

14 February 2011

MS. Ref. No. bg-2010-311

Title: Lack of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea

Dear Dr. Natascha Töpfer,
Copernicus Publications,
Editorial Support

We revised our manuscript, originally titled “**N-limited or N and P co-limited indications in the surface waters of three Mediterranean basins**”. Since one of the referees suggested us improve the title, we revised the title as “**Lack of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea**”.

By carefully reading the referees’ comments, we revised the manuscript and prepared our reply to the referees in the separate paper (see below).

We would be grateful, if you could kindly consider this revised manuscript for the publication in *Biogeosciences*.

Sincerely yours,

Tsuneo TANAKA on behalf of the authors

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Reply to Referee (Professor Olav Vadstein)

[Referee comment] It is claimed that uptake rate is measured, but this is strictly not true (pp. 8151-8152). The inverse of the turnover time (T) is the uptake rate constant, whereas the uptake rate would be PO₄ concentration multiplied with the uptake rate constant (or divided by T). This also has implications for the definition of specific affinity on p. 8146 in the Introduction.

[Author response] We deleted the term “uptake rate”. However, we kept the term “specific uptake rate” instead of “uptake rate constant”.

[Referee comment] However, the calculated DIN:PO₄ ratios should be deleted from Results (p. 8154), and the discussion of these ratios should be kept to a minimum (i.e. close to zero; pp. 8157-8158). As several of the measurements in Table 1 are below the detection limit, some strange arithmetic's is the results. E.g. for station A the DIN:PO₄ ratio is calculated as: $(34 + <20) : <10 = 6.5:1$. Thus the discussion on pp. 8157-8 is partly without data. This should be revised, even though it does not affect conclusions.

[Author response] We deleted the calculated DIN:PO₄ ratios from Results. We calculated the DIN:PO₄ ratio only for the data at Stn C.

[Referee comment] I would have like some more discussion of the fact that PO₄ concentrations increase considerably with time in the two treatments not receiving PO₄ for Stn B and C (Fig. 1), and at the same time turnover time of PO₄ decrease considerably (Fig. 2, notice log scale). Rough by eye calculations suggest that the uptake of PO₄ may have increased a factor 10 to 20. This emphasize that it is the flux and not the concentration that best describe the demand for a nutrient. Moreover, why did PO₄ and not NH₄ increase with time, and which consequences does this decoupling between N and P regeneration have for limiting factors of auto-and heterotrophs?

[Author response] PO₄ concentrations were below or close to the detection limit in the sample water. The molybdenum bleu reaction method does not necessarily measure only PO₄. Hence, it was difficult to determine the biologically available PO₄ pool.

Increases of PO₄ concentration in the Control and +N at Stn B during the incubation were unexpectedly high (up to ca. 6 times, Fig. 1e), and were not consistent with variations of organic P pools. We speculate that PO₄ contamination in these samples occurred outside rather than inside the microcosms. Because of these uncertainties, we do not direct our discussion to the relation between the decreases of PO₄ concentration and the increases of PO₄ uptake rate and the decoupling between N and P regeneration.

[Referee comment] p. 8159, l. 14-16: Hard to understand.

[Author response] We improved as follows: “Our results suggest that initially the availability of the PO₄ pool for osmotrophs was controlled by the availability of NH₄ (i.e., potential N-limitation) at the three stations. Hence, an NH₄ addition to the waters collected in this study enhanced the P requirement by the osmotroph community, by which turnover times of PO₄ and ATP decreased and APA increased in +N.”

[Referee comment] Sterner & Elser is missing in the References.

[Author response] Added.

[Referee comment] Table 1: This is not a list of Parameters (as stated), but Variables.

[Author response] We modified as follows: “Initial condition at the three sampling sites. Parameter values are shown as mean±SD (n=3) except for water temperature, nutrient stoichiometry, and heterotrophic nanoflagellates.”

We thank Prof. Vadstein for helpful comments.

Reply to Referee #2

[Referee comment] While there are evidence that the East Mediterranean is probably P-limited (Krom et al 1991, Zohary and Robarts, 1998) and that the West Mediterranean is probably N-limited (Raimbault and Cost 1990, Thingstad and Rassulzadegan 1995), several studies showed that limitation shifts from N to P and vice versa depending on the period of the year (Fiala et al 1976, Dolan et al 1995) or the area considered (Woodward and Owens 1989).

[Author response] We would like to point out that neither Raimbault and Cost (1990) nor Thingstad and Rassoulzadegan (1995) mention that the West Mediterranean is probably N-limited. Fiala et al. (1976) and Woodward and Owens (1989) show the evidence of N-limitation in Mediterranean coastal waters. Dolan et al. (1995) do not show the direct evidence of the limitation shift from N and P and vice versa.

[Referee comment] I suggest modifying the title “N-limited or N and P co-limited indications in the surface waters of three Mediterranean basins”. Here it says that you have only weak conclusions (indications) about the nutrient status of the Mediterranean. It is therefore not very attractive to your potential readers. Moreover, you should indicate what is limited: here, grammatically, the indications are limited or co-limited.

[Author response] We modified the title as follows: “Lack of P-limitation of phytoplankton and heterotrophic prokaryotes in surface waters of three anticyclonic eddies in the stratified Mediterranean Sea”.

[Referee comment] The English should be improved by a native speaker. Some sentences are very long and/or confusing and make the manuscript hard to follow here and there. Numerous abbreviations are not given in full letter at first use.

[Author response] A native English speaker did the linguistic correction of the revised manuscript. We improved the introduction of all abbreviations used in this manuscript.

[Referee comment] Abstract: L2-5: this sentence needs to be rewritten for English. Why using “respectively” at the end of this sentence?

[Author response] We improved this sentence.

[Referee comment] L9 “...were set up for the carboys”. Since you introduce the experiment as being manipulated in microcosm, I would keep this term instead of carboys. Moreover, this sentence should be rewritten as well.

[Author response] As suggested, we improved this point.

[Referee comment] L13: I don't think that “purely” is a well chosen term here. I suggest changing all over the manuscript.

[Author response] We deleted this word.

[Referee comment] L16: I think “the” is missing between “at” and “three study sites”

[Author response] Added.

[Referee comment] L16-17: See Figure 4: the stimulation of PP is much greater in +NP suggesting co limitation rather than N limitation. Please rectify. This is true for most of the parameters presented in this study and that should be mentioned.

[Author response] Our experiment is a 2x2 factorial design. When the stimulation of PP is significantly higher in +N than the Control, this indicates N-limitation. In this case, a much greater PP in +NP does not indicate N and P co-limitation. To clarify this aspect, we added our criteria to interpret the limiting nutrient in the revised manuscript (see Materials and methods, 2.8 Statistical analysis).

[Referee comment] L19-20: I don't understand this sentence. What do you mean?

[Author response] We improved this sentence as follows: "Our results demonstrated the gap between biogeochemical features (an apparent P-starved status) and biological responses (no apparent P-limitation)."

[Referee comment] Introduction: P8185 L24: I don't think that "ultra-oligotrophic" is necessary here.

[Author response] Deleted.

[Referee comment] P8185 L24 to P8185 L1: Can you provide references?

[Author response] Added.

[Referee comment] P8185 L1: I think "the" is missing between "understand" and "biogeochemical". Carbon should be written in all letters before introducing C.

[Author response] "the" was added. "C" was spelled out.

[Referee comment] P8185 L7: Particulate Organic Carbon (POC)

[Author response] Improved.

[Referee comment] L12: I would remove the brackets for "relatively"

[Author response] Deleted.

[Referee comment] L12-13: "The deep waters have..." – How deep?

[Author response] The water depth is variable depending on the region in the Mediterranean Sea. We modified the phrase as follows: "The waters below the epipelagic layer..."

[Referee comment] L16: "...the biogeochemical evidences..." - I suggest: "...these biogeochemical parameters suggest that..."

[Author response] We modified as follows: "The biogeochemical parameter of the N to P ratio..."

[Referee comment] L19: “in the lower part of the plankton food web,” – un-necessary, I would remove.

[Author response] Removed.

[Referee comment] P8147 L6: “P” - do you mean phosphate or phosphorus?

[Author response] Phosphate.

[Referee comment] P8148 L12: objectives...were...

[Author response] Corrected

[Referee comment] P8147 L15: One of the main objectives was to test “if the bypass and tunneling mechanisms for P exist”. Nevertheless, the authors did not answer this objective and just mention in the discussion “Hence it was impossible to test in this study if the bypass and tunneling mechanisms for P exist in different sites of the Mediterranean Sea.” I would thus consider removing this objective from the introduction and just discuss this point. Moreover, even if you introduced these concepts in the previous paragraph, you did not mention the terms of bypass and tunneling. If the reader has not read Thingstad et al (2005), they will not follow. Please define the terms of bypass and tunneling earlier.

[Author response] As suggested, we deleted this objective but explained the terms of bypass and tunneling in the previous paragraph.

[Referee comment] P8149 L1: Can you explain why did you choose this depth?

[Author response] We explained as follows: “The sampling depth located at the lower part of the surface mixed layer (13.5 m at Stn A, 8.5 m at Stn B, 11.5 m at Stn C: Moutin et al. in preparation).”

Moutin, T., Van Wambeke, F., and Prieur, L.: Introduction to the bBiogeochemistry from the Oligotrophic to the Ultraoligotrophic Mediterranean (BOUM) experiment, Biogeosciences Discussion, in preparation.

[Referee comment] L3: delete “volume”

[Author response] Deleted.

[Referee comment] L3-4: delete “Four different treatments were set up to examine the limiting nutrient for the plankton community.” But add “four” between “The” and “treatments” L5.

[Author response] As suggested, we improved.

[Referee comment] L7: Please explain why did you add twice as much N at Stn C?

[Author response] We added an explanation as follows: “The level of nutrient amendments were chosen with the aim of satisfying the requirement of either N or P or both by the natural Hprok and phytoplankton community during the experimental period (i.e., 3-4 days). The N:P

ratios (16 and 32) were used as a rough approximation of those below the epipelagic layer of the Western and the Eastern Basins, respectively (see Introduction).”

[Referee comment] L10: Please explain why did you add NH₄ only, while NO₃ is undetectable at StnA and lower than NH₄ at StnB in the initial conditions?

[Author response] We explained as follows: “Since previous studies have shown that Hprok and small phytoplankton (pico- and nanophytoplankton) use ammonium (NH₄) rather than NO₃ (e.g., Lipschultz, 1985; Wheeler and Kirchman, 1986), we used NH₄ as an inorganic N source in this study.”

[Referee comment] L12: “During the incubation, samples were taken...” – Please indicate the sampling frequency.

[Author response] As suggested, we added the sampling frequency for each parameter.

[Referee comment] P8150L9: “For each sample, samples (500 ml) were...” – I suggest: “For each sample, 500 ml were...”

[Author response] We modified this, as suggested.

[Referee comment] L19: remove “respectively”

[Author response] Deleted.

[Referee comment] P8151L9: Do you mean that you first pre-concentrated by gravity and then concentrated again in Utermohl chamber? This is unclear.

[Author response] We improved the description as follows: “Ciliates were pre-concentrated by gravity in the sample bottles, and then settled in Utermöhl chamber.”

[Referee comment] L16: What was the final TCA concentration?

[Author response] Added “5%” in the text.

[Referee comment] L20: What were your background levels?

[Author response] We added background levels for measurement in turnover time of PO₄ and ATP (10.7% and 8.5%, respectively).

[Referee comment] P8152L18-22: Why using all these conversion factors to get P-biomass from C while you measured POP? This is certainly a large source of error.

[Author response] We explained as follows: “The POP data present a sum of living particles and detritus so that we estimated P-biomass based on Chla and cell concentration. The estimated P-biomass was 45-98% (n=7) of POP except for two occasions (105 and 115% of POP).”

[Referee comment] P8153L21: Do you mean the supernatant was aspirated and the pellet was washed 3 times (washed with what?).

[Author response] We improved the description as follows: “The incorporation was stopped with the addition of TCA (final conc. 5%). Bovine serum album was added to each sample (final conc. 100 mg l⁻¹) prior to the first centrifugation. After aspirating the supernatant, 5% TCA was mixed with pellet, and then the sample was centrifuged again. Supernatant was discarded, and a last centrifuge treatment was done after addition of 80% ethanol. After removing the supernatant, and addition of scintillation cocktail, the sample was radioassayed (see Van Wambeke et al., 2010 for details).”

[Referee comment] P8154L12: What is DIN?

[Author response] We added a sentence “Concentrations of dissolved inorganic nitrogen (DIN) were calculated by summing concentrations of NO₃₊₂ and NH₄.” in 2.2 Dissolved and particulate nutrients in Materials and methods section.

[Referee comment] L13-17: Please indicate that you are providing ranges; it is unclear until we read the last sentence.

[Author response] We improved the description, as suggested.

[Referee comment] L17: Please remove “That is”

[Author response] Removed.

[Referee comment] P8155L1-7: Please provide numbers such as factors of decrease in NH₄ and PO₄ concentrations between Ti and Tf. PO₄ at Stn A treatment +N: how can you measure significant decrease since PO₄ was under the detection limit at the beginning of the experiment (<10 nM, Table 1).

[Author response] We added factors of increase or decrease of nutrient concentrations. We deleted the DIN:PO₄ ratios.

[Referee comment] L8-9: “No significant change of NO₃₊₂ concentration was detected between the start and the end of the incubation in 8 out of 12 cases (t-test, P >0.05, data not shown).” Can you specify in which treatments it changed and discuss this interesting result in the discussion?

[Author response] We specified the treatments in which NO₃₊₂ concentrations changed significantly during the incubation. We discussed on this aspect in Discussion section as follows: “With the analytical detection limit and the precision for the NO₃₊₂ measurement in this study, significant changes of NO₃₊₂ concentration remained to be explained.”

[Referee comment] P8156L1-2: Please remove; this is part of the Method section.

[Author response] Removed.

[Referee comment] L5-7: I do not understand this sentence

[Author response] We improved this sentence as follows: “An effect of nutrient addition on DOC concentration was detected only as a significantly lower concentration in +NP than the Control at Stn C (Fig. 3d, e, f).”

[Referee comment] L21: Please replace “smaller” by “lower”

[Author response] Replaced.

[Referee comment] L16-17: “PP was significantly higher in +N than the Control and +P, and highest in +NP at all stations (Tukey HSD test, $P < 0.05$).” Even though PP was significantly higher in +N, it was largely higher in +NP (Fig 4) and this result should be emphasized since the authors provide misleading conclusions in the abstract (i.e. “Primary production was consistently limited by N”).

[Author response] As mentioned above, in order to clarify this aspect, we added our criteria to interpret the limiting nutrient in the revised manuscript (see Materials and methods, 2.8 Statistical analysis).

[Referee comment] P8157L5: Please replace “cases” by “occasions”

[Author response] Improved.

[Referee comment] L1-15: I would remove this sentence as it is not really relevant. This study might be the only one to study the effect of nutrient additions on these parameters all together but it is far from being the first study to look at the limiting nutrients in the Mediterranean.

[Author response] Our intention was to emphasize our results are the first demonstrating that the lower part of the pelagic plankton food web did not experience P-limitation in the three anticyclonic eddies located in the Western Basin, the Ionian Basin, and the Levantine Basin during the stratified period. Even if previous studies report no P-limitation, these studies were done (1) in the coastal water (Fiala et al. 1976, Woodward and Owens 1989), (2) during the winter mixing period (Woodward and Owens 1989), or (3) for either phytoplankton (Fiala et al. 1976) or Hprok (Van Wambeke et al. 2002).

[Referee comment] L15-17: "are the first that"

Guerzoni et al 1999 Progress in Oceanography p169-70 or Handbook of environmental chemistry vol 5 - C. Migon p250: "Despite early observations of P limitation (Fiala et al 1976, Berland et al 1973, 1980), recent works suggest that the Mediterranean surface waters are N-limited (Andersen and Nival 1988, Owens et al 1980). In fact, there is growing evidence that the east Mediterranean is P-limited (Krom et al 1991) and that the West Mediterranean is probably N-limited (Raimbault and Cost 1990, Thingstad and Rassoulzadegan 1995), or that limitation shifts from N to P and vice versa depending on the period of the year (Fiala et al 1976, Dolan et al 1995) or the area considered (Woodward and Owens 1989)." Again, this study is not a first. See also in your discussion "phytoplankton were N and P co-limited in surface waters in May 2002 in the same area (Thingstad et al., 2005; Zohary et al., 2005)" Please remove "are the first that".

[Author response] We would like to underline that:

- Neither Raimbault and Cost (1990) nor Thingstad and Rassoulzadegan (1995) mention that the West Mediterranean is probably N-limited.
- Fiala et al. (1976) and Woodward and Owens (1989) show the evidence of N-limitation in Mediterranean coastal waters.

- Dolan et al. (1995) do not show the direct evidence of the limitation shift from N and P and vice versa.
- Andersen and Nival (1988) simulated the pelagic ecosystem of the coastal waters of the Ligurian Sea (NW Mediterranean Sea). Their model consists of phytoplankton, copepods, salps, chaetognaths, dissolved inorganic nitrogen, and particulate organic matter. However, they do not discuss on nutrient limitation.
- Thingstad et al. (2005) and Zohary et al. (2005) report that phytoplankton were N and P co-limited and Hprok were P-limited. In our study done in the same basin (Stn C), we found that phytoplankton were N-limited and Hprok were N and P co-limited.

[Referee comment] P8158L10: which depths?

[Author response] The water depth is variable depending on the region in the Mediterranean Sea. We used the following phrase: “waters below the epipelagic layer”

[Referee comment] L23-27: Again, why not using POP data?

[Author response] We explained as follows in Materials and methods: “The POP data present a sum of living particles and detritus so that we estimated P-biomass based on Chla and cell concentration. The estimated P-biomass was 45-98% (n=7) of POP except for two occasions (105 and 115% of POP).”

[Referee comment] P8159L29: What was different? Microcosm size (bottle effect?), final concentration of the amended nutrients? Other? Please describe. Please provide some hypothesis to explain these differences in plankton response to P additions.

[Author response] Nutrient concentration and microcosm size were different. However, we have no hypothesis to explain these differences.

[Referee comment] P8160L28: there is no "on one hand" so there should not be any "on the other hand". Please modify.

[Author response] Disagree.

[Referee comment] L29: PP and Chla were not measured at the trophic level

[Author response] We corrected to “the osmotrophs community level”.

[Referee comment] P8161L8: “...during the winter overturn, and annual phytoplankton...” Here we understand that the N and P inputs happen during the overturn AND the bloom. I think that you meant that the input during mixing event results in a bloom which exhausts PO₄ and NO₃ to residual levels?

[Author response] We modified this sentence as follows: “In the Eastern Mediterranean Sea, high amounts of NO₃ and PO₄ with N:P ratios of 22-28 is supplied to the epipelagic layer during the winter overturn, and annual phytoplankton bloom (November-March) results in an exhaustion of PO₄ and a NO₃ residual in the epipelagic layer when the water column stratification is established (Krom et al., 1991, 2003, 2010).”

[Referee comment] L12: Please remove “That is”

[Author response] Removed.

[Referee comment] P8161L25-30: This sentence is very long and hard to understand. I would suggest to write 2 sentences and to improve the English.

[Author response] We improved this sentence as follows: “A possible scenario for these seemingly disparate results would be that (1) as the stratified period progresses, the available pool of both inorganic N and P becomes very small in the surface mixed layer, (2) the plankton food web shifts to N and P co-limitation. Thus, growth of osmotroph groups is limited by different nutrients in a same system, and the most limiting nutrient for osmotroph growth shifts seasonally or sporadically among N, P and N+P (cf. Hecky and Kilham, 1988).”

[Referee comment] P8162L2-6: same comment

[Author response] We improved this sentence as follows: “Our results show that while the C:N:P ratio of particulate organic matter consistently indicated P-starved status compared to the Redfield ratio, whenever nutrient limitation was detected, phytoplankton and Hprok never experienced P-limitation in surface waters in the center of anticyclonic eddies at the three Mediterranean Basins.”

[Referee comment] L8-9: Since you are not the first one to raise this question, I would remove this sentence or rewrite it.

[Author response] We carefully explained that our study was done in three anticyclonic eddies (i.e., offshore waters) during the stratified period and found no P-limitation of phytoplankton and Hprok. This is the first report in the Mediterranean Sea.

[Referee comment] L10-11: “the skewed PON:POP ratio but the microbial food web being N-limitation or N and P co-limitation” – Please rewrite

[Author response] We improved as follows: “If indeed organic matter in the surface layer is P-poor (skewed ratios of DON:DOP and PON:POP), but the microbial food web is either N-limited or N and P co-limited, are conditions which suggests a turnover time of organic N longer than that of organic P (cf. Thingstad and Rassoulzadegan, 1995). A required condition that permits N and P co-limited Hprok but N-limited phytoplankton in the same water is that N:P ratio in phytoplankton community is much higher than that in Hprok community (i.e., greater P requirement per cell volume by Hprok) (reviewed by Vadstein, 2000; Sterner and Elser, 2002) in surface waters.”

[Referee comment] L19-20: This sentence is irrelevant here. I don’t understand the link with the previous and following ideas.

[Author response] Deleted.

[Referee comment] L20-21: Please explain how.

[Author response] Deleted.

[Referee comment] Table 1: I suggest the following title: “Initial conditions at the three sampling sites.” “Values are shown as mean \pm SD (n = 3) except for water temperature, nutrient stoichiometry, and heterotrophic nanoflagellates.” Do you mean that n=1 for the exceptions? ATP turnover time at Stn B is very high compared to Stn A and C. Are you confident that it is representative of the initial conditions at Stn B? Indeed it influences the results on Figure 2 where ATP turnover decrease while they were pretty much constant at Stn A and C. How can you calculate DIN:PO₄ when NO₃ and PO₄ are under the detection limit?

[Author response] We improved the table explanation. We deleted the DIN:PO₄ ratios at Stn A and Stn B. ATP turnover times at 5 m at Stn B measured at different time points also showed similar values. The datum was obtained from the surface mixed water (5 m) which was chronologically closest to the start of the microcosm experiment. We believe that the initial ATP turnover time at Stn B is similar to this datum. However, we modified Fig. 2e to clarify that this datum was not obtained in the same microcosm experiment (i.e., a different symbol without line connection to the other data points).

[Referee comment] Figures: I suggest adding A, B, C... letters to the different panels in order to help the reader in the result section.

[Author response] We added “a, b, c, ...” to the different panels in all figures.

[Referee comment] Figure 1: How do you explain the increase in PO₄ in the control and +N treatment at Stn B and C? Please comment in the discussion

[Author response] Commented in the discussion.

[Referee comment] Figure 3: It is too small

[Author response] We split this figure into two parts.

[Referee comment] Figure 4: the stimulation of PP is much greater in +NP suggesting co limitation rather than N limitation as the authors mention in the abstract.

[Author response] We explained our criteria to interpret the results in Materials and methods.

We thank Referee #2 for helpful comments.