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# *Interactive comment on* "Influence of increasing dissolved inorganic carbon concentrations and decreasing pH on chemolithoautrophic bacteria from oxic-sulfidic interfaces" *by* K. Mammitzsch et al.

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Comments to the review of Referee #1

We thank the Reviewer for the constructive and helpful comments, which have improved this manuscript. In the following we outline the changes we have made in response to the comments. Page and line numbers are according to the discussion paper.

General comment: As both Reviewers felt that the experimental conditions used to

examine the impact of pH and DIC on the growth of “Sulfurimonas gotlandica” strain GD1 were too far removed from the in situ conditions, we begin our response to their specific comments with a general remark addressing this point. “S. gotlandica” str. GD1 is currently the only representative isolate from this bacterial group, which inhabits anoxic water columns. It has been shown that in pelagic redoxclines of the Baltic and Black Sea related taxa within this group are key components, as they are responsible for chemolithoautotrophic denitrification (which is the major N loss process in the water column), sulfide detoxification, and chemoautotrophic production (Grote et al. 2008, 2012; Bruckner et al. 2012). Thus, these organisms mediate important redox transformation processes and link the carbon, nitrogen, and sulfur cycles at oxic-anoxic interfaces. We previously showed that “S. gotlandica” str. GD1 is an appropriate model organism to study the above-mentioned processes and to deepen our understanding of microbial adaptations to pelagic redoxclines at the genomic and physiological levels (Grote et al. 2012). The established batch culture conditions for this strain were applied in all previous experimental investigations and include temperature and substrate concentrations that are higher than in situ conditions. This is necessary to obtain measurable rates and activities, and to show significant and reliable effects in response to the environmental factors studied. Higher substrate concentrations are generally used in batch culture experiments with environmentally relevant microorganisms (see, e.g., Cardoso et al., 2006; Inagaki et al., 2003; Yamamoto et al., 2010). Probably the only alternative would be to work with chemostat cultures. Nevertheless, by comparing field and laboratory studies we could previously show that the results obtained under these experimental conditions provide novel and essential insights into metabolic pathways and process rates, and principally fit to the observations made in the environment (Grote et al. 2012, Bruckner et al. 2013). We strongly feel that there is an urgent need for this type of culture studies. As a true representative of a biogeochemically important bacterial group, “S. gotlandica” str. GD1 offers unique possibilities to gain important insights into the principal regulatory mechanisms of chemoautotrophs in pelagic redoxclines.



BGD
9, C9468–C9477, 2013





Reviewer comment: Growth rates are not presented within the manuscript and cannot be calculated from batch cultures that were sampled after 14 days.[. . .] thus steady state growth rates cannot be calculated.

Response: There are different ways to examine the response of a bacterial culture to abiotic factors. We admit that the measurement of growth rates is a very sensitive assessment. The reason that we did not choose this approach is that frequent subsampling of the anaerobically growing culture constitutes a potential disturbance, as sampling may result in subtle changes in the head-space gas pressure or even in a minute oxygen contamination, both of which are likely to influence bacterial growth. Based on our previous experience with Sulfurimonas cultures, the bacterium reacts very sensitively to such disturbances. The alternative approach to comparing the impact of certain abiotic factors is to assess the realized biomass at the end of the exponential phase. This resembles both the carrying capacity of the culture under the given conditions (as also stated by the Reviewer, below) and also the yield of the culture (if seen in relation to the substrate concentrations) and is an often-used parameter in laboratory experiments with pure or mixed cultures. Although this is less sensitive than growth rate measurements, it has the advantage that it reflects a summary effect, including the bacterial growth rate and the efficiency of using the available substrates and converting them into bacterial biomass. As seen in Figure 4, the stationary phase of our cultures was relatively stable (approximately from day 10 to day 14). Thus, maximal cell numbers could still be assessed at day 14. However, we have now more clearly defined our approach to assessing maximal cell numbers and replaced the term “growth” with “maximal cell numbers.” We also added the following sentence in the Methods (page 18376, line 2): “Bacterial cell numbers were quantified by counting DAPI (4’,6-diamidino-2-phenylindol)-stained cells by epifluorescence microscopy. The maximal cell numbers, which were reached at the end of the exponential growth phase, represent both the yield of the culture (with respect to the substrate concentrations) and the carrying capacity under the given conditions, reflecting the efficiency of using the available substrates and converting them into bacterial biomass.” Accordingly, we



BGD
9, C9468–C9477, 2013

could show the optimum pH range for the chemoautotroph growth of “S. gotlandica” str. GD1, with a drastic decrease of maximal cell numbers directly below and above the optimum range. This approach was also the basis for our selection of distinct pH values for growth experiments, in which a remarkable shift from nearly optimal growth at a pH of 6.6 to strongly suppressed growth at a pH of 6.55 was demonstrated (Fig. 4). In this experiment we also calculated the specific growth rates (0.4 $\pm$ 0.01 h-1 for the pH range 6.6–7.1, and 0.31 $\pm$ 0.02 h-1 at pH 6.5). The information on the growth rates for this experiment is now provided in the manuscript as follows (page 18381, line 12): “The growth rate was 0.4 $\pm$ 0.01 h-1 and did not differ between pH 6.6 and 7.1. At pHs 6.5, bacterial growth was somewhat slower, with a growth rate of 0.31 $\pm$ 0.02 h-1.” Using this approach we could also show that the current DIC concentration in the Baltic Sea is well within the saturation of “S. gotlandica” str GD1 and that a DIC concentration below 800 $\mu$M may be growth limiting.

Reviewer comment: On page 18381 line 9-12 the authors state exponential growth to be occurring between day 6 and 9 (Fig. 4). Actually, the culture should have been growing exponentially between the start of the experiment until about day 8, when thiosulfate became limiting.

Response: Exponential growth started after a lag phase, at about day 3. We chose days 6–9 from the exponential phase to calculate substrate utilization because during this time span differences in the substrate concentrations were the most significant, thus reducing methodological biases (which are larger when only small concentration changes occur). This is now more clearly described in the manuscript (page 18381, line 12). A similar time frame was used in previously published experiments with this strain (Bruckner et al., 2013). Even if at day 9 the exponentially growing culture was already in transition to stationary phase, this does not affect our conclusions, as the differences in substrate utilization between pH 6.6 and pH 6.55 were very large (see Table 1 and Figure 4).

Reviewer comment: Steady state growth rates could have been calculated with addi-



BGD
9, C9468–C9477, 2013





tional data on cell counts before the incubation as well as before growth limitation (e.g. day 6).

Response: See comment above regarding why we did not perform additional sub-samplings and cell counts. However, we have now added cell counts at the beginning of the incubation time (day 0) to the manuscript.

Reviewer comment: Substrate and treatment concentrations are insufficiently determined by measurements during and after incubations. While sulphur oxidation is over-determined with measurements of thiosulphate and sulphate, inorganic carbon species are hardly documented.

Response: We disagree that our substrate and treatment concentrations were insufficiently determined. We are aware of the usual uncertainties and limitations of those measurements, but in our opinion they do not hamper any of the conclusions drawn in this study. The methods used for our measurements were similar to those used in other recently published studies of this strain (see Grote et al., 2012; Bruckner et al., 2013). We did not monitor inorganic carbon speciation during the experiment in order to avoid the above-mentioned problems associated with frequent sub-samplings. Instead, we calculated the theoretical DIC speciation (with the CO2sys program after Lewis and Wallace, 1998) and have added the data to the manuscript (page 18376, line 1): "The distribution of the DIC speciation at pH 6.5 is 70.96 % hydrogen carbonate, 28.96 % carbon dioxide, and 0.07 % carbonate, whereas at pH 8.0 the distribution of the DIC speciation is 95.59 % hydrogen carbonate, 1.23 % carbon dioxide, and 3.17% carbonate." As already mentioned in the manuscript (page 18383, lines 9–13) we do not think that the different carbon speciation and the pH-dependent shift influenced bacterial growth since a DIC concentration of 800 $\mu$M proved to be more than sufficient to support maximal cell numbers.

Reviewer comment: Only potentiometric pH data (NBS-scale) is shown, which is not calibrated to any seawater scale. Those pH measurements are only reported for start-



BGD

9, C9468–C9477, 2013



ing conditions of the experiments. [. . .] It remains unclear how pH developed in the single treatments.

Response: We admit that it would have been more precise to calibrate the pH meter using a seawater scale. However, the NBS scale is currently the most commonly used one (see e.g., Krause et al. 2012, PLosOne; Wannicke et al, 2012, BG, Frommel et al., 2010, BG; Franke und Clemmensen, 2011, BG). Accordingly, its further use is the best way to allow comparisons between our data and those from other, similar experiments, including studies of other bacterial taxa (Takai et al., 2006; Inagaki et al., 2003; Scott and Cavanaugh, 2007). Although not all publications mention the method used for measuring pH, the NBS scale seems to be the most frequently chosen one. We report measurements not only for the starting conditions of the experiments, but also for the end of the incubation time (see page 18379, line 22; page 18380, lines 14–16). The pH change during the experiment (shown in Figure 4) is now included (page 18381, line 19). Although we did not measure the pH during the incubation time and thus cannot show its corresponding development, we want to emphasize that neither an increase in the DIC nor a decrease of pH of about 0.2 units (from pH 7.1 to pH 6.9) would have influenced the maximal cell numbers of "S. gotlandica" str. GD1 (page 18382, lines 4–11), according to our results. Thus, the predicted changes in response to ocean acidification still result in DIC ranges and pH conditions that are optimal for the growth of this bacterium.

Reviewer comment: Substrate concentrations appear unrealistically high.

Response: Compared to environmental conditions, the substrate concentrations in the batch culture experiments are indeed high. However, our aim was not to mimic the natural situation in the redoxcline (in that case we would have used seawater as the medium) but to use this model system to reveal the principal mechanisms regulating the growth of "S. gotlandica" str. GD1. A certain cell density and growth are currently required to examine those mechanisms. Similar substrate concentrations were used successfully in previous experiments to examine the utilization of different electron donors



BGD
9, C9468–C9477, 2013

and acceptors, including organic carbon (Grote et al. 2012), and the stoichiometry of chemoautotrophic denitrification (Bruckner et al. 2013). Although conditions more closely resembling the environmental ones are desirable, there is currently no evidence that the results obtained at higher substrate concentrations cannot be applied to in situ conditions. However, as this is a critical point that should be considered in future experiments, we have added the following sentence in the Discussion (page 18384, line 20): "Since substrate utilization in the batch culture experiments was significantly higher than under environmental conditions, the results rather reflect the carrying capacity of "S. gotlandica" str. GD1 at the given conditions. Future studies should aim to more accurately simulate in situ conditions (e.g., with chemostat cultures). However, at present we have no evidence that the results concerning either substrate utilization or the impact of pH and the DIC concentration are not relevant to in situ conditions."

Reviewer comment: References indicating whether a conversion of _1mM of nitrate with 1mM of thiosulphate, leading to this pH shift; is a realistic scenario for Baltic Sea deep water are missing. This pH shift is discussed as if it naturally accompanies sulphur oxidising denitrification (page 18382 line 4-11).

Response: The pH shift that is expected also in deep water under future ocean acidification conditions is not due to the conversion of 1mM nitrate and 1 mM thiosulfate, and chemolithoautotrophic denitrification under natural substrate concentrations will not substantially contribute to possible shifts in environmental pH. The major mechanism would be a transfer of acidified conditions from the surface to deep water. We apologize if the text did not make this scenario sufficiently clear. At the point in the manuscript to which the reviewer refers, our aim was to discuss whether the pH shift in the experimental incubations would constitute a problem for the interpretation of the results. Nonetheless, to further clarify this issue we have added the following sentence to the Discussion (page 18382, line 11): "However, a decrease in the pH of the unbuffered medium during the incubation time does not occur naturally under environmental conditions but it was observed as an effect in batch cultures during growth at



BGD
9, C9468–C9477, 2013

high substrate concentrations (Trouve et al, 1998; Javor et al. 1990)."

Reviewer comment: Direct measurements of DIC and phosphate are lacking completely.

Response: The measurement of phosphate was not considered in the experimental design because our aim was to focus on the two major parameters of ocean acidification (decreasing pH and increasing DIC). To our knowledge, phosphate concentrations affect neither the metabolism of "S. gotlandica" str. GD1 nor the investigated pH and DIC ranges. DIC measurements would have been desirable but it was not possible to measure DIC directly within this experimental design as our analytical method (coulometric, SOMMA system) would have required at least 500 mL of medium to measure DIC correctly. Removing this amount of medium would have resulted in a huge headspace in the sample and accordingly a shift in the DIC species between medium and headspace (e.g., at pH 7.0: 2.7 % of the DIC compounds were converted into CO2 gas in the headspace; and at pH 7.5: 7.8 %). Thus, we decided to calculate the DIC concentration only.

Reviewer comment: High cell densities are in these experiments combined with large nutrient resources (9_M PO4, 91_M NH4, 1mM NO3, 1mM thiosulphate) so that substantial consumption of DIC can be expected before substrate limitation is reached. Cells grown at various DIC concentrations presented in Figure 2 can rather be interpreted for carrying capacities of the growth media than used to infer on cellular growth rates. Here, DIC below 500 _M might as well be ultimately limiting the amount of biomass produced, while thiosulphate might have been limiting at DIC above 800_M (page 18381 line 1-2).

Response: We agree that our data can be interpreted as carrying capacities at the given nutrient conditions rather than as growth rates. This is outlined also in the response above and the respective changes were made throughout the revised manuscript. Nevertheless, the results from the DIC experiment produced valuable in-



BGD
9, C9468–C9477, 2013





formation regarding the range of DIC concentrations that impact the growth of "S. gotlandica" str. GD1, for example a DIC below 500 $\mu$M would obviously be growth-limiting. Although thiosulfate seemed to be entirely consumed at the end of the experiment (see Fig. 4), this did not interfere with conclusions regarding the role of sub-optimal DIC concentrations. Furthermore, based on calculations using the stoichiometry for chemoautotrophic denitrification given in Bruckner et al. (2013), the carrying capacity with the substrate concentrations used in this experiment would be approximately 5 $\times$ 108, 6 $\times$ 107, and 3.5 $\times$ 107 cells mL-1 for DIC, thiosulfate, and nitrate, respectively. According to this stoichiometry, nitrate would be the first limiting factor. Earlier experiments with this strain did not produce higher cell numbers with higher thiosulfate concentrations (Bruckner et al., 2013; unpublished data). Therefore, other potentially limiting factors, related to cell concentration, have to be considered, such as the accumulation of inhibitory metabolic products. For this reason in the revised manuscript we have defined maximal cell numbers also as the carrying capacity (page 18382, line 26): "Although thiosulfate seemed to be entirely consumed at the end of the experiment (see Fig. 4), earlier experiments with this strain did not produce higher cell numbers with higher thiosulfate concentrations (Bruckner et al., 2013; unpublished data). Therefore, other potentially limiting factors, related to cell concentration, have to be considered, such as the accumulation of inhibitory metabolic products. For this reason, we defined maximal cell numbers also as the carrying capacity."

Reviewer comment: The only data presented within this manuscript that could be used to infer on growth and substrate utilisation are the ones presented in Figure 4, within the first four to fife days, when DIC concentration and pH can be assumed to be close to initial values.

Response: The experiment, for which results are presented in Fig. 4, is indeed the only one in which growth and substrate utilization were assessed. However, although the Reviewer suggests that the DIC concentration declines to limiting values, we disagree, since DIC was added in saturation (2000 $\mu$M, see also Fig. 2). According to Bruckner



BGD
9, C9468–C9477, 2013





et al. (2013), the bacterium fixes on average 4.16 fmol DIC per cell. Thus, cell growth of up to 2 $\times$ 107 cells mL-1 would equal a DIC consumption of 4.16 % of the initial 2000 $\mu$M DIC and therefore an only negligibly lower DIC concentration. The pH was kept constant by using the buffer HEPES. Thus, we can assume that pH and DIC remained close to the initial values during the incubation time.

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Interactive comment on Biogeosciences Discuss., 9, 18371, 2012.