1 **Letter to Editor and response to referee comments for revised version of BG-2016-313,**
2 **Hrustić et al. „Exploring the distance between nitrogen and phosphorus limitation in**
3 **mesotrophic surface waters using a sensitive bioassay“**
4
5 **General response from authors**: We want to thank referee#1 for his positive
6 evaluation and referee #2 for her/his obviously careful reading and helpful comments.
7 We have given special attention to referee #2's concerns on accessability and readability
8 and hope that our revisions have improved the text in this respect. Several of the
9 referee's comments refer to the underlying mechanisms for the response observed and
10 which consequences this has for the interpretation. We absolutely agree that this is
11 interesting and important and we have expanded both the introduction and the
12 discussion on this issue. We also agree with the referee that this bioassay gives results
13 that are likely to be different from N* and P*, and therefore introduced the symbols N^+
14 and P^+ to distinguish bioassayed surplus nutrients from the traditional, chemically
15 determined N* and P*.
16 Since our measurements of the processes in our incubation tubes were restricted to the
17 APA determinations, any discussion on processes, populations etc. remain hypothetical.
18 We have therefore tried to find a balance between the need to point out possible
19 processes influencing our results, and the danger of long arguments that may be appear
20 too speculative to some readers.
21
22 **Referee's comment:** „Both conceptually and in terms of practical calculation the estimates
23 of ‘N*’ and ‘P*’derived from the experimental protocol described differ from the typical
24 usage, which has in the past been based on a strict definition (e.g. N* = Nitrate – 16 x
25 Phosphate). In order to avoid confusion within the literature I would prefer the use of
26 different terminology (e.g. maybe ‘apparent excess N or P’) or, at the very least, a strict
27 definition of the terms used“.
28
29 **Authors' response:** We agree that one should distinguish our «apparent excess nutrients» as
30 based on bioassays, from the previous definitions of N* calculated from nitrate and
31 phosphate. We do, however, wish to keep the analogy between the two concepts present in
32 the readers' mind and therefore now introduce the symbols N^+ and P^+ for what the
33 organisms «see» according to the bioassay. We have modified the introduction to
34 accomodate the definition of N^+ and P^+.
35
36 **Referee's comments:** „In particular, at present I remain unsure how and whether the
37 resulting values of derived ‘N*’ and ‘P*’ might depend on system characteristics, e.g.
38 including the biological characteristics of the system.
39 Indeed, given the time dependence of the results, it seems likely that derived values are a
40 complex and variable system property. Additionally, I would like to see the authors’ thoughts
41 on whether the variable sizes and turnover times of bioavailable dissolved organic N and P
42 pools might influence their results?
43 Additionally, I was unclear how the presence and turnover of natural DOP pools, both AP-
44 hydrolysable and otherwise might be influencing the results?
45
46 **Authors' response:** We have expanded both the introduction and the discussion to make the
47 reader aware of what we think are the most likely processes influencing our results. We

have tried to balance this against the danger of too extensive, experimentally unsupported and speculative discussions.

Referee's comment: Related to the above, it would be useful to have some additional information on the systems studied, e.g. including inorganic and organic N and P concentrations and potentially some characteristics of the planktonic community?

Authors' response: Our main reason for choosing the two environments was the general expectation of N versus P limitation in the Baltic and in the Norwegian fjord environment, respectively. The response is a system-level response and, as discussed above, a deeper analysis of what happened in the assay would need a lot of information both on chemistry and community composition in the system. This was one argument for doing the assays on samples in mesocosms where there would be huge independent efforts on characterizing different ecosystem aspects. For the Tvärminne experiments, interested readers can find a lot of this in doi:10.5194/bg-12-6181-2015 (Paul et al., 2015) and doi:10.5194/bg-13-3035-2016 (Nausch et al., 2016), both in the reference list. The Espegrend mesocosm experiment is not yet published.

Referee's comment: Given the focus of the paper, I would like to see some more details on the APA measurements. In particular what substrate was used (MUFF-P?) and at what concentration. Also how long were the APA incubations and were multiple timepoints measured within the incubation? Concentrations of substrate are potentially particularly important and I would be interested in the authors' opinion on whether it would matter if the substrate concentration was saturating or not for the derived response surface?

Authors' response: We have included a more detailed description in M&M, the use of 3-o-methylfluorescein-PO_4 at final concentration 0.1 µmol L^{-1}. Modifications of the original method of Perry (1972) were mainly in volume adjustments to match the characteristics of the plate reader in Bergen, while for the Tvärminne all the details of measuring APA were indentical to Perry (1972). In Bergen, the Perkin-Elmer plate reader was programmed to read each well 15 times within 70 minutes (repetition interval 5 minutes).
In Tvärminne, fluorescence on an initial sample was measured after 0, 10, 30 and 60 minutes of incubation with substrate and APA calculated as the slope obtained by linear regression. Based on this, the slope for reported samples are based on a single incubation time (30 minutes).
In another western Norwegian fjord, we have estimated the half-saturation constant (K+Sn) for APA with this substrate to be ca. 300 nM (Thingstad et al., 1993; see the manuscript). Our substrate concentration was therefore probably not saturating. A higher substrate concentration is therefore likely to increase the parameter A „lifting the roof“ of the response surface. Since this parameter is not used in the evaluation of the N:P-kinetics, this would not in itself affect the interpretation of the results. If the concentration effect is not proportional throughout the transition zone between P and N limitation, however, other parameters (N_0, r, s) could in principle be affected. This has not been investigated.
A too low substrate concentration would create a risk for substrate depletion during incubation. With our measurement protocol (above), this should have been detected as a decrease in rate during incubation with substrate and is therefore not likely to have been the case.

Referee's comment: Some of the measured APA values are negative, what does this represent? I assume some form of blank correction within the fluorometric measurements? Please be more specific on methods.

Authors' response: Some measurements were close to, equal to, and even below the blanks (triplicates for each incubation), whilst the presented measurements (Fig. 2A; Fig. 2B; Supplement) are corrected for blanks. Samples in the N-limited region with no or neglible APA therefore get negative values. This will affect the parameter B describing the „floor“ of the response surface. B is not used in the interpretation of N:P relationships.

Referee's comments and suggestions how to rewrite the manuscript (authors' responses are below each comment)

Abstract:

Referee: Line 19: I suggest '. . .primary limiting nutrient. . .'

Authors: We have corrected the Lines 18–22 into: This assay not only provides information on which element (N or P) is the primary limiting nutrient, but also gives a quantitative estimate for the excess of the secondary limiting element (P^+ or N^+, respectively), as well as the ratio of balanced consumption of added N and P over short time scales (days).

Section 1:

Referee: Line 16: suggest '. . .fixers potentially having a competitive. . .' i.e. the evidence in the cited study is largely on the basis of hypothetical modelling

Authors: „potentially“ is included in the sentence.

Referee: Line 22: define N* and P* (e.g. N* = Nitrate – 16 x Phosphate) on first use (also note
above, I would prefer use of different terminology for derived apparent excesses of N and P)

Authors: Done

Referee: Line 44: It was not immediately clear to me that the assays described can be used to quantitatively asses the excess of overall bioavailable N or P in a system and, despite spending some time with the authors' code and data (which are very usefully provided within the supplement), I still remain to be entirely convinced. Firstly, there is clearly an assumption that the organisms in the community react to P limitation through increasing APA (see abstract of Thingstad and Mantoura 2005). While this may be the case, it remains an assumption and should probably be explicitly stated as such. I think there is also an underlying assumption that the community level APA response is directly proportional to the overall availability of all forms of bioavailable N and P within the system.

Authors: It is a standard assumption based on the literature that the organisms within the microbial community react to P-limitation by inducing production of the alkaline phosphatase enzyme. A standard measurement of APA (without prior incubation with N and P) is thus a convenient and frequently used way to determine whether the organisms „feel" P-limited (have de-repressed the Pho-operon). We have re-read and slightly adjusted the wording of our introduction and feel that the point that microbes react to P-limitation by inducing AP synthesis now should be relatively clear. Our assay thus basically should measure when organisms have consumed the available forms of P in the incubation tubes, but still have N available to produce the enzyme. The result therefore should depend on the structure of the community: E.g. a community that binds a lot of P relative to N will give a larger P-limited region in the N:P matrix. We are not sure that linearity in the response to bioavailable N and P is an assumption. With strict linearity, the transition between the N and P limited regions should probably be linear and not sigmoid (?) The sigmoidal shape may originate from flexibility in the biological response (variability in the response of individuals or species or functional groups). Our sigmoid response surface was originally choosen from the analogy to LC_{50} analysis in toxicology (see Thingstad and Mantoura, 2005) to fit the observed response, not from a mechanistic model of AP-production.

Referee: The authors can correct me if this is wrong, however, I think that the equation on line 2, page 4 suggests there is this assumption of a linearity of response? Overall however I will admit that I struggled to follow some of the authors' arguments, particularly on the first read through. I would therefore suggest that the authors could provide a fuller treatment and explanation for their analysis method for the data presented.

Authors: The line does not represent linearity in the particular response of APA in a certain microcosm (i.e. falcon tube). It represents an assumed linearity in the consumption of N and P by the community. The validity of this assumption is confirmed by our results. If N and P were used in a different ratio in the upper right corner of the addition matrix (high additions) than in the lower left corner (low additions) this should have been visible as a systematic pattern in the residuals shown in Fig.2.

Referee: Related, it would be good to fully separate out the equations, number them and explicitly refer to them as/when required. Some additional simple graphical schematic plots and further graphical presentation of the extensive data set might also aid accessibility.

Authors: We usually agree in the strategy to take equations out of the text and place them in a separate box/table. In this case, however, there are only three equations, and they are only used in this already technical section. We feel that the box/table solution therefore is not optimal here.
We have instead tried to make the text a bit more readable by separating the three equations out as separate paragraphs and numbering them, allowing later reference. We have also added a bit more text to hopefully help the reader to what is probably a more unconventional than a particularly difficult algorithm.

We have also re-coloured the fitted response-surfaces in Fig. 2, giving them a gradient in colour. Hopefully this helps the intuitive impression of the shape of these surfaces.

1 The two figures in Fig. 2A and 2B represent the N- and P-limited situations, respectively.
2 Similar plots of the other data seem to us to give minor additional information only. We
3 have not been able to come up with other graphical representations that provide
4 fundamental new insights into the data.
5
6 **Section 2:**
7
8 **Referee:** Line 4: Rephrase. Please provide some background to the experiment. At this stage
9 the experimental mesocosms have not been described/introduced.
10
11 **Authors:** We have added an introductory line about the mesocosms and their main purpose
12 (acidification effects). Since we found no significant acidifaction effects on our assays, we
13 haave chosen not to go into details. For the Tvärminne experiment, the interested reader
14 can find this in the references cited. Documentation of the Espegrend mesocosm experiment
15 is so far in preparation and will be available to future readers.
16
17 **Referee:** Line 30 (section 2.3): please provide information on both the incubation time for
18 APA, substrate used, concentration etc. (see above).
19
20 **Authors:** Done
21
22 **Referee:** Page 6, lines 1-12: given that the value of r has a direct impact on the calculations
23 of any excess bioavailable N or P within the system, I was left wondering how the lack of a
24 consistent explanation for the variability in this derived parameter between the different
25 sets of experiments could potentially influence the authors' conclusions.
26
27 **Authors:** The variation and the seemingly consistent difference in r between N and P limited
28 environments is interesting. We again feel that the finding itself is properly reported and
29 discussed. To determine the underlying mechanisms and the consequences for our
30 understanding of the system assayed would require research beyond the scope of the
31 present work.
32
33 **Referee:** Overall I might argue that caution should be applied to quantitative conclusions
34 based on the technique described until a more complete understanding of the responses
35 is available. Related, does 'r' really represent a consumption ratio? I think it likely
36 represents the equivalence ratio between the influence of added N and P on APA rates
37 within each individual system studied, but it isn't immediately obvious to me that this
38 would be the same as the overall nutrient consumption ratio, in particular due to the
39 potential for variable turnover rates of different pools.
40
41 **Authors:** As a response to this comment we have tried to increase the precision of our
42 language by changing „consumption" to „net consumption" which is what determines the
43 relative sizes of the pools of free N and P. We have also added a caution in the final
44 conclusion that the underlying mechanisms need further investigation.
45
46 **Referee:** Figure 1: this was useful in helping to understand the technique, however a similar
47 schematic of the opposite case (i.e. where No is negative) would be useful.

1
2 **Authors:** This is now explained in the legend of Fig. 1, whilst Fig. 2 contains examples of the
3 two cases. We feel that adding more information (e.g. a line with negative Y-intercept)
4 would clutter the presentation. The alternative of a Fig. 1B with the line shifted to give a
5 negative intercept is indeed possible, but was felt to give marginal new information.
6
7 **Referee:** Figure 2: I found this figure difficult to interpret on first sight. After using the
8 authors' code to analyse their data I eventually got the hang of what was being presented,
9 however I wonder whether there might be a more simple graphical way to display the data.
10 e.g. could a similar plot to figure 1 be produced with the responses to individual values
11 within experiments contoured/coloured rather than presented as a surface? This may
12 aid a reader in picking out the N = No + rP line and associated intercepts etc. Maybe
13 such a presentation could be used in addition to the format in Figure 2, as the latter
14 admittedly does have the benefit of displaying the magnitude of residuals. Overall I would
15 encourage the authors to consider a wider presentation of the data, as the manuscript
16 is currently short and hence there is ample space available. Related, a key strength of
17 the data appears to be the repeatability of results, so I would suggest presentation of
18 some more individual experiments would be useful to the reader.
19
20 **Authors:** We chose this representation exactly from the reason identified by the referee: the
21 possibilty to represent the residuals. We see these as important for demonstrating that the
22 fitting function chosen is suitable (in terms of representing the observed response). A
23 contour plot would be a series of lines parallel to the $N=N_0+rP$ line and did not seem very
24 informative to us. We have tried to meet the need for an intuitively easier graphics by
25 changing the previously uniform gray response surface to a colour gradient (blue to red) .
26
27 **Referee:** Finally, running the authors MATLAB codes on the data provided I found some
28 discrepancies with the information provided in table 1. I haven't checked all the
29 experiments, but, for example, for the 'M7' experiment for Tvärminne I get:
30 General model: myfit(x,y) = A/(1+exp(s*(1/sqrt(1+r^2))*(r*x+No-y)))-B Coefficients
31 (with 95% confidence bounds): A = 8.027 (7.681, 8.373) s = 20.67 (19.18, 22.15) r
32 = 1.616 (1.555, 1.677) No = 0.6882 (0.676, 0.7004) B = 0.1094 (0.06078, 0.158)
33 Thus the estimate for No appears different (albeit only minor) to that stated in the table,
34 as does the confidence interval for s?
35 Similarly, for 'Fjord' I get:
36 General model: myfit(x,y) = A/(1+exp(s*(1/sqrt(1+r^2))*(r*x+No-y)))-B Coefficients
37 (with 95% confidence bounds): A = 7.41 (7.156, 7.664) s = 37.87 (34.98, 40.76) r
38 = 2.482 (2.39, 2.573) No = 0.6771 (0.6676, 0.6867) B = 0.2197 (0.1826, 0.2569)
39 Which again seems different to info in Table 1?
40
41 A = 16.05 (13.34, 18.76)
42 s = 29.19 (23.11, 35.27)
43 r = 2.52 (2.214, 2.825)
44 No = 0.7897 (0.7473, 0.8321)
45 B = 0.2284 (0.08769, 0.369)
46

1 **Authors:** Re-running our codes we find the values given in Table 1. To prevent a possible
2 mixup of file-titles we will re-load the files in SI if the article is accepted.
3
4
5 Please find below our manuscript with the track-changes option on, showing the main
6 changes from the original ms.

1
2
3

4 # Exploring the distance between nitrogen and phosphorus
5 limitation in mesotrophic surface waters using a sensitive
6 bioassay

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16 **Abstract**

17 The balance in microbial net consumption of nitrogen and phosphorus was investigated
18 in samples collected in two mesotrophic coastal environments: the Baltic Sea
19 (Tvärminne field station) and the North Sea (Espegrend field station). For this, we have
20 refined a bioassay based on the response in alkaline phosphatase activity (APA) over a
21 matrix of combinations in nitrogen and phosphorus additions. This assay not only
22 provides information on which element (N or P) is the primary limiting nutrient, but
23 also gives a quantitative estimate for the excess of the secondary limiting element (P^+ or
24 N^+, respectively), as well as the ratio of balanced net consumption of added N and P over
25 short time scales (days). As expected for a Baltic Sea late spring-early summer situation,
26 the Tvärminne assays (n=5) indicated N-limitation with an average P^+=0.30±0.10 µM-P,
27 when incubated for 4 days. For short incubations (1–2 days), the Espegrend assays
28 indicated P-limitation, but the shape of the response surface changed with incubation
29 time, resulting in a drift in parameter estimates toward N-limitation. Extrapolating back
30 to zero incubation time gave P-limitation with N^+≈0.9 µM-N. The N:P ratio (molar) of
31 nutrient net consumption varied considerably between investigated locations; from
32 2.3±0.4 in the Tvärminne samples to 13±5 and 32±3 in two samples from Espegrend.
33 Our assays included samples from mesocosm acidification experiments, but statistically
34 significant effects of ocean acidification were not found by this method.

35 Keywords: alkaline phosphatase activity, bioassays, mesotrophic temperate seas,
36 nutrient limitation, phytoplankton

37 ## 1 Introduction

N to P balance is a core biogeochemical feature of aquatic systems as highlighted in Redfield´s classical question of whether it is the chemistry of seawater that has determined the stoichiometry of the marine organisms, or biology is the cause for the "normal" 16:1 (molar) ratio between N and P in seawater (Redfield et al., 1963). The issue of surface ocean nutrient limitation is as acute as ever (Moore et al., 2013), since it has bearings on phenomena ranging from the global carbon cycle, where it plays a key role in the dynamics of the ocean´s biological pump (Ducklow et al., 2001); via basin scale issues such as N deficiency in Arctic water of Pacific origin (Lehmann et al., 2005), P deficiency in Eastern Mediterranean deep waters (Krom et al., 1991) and the North Atlantic gyre (Mather et al., 2008); via regional issues such as the question of P and/or N removal from the Baltic Sea (Elmgren and Larsson, 2001; Granéli et al., 1990; Räike et al., 2003); to local ecosystem characteristics such as P-deficient brackish layer overlaying potentially more N-limited marine waters in the fjords of western Norway (Thingstad et al., 1993). The classical idea of predominantly N-limitation in marine systems (as opposed to predominantly P-limitation in limnic systems) (Hecky and Kilham, 1988) has also become considerably more nuanced, not only due to the cases mentioned above, but also with the identification of the High Nutrients Low Chlorophyll (HNLC) areas as being iron-limited (Franck et al., 2003), phosphorus and iron as co-limiting elements of nitrogen fixation in the tropical North Atlantic (Mills et al., 2004) and N_2 fixers potentially having a competitive advantage in oligotrophic P-starved regions (Landolfi et al., 2015). While some of the mechanisms behind these apparent deviations from Redfield stoichiometry seem to be well understood, there are others which lack generally accepted explanations.

In deep waters with most of the bioavailable N and P converted to NO_3 and PO_4, the chemical determination of N or P in excess of the Redfield ratio may be relatively straight forward. In biogeochemistry this excess is calculated on the basis of measured nitrate and phosphate, and is referred to as N* and P*, e.g. $N^*=NO_3-16PO_4+2.9$ mmol m^{-3} (Sarmiento and Gruber, 2006). In productive surface waters this is a more complex issue. A potential solution to the chemically intractable problem of measuring a large suite of presumably bioavailable pools is to use a quantitative bioassay, i.e. to ask the organisms how much of the primary and secondary limiting elements they can "see". In productive waters, both N and P may accumulate over time in pools of DON and DOP with different grades of bioavailability. Microbes have flexible stoichiometry as their content of storage materials, structural carbohydrates, nucleic acids and lipids vary with growth conditions (Bertilsson et al., 2003; Geider et al., 2002; Krauk et al., 2006). There are also differences in the stoichiometry of different functional groups of organisms, where e.g. bacteria (Fagerbakke et al., 1996) tend to have N:P ratio significantly lower than 16. Which element that first becomes limiting, and how much of the secondary limiting element then remains in excess may thus depend not only on the total pools as conceptually expressed by N*, but to vary as a function of the biological structure of the food web and its pre-history. Although conceptually related to N*,P*, the answer to what excess nutrients the organisms "see" may therefore differ even between systems with the same, chemically defined N*. We therefore have chosen to use the symbols N^+ and P^+ for surplus nitrogen and phosphorus as determined by bioassays, to distinguish these numbers from their chemically defined analogs N* and P*.

Microorganisms have evolved sophisticated physiological mechanisms to adapt to the different forms of nutrient limitation (Geider et al., 1997; Ivančić et al., 2012; Thingstad et al., 2005; Van Mooy et al., 2009; Lin et al., 2016), including the induction of extracellular enzymes such as alkaline phosphatase (AP) catalyzing the hydrolysis of

1 phosphomonoesters within DOP (Hoppe, 2003). A well-studied model system is the
2 induction of the Pho-regulon in *Escherichia coli*, which leads to expression of a series of
3 P-starvation related genes, including *phoA* coding for AP synthesis (Torriani-Gorini,
4 1994). The induction of AP synthesis seems to be more coupled to a low internal cell
5 quota of P, than directly to low external concentrations of inorganic P (Lin et al., 2016),
6 thus presumably providing a main signal when both external pools and internal storage
7 reserves of P have been depleted below the certain level (Boekel and Veldhuis, 1990;
8 Chróst and Overbeck, 1987). Inducible AP synthesis is wide-spread feature in
9 microorganisms (Jansson et al., 1988). It is easily measured as AP activity (APA) (Perry,
10 1972, Hoppe, 2003), and thus it has been frequently used as an indicator of P-stress
11 (Jansson et al., 1988; Dyhrman and Ruttenberg, 2006; Lomas et al., 2010).
12 This method was further exploited by Thingstad and Mantoura (2005) in the
13 oligotrophic Eastern Mediterranean, showing that the concentration of added PO_4
14 needed for APA to disappear in a P-limited system, or alternatively the NH_4 needed to
15 induce APA in an N-limited system, could be used as a bioassay to quantitatively
16 estimate $N^{\pm}$ and P^{+}, respectively. We here expand this technique by using a matrix-setup
17 including simultaneous gradients in both PO_4 and NH_4 additions. This is applied to
18 samples from the coastal waters of western Norway and the Baltic Sea, confirming that
19 the assay gives informative results also in temperate, mesotrophic environments.

20 2 Material and Methods

21 2.1 Study areas and sampling

22 Part of the sampling for this study was performed in mesocosms designed to study
23 acidification effects. In the Baltic, the water was collected as integrated samples (depth
24 0–10 m) in Storfjärden near Tvärminne field station (59° 51.50' N, 23° 15.50' E) on 6
25 August 2012. The collection was performed 45-30 days after the first-last CO_2
26 treatments and 50 days after the mesocosm closure (Paul et al., 2015). Samples were
27 collected from the fjord (417 µatm) and mesocosms M1 (365 µatm), M3 (1007 µatm),
28 M6 (821 µatm) and M7 (497 µatm); where numbers in parentheses are average $f(CO_2)$
29 over the period Day 1–Day 43. The mesocosms received no nutrient manipulations
30 except the CO_2 treatments. Further details about location and the mesocosm experiment
31 can be found in Paul et al. (2015) and Nausch et al. (2016).
32 The samples from western Norway were collected during a similar mesocosm
33 experiment in Raunefjorden close to Espegrend field station (60° 16.2' N, 5° 11.7' E).
34 From one mesocosm (MR) an integrated (depth 0–20 m) sample (1165 µatm) was
35 collected on 25 May 2015 corresponding to Day 22 after acidification treatment. The
36 fjord sample was collected at nearby landlocked location Kviturspollen (60° 15.8' N, 5°
37 15' E) at the depth of 1 m using a Niskin sampler on 3 June 2015. Samples were pre-
38 filtered through gauze of 112 µm mesh size to minimize the variability due to the
39 occasional large zooplankton.

40 2.2 Matrices of nitrogen and phosphorus additions

41 Samples were distributed in 15 mL Falcon® polypropylene tubes (BD Biosciences®)
42 organized in 10x10 or 8x8 columns x rows (Tvärminne and Espegrend, respectively).
43 PO_4 (KH_2PO_4 10 µM) was added in final concentrations from 0 to 290 nM-P in steps of
44 32.2 nM (Tvärminne) and from 0 to 105 nM-P in steps of 15 nM (Espegrend). Each of the

columns received additions of NH_4 (NH_4Cl 200 µM) in final concentrations from 0 to 964 nM-N in steps of 107 nM-N (Tvärminne) and from 0 to 2100 nM-N in steps of 300 nM-N (Espegrend). The tubes were incubated in light:dark (16 h:8 h) at 17–18°C (Tvärminne) and in light:dark (12 h:12 h) at 16.5°C (Espegrend), both at irradiance of 78 µmol photons m^{-2} s^{-1}. Incubation at Tvärminne lasted 4 days for all samples, whilst APA assays for Espegrend were repeated as given in each case.

2.3 Alkaline phosphatase activity

Measurements of APA were done according to Perry (1972) using 3-o-methyl-fluorescein-PO_4 (final concentration 0.1 µM) as the substrate. Volumes were modified to the use of fluorescence plate reader by pipetting 200 µL subsamples from each Falcon tube into the wells containing the substrate. Results are expressed as increase in relative fluorescence units per hour (RFU h^{-1}). APA in the coastal waters of the western Norway was measured using a PerkinElmer Enspire 2300 plate reader programmed to do 15 repeated measurements (time interval 5 min) over a total incubation time of 70 minutes. APA was calculated as the slope of the fitted linear regression line. APA in the Baltic Sea was measured by Varian Cary Eclipse fluorometer after 30 minutes incubation with substrate.

2.4 Fitting the response surface

To interpret the data obtained by this method, an objective algorithm is needed to define the transition between subsamples with high (P-limited) and low (N-limited) post-incubation APA. Thingstad and Mantoura (2005) did this by fitting sigmoidal functions to the observed APA-responses; either a decreasing function parallel to the P-addition axis in the case of a P-limited system, or an increasing function parallel to the N-addition axis in the case of N-limitation. To avoid this pre-fitting choice of function, we here have instead started with the assumption that the *P,N*-plane is split into a P-limited and an N-limited region by the straight line:

$$N = N_0 + rP \quad \text{Eqn. 1}$$

where a negative value of the intercept N_0 corresponds to the excess-N (N^+) present in a P-limited system and $P_0 = \frac{-N_0}{r}$ is the amount of phosphate needed to shift the system to N-limitation. Conversely, a positive value of the intercept N_0 would correspond to the amount of N required to shift an N-limited system into P-limitation, while $P_0 = \frac{N_0}{r}$ then corresponds to the excess-P (P^+) in this N-limited system. The shift from P- to N-limitation, and therefore the expression of APA in a point *P,N* is assumed to be a function of the distance *Z* between this point and the line (Fig. 1). The sigmoidal function fitted is:

$$APA_{est} = \frac{A}{1+e^{sZ}} - B \quad \text{Eqn. 2}$$

From the geometry of Fig. 1 one can calculate the perpendicular distance Z from the point *P,N* to the line defined by Eqn.1 as

$$Z(P,N) = \frac{1}{\sqrt{1+r^2}}(rP - (N - N_0)). \quad \text{Eqn. 3}$$

Here, the exponential function in the denominator of Eqn. 2 replaces the term $\left(\frac{Z}{Z_0}\right)^s$ adopted by Thingstad and Mantoura (2005) from standard calculation of lethal concentration (i.e. LC_{50}) in toxicology. This standard expression is undefined for Z_0=0

and therefore not applicable with our approach where Z = 0 along the line defined by Eqn. 1. Visual inspection of residuals in graphs (see Fig. 2A, B) did not suggest systematic deviances between response surfaces fitted with this function and the observed data. Alternative fitting functions have therefore not been explored. With five parameters to fit (r, N_0, s, A, B), this leaves 95 and 59 degrees of freedom for the Tvärminne and Espegrend set-ups, respectively. The fitted surface APA_{est} has a maximum A-B obtained for co-ordinates combining low P with high N (large negative Z) and APA_{est}=(A/2)-B along the line N=N_0+rP separating the P- and N-limited regions. The parameter s defines the steepness of transition between the two regions perpendicular to this line. B is the background APA_{est} found for high-P, low-N (large positive Z) co-ordinates. The fitting was done using the "fit" function in Matlab® with its default Levenberg-Marquardt algorithm providing the parameter estimates with 95% confidence intervals (c.i.) (code included in SI).

3 Results

Two examples of the fitted response surface, one from Tvärminne (Fjord) (Fig. 2A) and one from Espegrend (MR) (Fig. 2B) are shown to illustrate the difference in shape of the response in situations apparently N-limited (Tvärminne) and P-limited (Espegrend), with estimated $P^{\pm}$=0.3 µM-P and $N^{\pm}$=0.4 µM-N, respectively . All assays are summarized in Table 1.

For the two Espegrend samples, the change in shape of the response surface with incubation time was explored (Fig. 3). For both samples, N_0 increased with incubation time ($p \leq 0.05$, Table 2), i.e. the assay results drifted towards increasing N deficiency when using longer incubation times. In the sample MR, r and s decreased significantly over time (Table 2). Using linear regression, the parameter estimates can be extrapolated back to zero incubation time. With this technique the average $P^{\pm}$ for the Tvärminne samples, based on a single incubation time, was 0.3 µM-P, and the average $N^{\pm}$ for the two Espegrend samples, based on backward extrapolation, was 0.9 µM-N.

The assays from Tvärminne mesocosms include an f(CO_2) gradient. Linear regressions of N_0 (p=0.55), r (p=0.63) (Fig. 4) and s (p=0.19) (not presented) on f(CO_2) gave no indication of any statistically significant effect of the 45 days exposure of the systems to different CO_2-levels. Compared to a Redfield N:P value of 16, all the Tvärminne samples gave low r (2.3±0.5; mean over samples±sd), while the two Espegrend samples gave r of 13±2 (Kviturspollen) and 32±3 (MR) (mean±sd, both over incubation times).

4 Discussion

This study extends the demonstrated applicability of this type of assay from its previous use in warm oligotrophic waters (Thingstad and Mantoura, 2005) to mesotrophic temperate environments. We modified the technique so that no *a priori* assumptions are now required as to whether the system investigated is N- or P-deficient. Note that the function used to fit the response (Eqn. 2) was not derived from explicit assumptions on biological mechanisms producing the response, but as a convenient statistical model that fitted the observed responses without obvious systematic patterns in the residuals (Fig. 2). It may, however, be of biological relevance to observe that, with this description, the initially three-dimensional description (P, N, APA) is reduced to two dimensions (Z, APA): all combinations of P and N that have the same perpendicular distance Z (Eqn. 3)

to the line representing N:P balance (Eqn. 1) develop the same APA (Eqn. 2). Contour plot representations of the fitted surfaces in Fig. 2 A and B would thus consist of straight lines parallel to the line described by Eqn. 1.

We explored the use of this modified assay in two environments with anticipated differences in ambient N:P stoichiometry. The Tvärminne mesocosm experiment was planned with the expectation of an N-limited spring-summer situation as characteristic in the Baltic Sea (Granéli et al., 1990; Rolff and Elfwing, 2015; Thomas et al., 2003), subsequently transiting from N-limitation towards an N- and P-co-limited situation as the result of "new" N being added through late summer blooming of diazotrophic cyanobacteria (Lignell et al., 2003). This bloom did not occur during the whole Tvärminne experiment and N-limitation at the time of sampling has been confirmed by Nausch et al. (2016) who studied the microbial P-cycle just before our experiment. Nutrient concentrations were not significantly changing throughout the whole acidification experiment (Paul et al., 2015). DIN and DIP equalled ~0.25 µmol L^{-1} and ~0.15 µmol L^{-1}, respectively, giving a ratio of 1.67 (Paul et al., 2015)~~.~~ Our finding of positive N_0-estimates for all 5 samples (Table 1) is in line with this. The Tvärminne assays were performed after the 4 days of incubation ~~when~~ needed for the APA-responses to emerge~~d~~. The conclusion of N-limitation is therefore confounded by the potential drift in parameter estimates as was later observed for the Espegrend samples (Fig. 3). The drift obviously complicates the use of this assay since there may be no single incubation time that gives a "correct" set of parameter values~~.~~ Since the drift seems to be reasonably linear for all parameters (Fig. 3), we see it as a promising option ~~is~~ to extrapolate the linear regressions back to time 0, assuming this to give values representative for the initial conditions in the water sample. In our case this gives negative N_0 values of -0.8 (-1.4,-0.2) and -1.0 (-2.4,0.4) for the Espegrend samples from MR and Kviturspollen, respectively (intercept with 95% c.i.); suggesting initial P-limitation. This conclusion is in accordance with ~~the~~ our expectation since the top layer of the fjords in western Norway has been shown to be P-deficient (Thingstad et al., 1993).

The mechanisms behind the drift in parameter estimates have not been studied further here. Three~~Two~~, not mutually exclusive, scenarios may, however, illustrate some of the ~~options~~theoretical possibilities: 1) The microbial food web in the incubated tubes remineralizes P faster than N (Garber, 1984). The assay may then correctly reflect the succession of the limiting nutrient in the sense that the bioavailable pools in the tubes change over time as N becomes immobilized in slowly degradable detritus to a larger extent than P.~~;~~ 2) N added in excess of P in the upper P-limited part of the *P*,*N*-plane is used by the organisms to produce alkaline phosphatase (rather than biomass). This would lift the response surface for high values of added N which may move the fitted line towards higher N_0, i.e. towards N-limitation. The use of extra N to produce exo-enzymes for acquisition of P from DOP has recently been argued for, but then with N_2-fixation as the N source (Landolfi et al., 2015). 3) Successions in the microbial food web move towards organism groups that require more N relative to P, although an increasing dominance of P-rich bacteria (Fagerbakke et al., 1966) would in this scenario produce a drift in the direction opposite to that observed. The *r* values representing the ratio of N- and P-net consumption are comparable between all the Tvärminne samples (2.3±0.5, n=5 different samples), indicating good reproducibility of the assay for similar water samples. This low value compared to the Redfield value of 16 was, however, strikingly different from the Espegrend samples with one Redfield-like 13±2 (Kviturspollen) and one high 32±3 (MR) value, both averaged over incubation times. A similar phenomenon

was noted by Thingstad and Mantoura (2005) using this method to study in-out differences in a Lagrangian experiment where orthophosphate was added to the P-deficient surface system in the Eastern Mediterranean. While their P-limited out-sample gave an r=15±2, the inside system, when driven to N deficiency by the *in situ* phosphate addition, gave a much lower r=3.0±0.2. Interestingly, we also here found the lower-than-Redfield r values in the probably N-limited samples from Tvärminne. From the limited number of assays available, the linkage between N deficiency and low r values thus seems consistent~~,~~. In microorganisms, C:P-ratios are usually more flexible than C:N-ratios (Gismervik et al., 1996; Fagerbakke et al., 1996). P-rich microorganisms in N-deficient environments may thus seem a potential explanation to the observed low r-values in N-limited situations.~~ – but the underlying mechanism is not immediately obvious. One could argue that, in a P-deficient system, the organisms present would be expected to have marked luxury consumption of any added P (Thingstad, 2005) (and *vice versa* for N) (Leonardos and Geider, 2004). As r represents the ratio between utilization of added N and added P, luxury uptake seems to lead to an expected effect on r opposite to that observed. One could speculate that organisms in N-deficient environments are selected for (or adapted to) low N:P requirements. Although this may be in qualitative agreement with our data, it seems doubtful that the large range in r (~2 to ~30) can be explained in this manner.~~

Considering the difference in sigmoidity (s) for the MR and Kviturspollen samples (Fig. 3) it seems that s represents a characteristic of the initial water sample. While s reflects the stoichiometric flexibility in the community response, it would require further investigations to determine whether this flexibility is at cell level and would be seen also in axenic cultures, or is a reflection of differences between species present.

5 Conclusions

We have demonstrated the extension of the APA assay from its previous use in warm oligotrophic, to temperate mesotrophic surface waters. The primary advantage of this technique over traditional nutrient-limitation bioassays is that it indicates which of the elements N or P that is the most limiting, while simultaneously providing estimates of the excess in bioavailable forms of the secondary limiting element (N^+, P^+) along with the ratio between net consumption of the two elements (r). The assay does not require determinations of the large variety of chemical and/or physical forms in which the primary and secondary limiting elements may exist. The assay was found to be complicated by a drift in parameter estimates with incubation time. A backward extrapolation to zero incubation time appears promising. Further work is needed to fully understand the ecological processes creating this drift and also the mechanisms that in some cases generate large deviations in r from the Redfield value of 16. The consortium of ecological processes that create the APA response during incubation are likely to be relevant to processes shaping nutrient limitation in natural aquatic systems,. The experimental setup used in this assay thus seems also to have a potential as a tool for future studies on the ecological stoichiometry of aquatic microbial food webs.

Data availability

1 Original data are given in Supplementary Information (SI) for each assay in the form of a
2 Matlab® program that will also fit the response surface as shown in Fig. 2.

3 Acknowledgements

4 The cooperation between Enis Hrustić and Professor Tron Frede Thingstad was realized
5 through the Erasmus+ training at University of Bergen, Norway and EU project OCEAN-
6 CERTAIN FP7-ENV-2013.6.1-1. Project nr. 603773. The mesocosm studies in Tvärminne
7 and Espegrend were funded by BMBF projects SOPRAN Phase II (FKZ 03F0611) and
8 BIOACID II (FKZ 03F06550). MSc Johanna Oja is thanked for laboratory assistance in the
9 Tvärminne experiment.

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3

1 Table 1: Estimates (with 95% c.i.) of the intercept (N_0) and the slope (r) of the line
2 $N = N_0 + rP$ separating the N- and P-limited regions as illustrated in Fig. 1. s represents the
3 steepness of transition from N- to P-limitation, perpendicular to the line. R^2-values are
4 for the fitted response surfaces.
5
6

	N_0 (μM-N)	r (μM-N:μM-P)	s (μM^{-1})	R^2
Tvärminne (Baltic Sea): (incubation time 4 days)				
Fjord	0.79 (0.75, 0.83)	2.5 (2.2, 2.8)	29 (23, 35)	0.674
M1	0.34 (0.30, 0.38)	2.9 (2.7, 3.2)	43 (31, 56)	0.622
M3	0.76 (0.69, 0.83)	2.2 (1.9, 2.5)	19 (15, 24)	0.664
M6	0.64 (0.56, 0.72)	2.2 (1.9, 2.6)	19 (13, 25)	0.569
M7	0.70 (0.66, 0.75)	1.6 (1.4, 1.8)	20 (16, 25)	0.635
Mesocosm Raunefjorden				
Incubation time (days):				
1	-0.41 (-0.60, -0.22)	40 (36, 44)	64 (52, 76)	0.965
2	-0.63 (-1.02, -0.25)	34 (28, 40)	50 (31, 70)	0.764
3	-0.21 (-0.47, 0.06)	31 (27, 36)	48 (33, 63)	0.843
4	-0.13 (-0.34, 0.08)	31 (27, 34)	47 (35, 59)	0.933
4.5	-0.13 (-0.30, 0.04)	30 (27, 33)	51 (40, 63)	0.951
5	0.42 (0.15, 0.69)	26 (23, 29)	36 (25, 47)	0.907
Kviturspollen				
2	-0.09 (-0.26, 0.08)	9.9 (8.5, 11.4)	25 (17, 33)	0.940
3	0.58 (0.46, 0.70)	12 (11, 13)	20 (15, 25)	0.972
4	1.83 (0.72, 2.95)	14 (13, 16)	15 (8, 22)	0.946
5	1.65 (1.32, 1.99)	15 (14, 17)	25 (20, 30)	0.963
7	2.84 (0.10, 5.59)	14 (11, 16)	18 (9, 27)	0.855

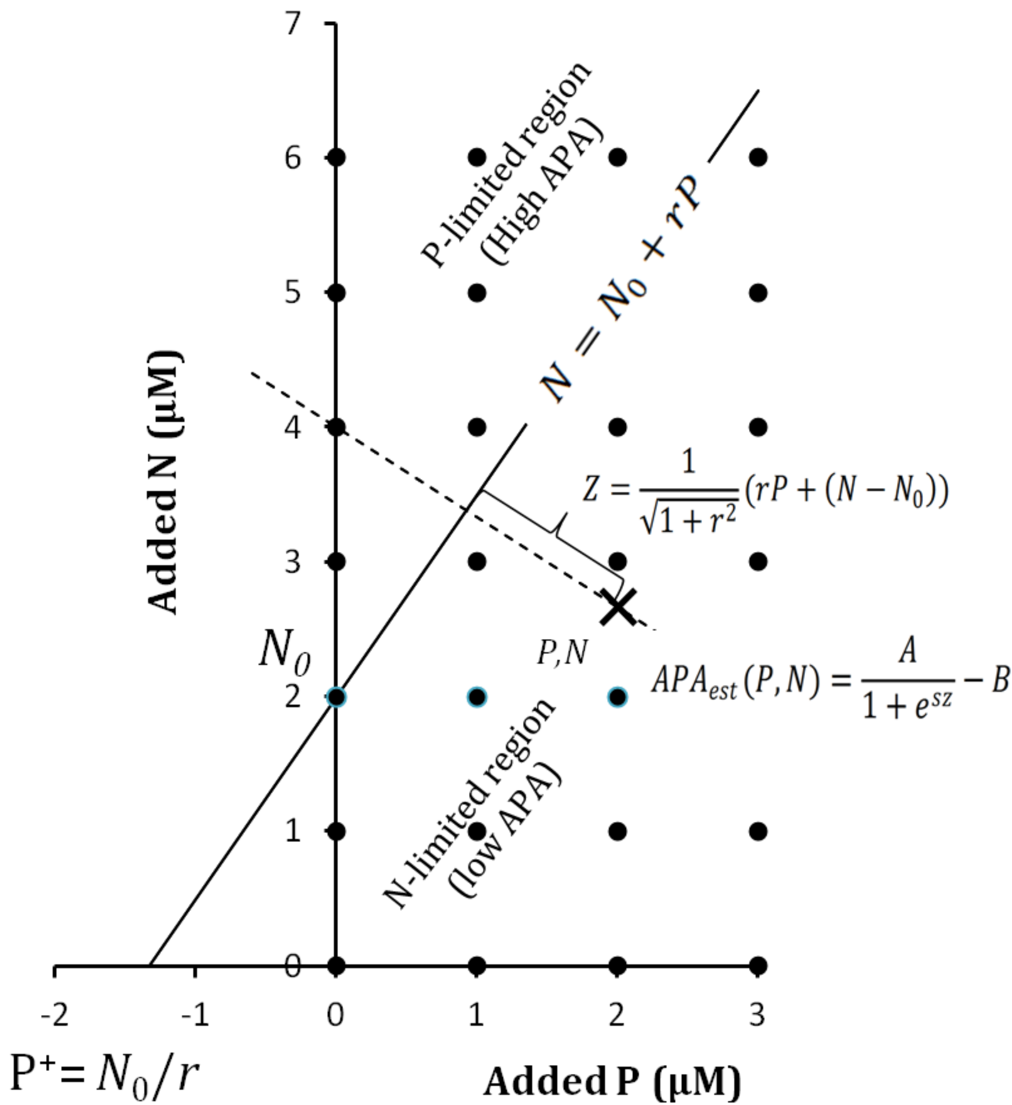
7

1 Table 2. Linear regressions of parameter estimates against incubation time for the
2 Espegrend samples (see Fig. 3). Extrapolation to zero time is given (Day 0).

	Intercept (Day 0)	Slope of linear regression	p (H_0: slope≠0)	R^2
		MR		
N_0	-0.8 (-1.4,-0.2)	0.19 (0.01,0.37)	0.05	0.674
r	41 (36,46)	-2.8 (-4.1,-1.5)	0.004	0.896
s	65 (48,81)	-4.7 (-9.4,0.0)	0.05	0.656
		Kviturspollen		
N_0	-1.0 (-2.4,0.4)	0.6 (0.3,0.9)	0.01	0.917
r	10 (3,16)	0.8 (-0.7,2.3)	*0.2*	0.479
s	24 (6,42)	-0.8 (-4.7,3.1)	*0.6*	0.121

3 H_0 null hypothesis

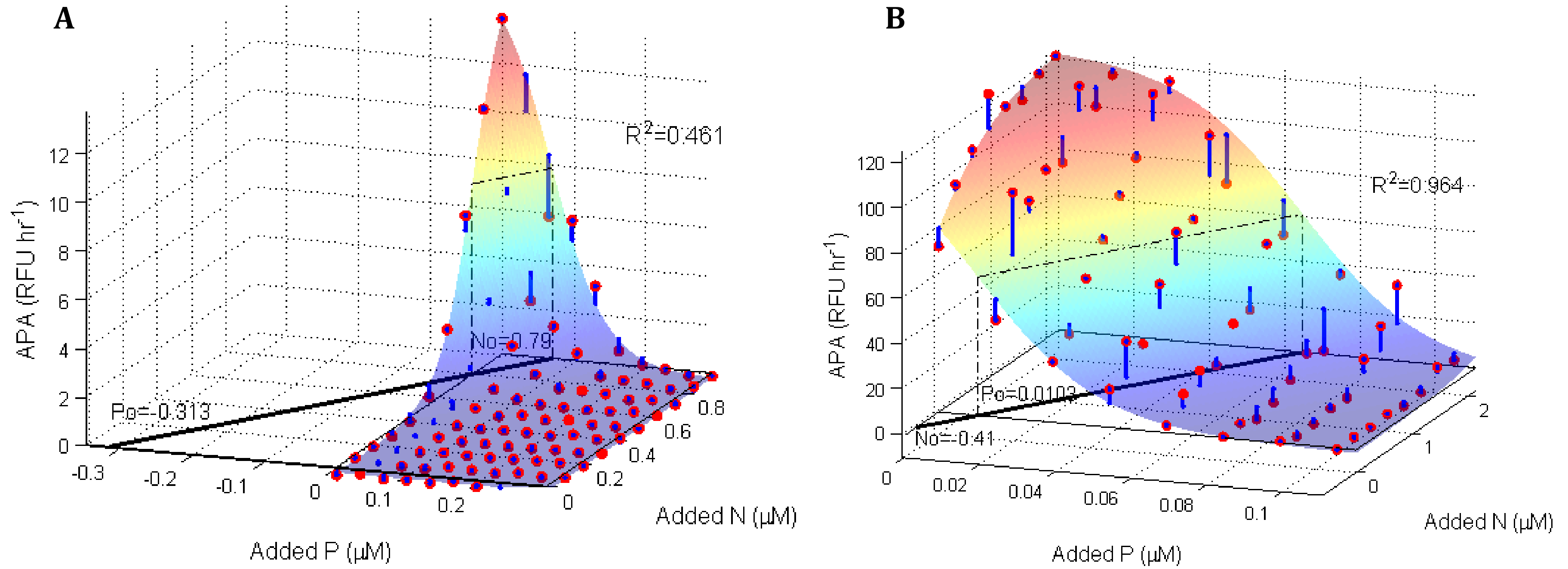
1
2
3



4
5
6

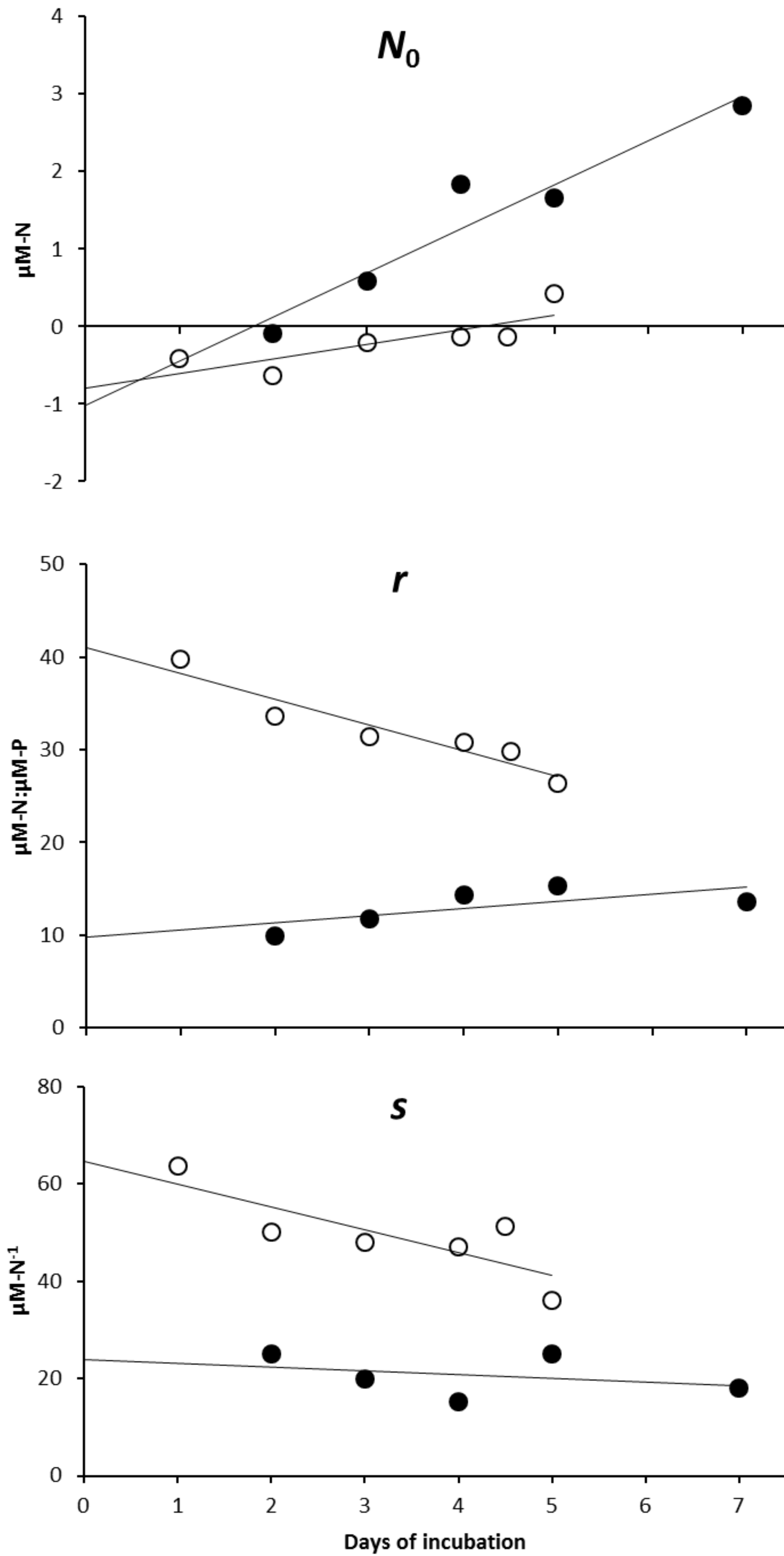
7 Figure 1. Illustration of the fitting algorithm used. With APA measured over a (here) 4x7 matrix of
8 combinations in additions of P and N (black dots), the objective is to find the line that splits this *P*,*N*-
9 plane in an upper P-limited region with high APA and a lower N-limited region with low APA. This
10 is done by least square fitting of the surface $APA_{est} = \frac{A}{1+e^{sZ}} - B$ to the APA-values measured in each
11 grid point. $APA_{est}(P,N)$ is a sigmoidal function of the distance $Z = \frac{1}{\sqrt{1+r^2}}(rP + (N - N_0))$ from the
12 point *P*,*N* (marked X) to the line. The situation illustrated represents an N-limited system with the
13 positive *N*-axis intercept (N_0) and excess-P ($P^{\pm}$) represented by the negative *P*-axis intercept N_0/r. A
14 P-limited system would be characterized by a negative intercept with the N-axis (negative N_0),
15 while a balanced system following Redfield stoichiometry would have a line with zero intercept
16 (N_0=0) and slope *r*=16.

1

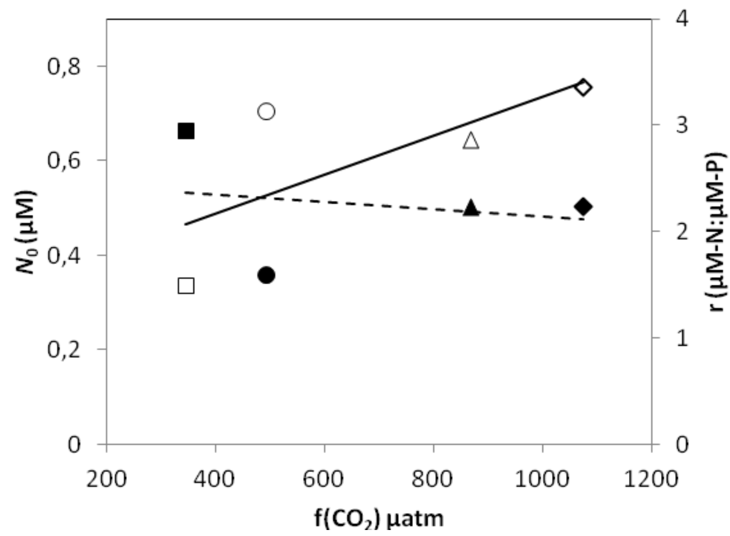


2

3 Figure 2. Measured APA values (red), fitted response surface (blue – red gradient from N- to P-limited) and residuals (blue) for assays:
4 (A) Fjord from Tvärminne and (B) MR from Espegrend (Day 1); illustrating situations interpreted as N-limited with P^{+}=0.3 μM-P and a P-
5 limited with N^{+}=0.4 μM-N, respectively.



1
2 Figure 3. Change in parameter estimates with incubation time for the two samples from western
3 Norway. Kviturspollen has filled symbols, mesocosm MR has open symbols.
4



1
2 Figure 4. Scatterplots between f(CO_2) and estimates of N_0 (open symbols, solid regression line) and
3 *r* (closed symbols, dotted regression line) for Tvärminne mesocosms M1 (squares), M3 (diamonds),
4 M6 (triangles) and M7 (circles). Regression slopes are not significant ($p=0.27$ and 0.79,
5 respectively).