction, the 0.22 µm pore sized Millipore filters (see above) were flash-frozen…'

Line 123, one from each station? Please clarify how you came up with 15. Which depth?

AR: We hope, this will now be clear from the new table added, where we indicated, which samples we referred to.

Lines 132-135, why these random depths?

AR: We chose the depths based on the water column biogeochemistry, this included the chlorophyll max, the halocline etc. We added a table showing the samples taken and the additional parameters.

Line 149, the statistics section is usually found at the end of the method section?

AR: We moved it to the end of the methods part.

Line 150, I think you need to provide more details on the PCA here, what are the different components in the figure? What is the difference between figure A and B?

AR: See also our comment above, we added the following explanation:

'To maximize the amount of explained variance of the dataset, the figure included both component one versus two (Fig. 9A), and one versus three (Fig. 9B).'

Line 155, why these stations and which depths?

AR: The stations were chosen so to cover all different regions covered by the cruise track to obtain information on N_2 fixation throughout the various regimes present there. Because incubations are run over 24 hours, some stations couldn't be covered for practical reasons including a limitation of time and manpower. The exact depths are now also included in the newly added sampling table.

Lines 156-157, I do not fully agree with this since lately this method has not been used due to underestimation risks (e.g., Klawonn et al. 2016).

AR: Please see our response above, we generally agree that there are better methods available, and we should indeed next time use the bubble addition method and the pre-dissolution method in parallel. However, this was not possible during this particular survey for practical reasons, mostly because we had only one person on board.

Line 157, top-filled?

Yes, the protocol was the same as in Löscher et al., 2014, an information that has been added, now.

Line 160, did you measure this or is it based on calculations?

AR: Calculated, the information has been added.

Line 162, were they incubated for the same time? Any labelled T0 samples collected to ensure free of contamination?

AR: We didn't collect labelled T0 samples, and this is also never part of our typical protocol. However, we collected unlabeled T0 samples.

Line 168, feels like a few words are missing, the typical Baltic Sea gradient I guess?

AR: Yes, we changed it to '…the typical salinity gradient in the Baltic Sea…'

Line 170-171, this is very low temperatures for Nodularia.

AR: Correct, still they are around.

Line 178, lower than what? Expected?

AR: This is a comparison to the south-western part. We clarified this in the text.

Line 180, you refer to DIN in the methods? Please be consistent.

AR: We corrected this throughout the manuscript to keep it consistent.

Lines 184-185, higher than the report or as compared to other locations?

AR: Higher than the report, this has been changed to 'In comparison to those reported concentrations, some of our observed nutrient concentrations in the Bornholm and Eastern Gotland Basins….'

Line 195, or temperature?

AR: Changed.

Line 196, that does more sound like they have more N than C not N depletion?

AR: Exactly, that's why we talk about N repletion.

Line 199, you have another order in the methods, with N2 fixation and community, consider keeping it consistent?

AR: True, we moved the rate measurements in the methods section to an earlier place.

Line 203, what is your detection limit?

AR: N_2 fixation rates were calculated with the equations of Montoya et al. (1996). Considering the particulate nitrogen linearity limit of the mass spectrometer (2. 2 µg N), 3 times the standard deviation of our background (time zero atom % 15N) values, our usual filtration volume, and incubation time, our volumetric N_2 fixation rate detection limit would be 0.03 nmol N L^{-1} d^{-1}.

Line 204, did this occur together with high Chl a concentration or cyanobacteria abundance?

AR: No, not clearly, but indeed it might be that we caught one 'loaded' filament on our filter. This is, however, only a speculation.

Line 208, lower the "2".

AR: Done.

Line 208, maybe expand here connect with lines 212-218? Maybe move the isotope data to the niche discussion?

AR: Done.

Line 209, stick to past tense, "was supported".

AR: Done.

Line 216, abbreviate to N. spumigena.

AR: Done.

Line 217, low light when? As compared to what?

AR: We changed this to '…lower light intensities during fall and winter…'

Line 219, if you state low rates, maybe give examples of high rates from the area or put in some kind of perspective.

AR: We rephrased this to 'low C fixation rates compared to rates in the micromolar range as typically detected in the Baltic Sea (e.g., Klawonn et al., 2016) …'

References:

Klawonn, I., Nahar, N., Walve, J., Andersson, B., Olofsson, M., Svedén, J.B., Littmann, S., Whitehouse, M.J., Kuypers, M.M.M. and Ploug, H. (2016), Cell-specific nitrogen- and carbon-fixation of cyanobacteria in a temperate marine system (Baltic Sea). Environ Microbiol, 18: 4596-4609. https://doi.org/10.1111/1462-2920.13557

Line 222, maybe mention where the rest of the N might come from?

AR: We cannot make a clear statement, here, but assume it would be either recycling, land runoff, riverine or atmospheric sources.

Line 236, it is very common in the Baltic Proper, starting early in the season, with salinity of 5-6 (e.g., Svedén et al. 2015 in FEMS) and dominating N2 fixation (Klawonn et al. 2016). Saying that it prefers as low as 0-2 is not really true here.

AR: Changed.

Line 238, I think you rather mean Dolichospermum? Although it used to be called Anabaena it has been referred to as Dolichospermum since 2009 (Wacklin) and then you can also refer to Olofsson et al. 2020 and Klawonn et al. 2016 here. You refer to it in line 66.

AR: Changed.

Lines 423-427, how does gene copies relates to cell numbers?

AR: We are not sure if the line number is correct here (maybe 243-247?). However, the assumption is that one gene copy is present in one cell. There are some known deviations from this, but we are not aware of such deviations being described for the clades we identified.

Line 261, check the reference layout.

AR: Changed.

Line 276, how can genes and transcript be related to cell numbers?

AR: Gene copy numbers are related to cell numbers; transcript abundances are not.

Line 298, do you mean Olofsson et al. 2020 here? Karlberg and Wulff was a laboratory study. Or if you refer to several studies in the sentence then include them, now it sounds like Karlberg and Wulff has done everything in the sentence including modeling.

AR: We added Olofsson et al.

Line 313, maybe specify where this freshening would be beneficial to Nodularia? Since freshening in the northern Baltic sea will have an opposite effect since its already very low.

AR: We changed this sentence (see also comments of reviewer 2) to

'In case of a potential future freshening of the upper water column (Liblik and Lips, 2019) in combination with increased PO_4^{3-} availability both through land-derived influx and through phosphorous mobilization via bottom-water anoxia (Ingall and Jahnke, 1997; Vahetra et al., 2007; Gustafsson et al, 2017; Stigebrandt and Anderson, 2020), N_2 fixation by e.g. Nodularia will likely increase in the region covered by our cruise.'

Line 324, maybe include south here?

AR: Done.

Line 725, Olofsson et al. 2021 is missing from the reference list?

AR: We included the reference now.

Figure 1, maybe have the three figs in a row in the same size instead? Why is it called last? Can you mark those stations where N2 fixation were measured? And explain in the legend.

AR: We re-arranged the figure. With 'last' we meant the deepest samples measured at each station. It is now clarified in the figure legend.

Figure 2, maybe include 20°E as well since this on the map in Fig. 1? Number of samples?

AR: Done.

Figure 3, "pres [db]" need to be better described. This figure feels a bit redundant, maybe move to supplementary? Number of samples?

AR: The description has been changed to 'Distribution of chlorophyll (Chl [µM] along the natural pressure gradient (press [db]) over the year, data derived from…''. The figure has been moved to the supplement. The number of samples was 5 depths x 12 months = 60 samples per basin, this information has been added.

Figure 4, the text on the axes is far too small. Also, the information within the graph-window. A lot of the discussion part of the text in the legend should be in the manuscript instead. Why is it limited to those stations? Number of samples?

AR: We increased the size of the text on the axes, and of the information text. The information on numbers of samples has been added. The aim was to follow the salinity gradient. However, as it was a short cruise (11 days, with 1.5 day docking at Gotland) we needed to reduce the number of stations.

Figure 5, consider stretch the x-axis so that the numbers is visible, now it almost overlaps. I would suggest that you write out carbon fixation in the legend, now it looks like it say Figure 5 C. This legend also consists of much results that should go into the manuscript instead. Why limited to those stations? Number of samples?

AR: Done. We left the result description in the legend, it might be a bit repetitive, but we believe it can be helpful to read the figure without jumping back to the text. The number of samples has now been added. Same explanation as above, regarding the limited amount of station.

Figure 6, the text is too small to be able to read, can you make it larger? Number of samples? Move to supplementary since you already have so many figures?

AR: The text size has been increased; the number of samples has been added to the legend. The figure is, however, a key figure to the manuscript, we would therefore like to keep it in the main text. The missing information has been added to the legend.

Figure 7, move to supplementary since you already have so many figures?

AR: We would like to keep this figure in the main text as it is a key figure. Figure 3 has been moved to the supplement.

Figure 8, consider using colors instead, now its hard to distinguish. Can you maybe make also a graph with transcripts per gene copies so its normalized to abundance? Number of samples?

AR: Done.

Figure 9, what is components one, two, and three? I don't see the differences between the figures and what has been done? Also how can for example N2 fixation and Nodularia point in different directions on B while being in the same directions in A? What do you mean by the center of each station?

AR: See also our comment above. N_2 fixation and Nodularia point in different directions based on two different angles. Yes, it might be contradictory which suggests that Nodularia is not solely responsible for N_2 fixation. "center of each station" is the larger dots which makes up a center for the three datapoint from each station.

Table 1, how were these numbers extracted? For example, 0.36 and 0.37 mmol N m-2 d-1 in the table is much lower than reported in Olofsson et al. 2021 figure 5? Provide ranges when you have, Klawonn et al. 2016 for example also have many measurements so you should be able to provide a range or mean value with SD. Why does some have ranges and some means and some just one value? Also use the same number of significant digits where you can, Rinne et al. for example has an absurd number of digits?

AR: The table has been revised according to your suggestions.

Also, numbers before 2001 were extracted from Wasmund et al. 2001 and calculated into mmol N L^{-1} d^{-1}. Numbers from 2001 to present were collected by literature search and converted into mmol N L^{-1} d^{-1} for comparability. The numbers from Olofsson et al. 2021 were calculated from e.g., 384 ± 74 kt yr^{-1} to mmol N L^{-1} as follows (a dimensional error was corrected, too):

$$\frac{374 \text{ kt year}^{-1}}{(210000\ km^2 * 1000000\ m^2\ km^{-2})} = 1.82 * 10^{-9}\ kt\ m^{-2}\ year^{-1}$$

$$\frac{1.82 * 10^{-9}\ kt\ m^{-2}\ year^{-1}}{365\ days\ year^{-1}} = 5 * 10^{-12}\ kt\ m^{-2}\ d^{-1}$$

$$5 * 10^{-12}\ kt\ m^{-2}\ d^{-1} * 10^{9}\frac{g}{kt} = 0.05\ g\ m^{-2}\ d^{-1}$$

$$\frac{0.05\ g\ m^{-2}\ d^{-1}}{14\ g\ mol^{-1}} = 0.0035\ mol\ m^{-2}\ d^{-1}$$

$$0.0035\ mol\ m^{-2}\ d^{-1} * 1000\ mmol\ mol^{-1} = \mathbf{3.5\ mmol\ m^{-2}\ d^{-1}}$$

Wasmund et al 2001, Evidence of nitrogen fixation by non-heterocystous cyanobacteria in the Baltic Sea and re-calculation of a budget of nitrogen fixation

Table 2, is these mean values for those studies? Provide more details in the legend. There should be more studies providing this for the Baltic Sea? Are they from the same season? Species?

AR: Surely there are other studies from the Baltic Sea, which are collected in Table 1. The purpose of this table was to set our data into a more global context for which we used studies having provided this exact analysis. We added the information on the season to the table. We also changed the figure legend to 'Contribution of N_2 fixation to primary production (PP) in different ocean basins, values represent mean values for the respective regions.'

Additional changes: We added a personal acknowledgment to Dr Kuosa and an anonymous reviewer.