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Influence of increasing dissolved inorganic carbon concentrations and decreasing pH on chemolithoautrophic bacteria from oxic-sulfidic interfaces

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Received: 6 December 2012 – Accepted: 7 December 2012 – Published: 17 December 2012

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Published by Copernicus Publications on behalf of the European Geosciences Union.

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Abstract

Increases in the dissolved inorganic carbon (DIC) concentration are expected to cause a decrease in the pH of ocean waters, a process known as ocean acidification. In oxygen-deficient zones this will add to already increased DIC and decreased pH values. It is not known how this might affect microbial communities and microbially mediated processes. In this study, the potential effects of ocean acidification on chemolithoautotrophic prokaryotes of marine oxic-anoxic transition zones were investigated, using the chemoautotrophic denitrifying ε -proteobacterium “*Sulfurimonas gotlandica*” strain GD1 as a model organism. This and related taxa use reduced sulfur compounds, e.g. sulfide and thiosulfate, as electron donors and were previously shown to be responsible for nitrate removal and sulfide detoxification in redox zones of the Baltic Sea water column but occur also in other oxygen-deficient marine systems. Bacterial cell growth within a broad range of DIC concentrations and pH values was monitored and substrate utilization was determined. The results showed that the DIC saturation concentration for growth was already reached at 800 μ M, which is well below in situ DIC levels. The pH optimum was between 6.6 and 8.0. Within a pH range of 6.6–7.1 there was no significant difference in substrate utilization; however, at lower pH values cell growth decreased sharply and cell-specific substrate consumption increased. These findings suggest that a direct effect of ocean acidification, with the predicted changes in pH and DIC, on chemolithoautotrophic bacteria such as “*S. gotlandica*” str. GD1 is generally not very probable.

1 Introduction

The report of the Intergovernmental Panel on Climate Change (IPCC) includes predictions for numerous climate-related scenarios, e.g. “business as usual” and an increase in the dissolved inorganic carbon (DIC) concentration of the oceans (Houghton et al., 2001). According to this scenario, a decrease of 0.3 pH units is predicted by the year

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2100 and a decrease of 0.77 pH units by the year 2300 (Caldeira and Wickett, 2003). The lower oceanic pH will cause a shift in DIC speciation, to higher concentrations of hydrogen carbonate and CO₂. However, these forecasts are relevant to the surface layers of the oceans, not for their deeper waters. Though, Karstensen et al. (2008) as-

5 assume that changes of surface water might reach the deeper parts of the oceans but only with a time delay of decades or a century. Evidence of ocean acidification has already been obtained regarding the harmful effects of high CO₂ partial pressure ($p\text{CO}_2$) on calcifying organisms, e.g. foraminifera and pteropods (Riesebell et al., 2000; Fabry et al., 2008), and of its stimulating effects on CO₂-fixing organisms (Iglesias-Rodriguez et al., 2008).

10 Another predicted consequence of global warming is an extension of the hypoxic areas of the oceans, i.e. where the oxygen concentration is less than 2 mL L⁻¹ (Stramma et al., 2008; Conley et al., 2011). It has been shown that ocean acidification is amplified in the hypoxic regions, where $p\text{CO}_2$ are already at much higher values than in surface

15 waters (Melzner et al., 2012). The most severe form of anoxia, with fatal consequences for higher life, constitutes the development of hydrogen sulfide containing bottom waters. These so called “dead zones” have expanded due to eutrophication in coastal ecosystem (Díaz and Rosenberg, 2008) but occur also in semi-enclosed basins with reduced water circulation such as the Black Sea, Cariaco Basin and the Baltic Sea.

20 The Baltic Sea is one of the largest hypoxic marine systems and it is intensely influenced by anthropogenic activities (Conley et al., 2011). The DIC concentration of the deeper anoxic zones in the central Baltic basins is already around 2 mM and the pH is 7.1 (Beldowski et al., 2010; Schneider et al., 2011). Due to the slow ventilation and pre-existing high $p\text{CO}_2$ and low pH values of anoxic zones, the impact of ocean acidification at these depths is expected to be minimal and gradually occurring (Hutchins et al., 2009).

25 However, although expanded zones of oxygen deficiency occur nearly permanently in the deep central basins, they appear periodically also in many shallow coastal areas (Conley et al., 2011, Melzner et al., 2012) where changes in surface conditions propagate more rapidly to the bottom layer.

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consistent picture yet (see review by Liu et al., 2010). Only few studies have examined the impact of pH and DIC on growth of chemolithoautotrophic bacteria, mostly with focus on carbon concentrating mechanisms, and using isolates derived from hydrothermal vent habitats (Scott and Cavanaugh, 2007; Dobrinski et al., 2005). However, the 5 effects of DIC and pH were generally not investigated separately, and representative organisms of pelagic anoxic zones were not available until now. Therefore, in the present work we first investigated whether there is an influence of both aspects of ocean acidification, increasing DIC concentration and decreasing pH. Second, in order to deduce single regulating factors, we examined the influence of these two factors for growth and 10 substrate utilisation of “*S. gotlandica*” str. GD1 separately.

2 Material and methods

2.1 Cultivation

“*S. gotlandica*” str. GD1 was grown in anoxic artificial brackish water with the following components: 95 mM NaCl, 11.23 mM MgCl₂, 2.28 mM CaCl₂, 2.03 mM KCl, 10 mM 15 HEPES, 192 µM KBr, 91 µM H₃BO₃, 34 µM SrCl₂, 91 µM NH₄Cl, 9 µM KH₂PO₄ and 16 µM NaF. Resazurin served as the redox indicator. To remove oxygen from the medium, the deionized water used in medium preparation was boiled for at least 10 min and then purged with N₂ for at least 45 min. After autoclaving the medium, vitamins 20 (Balch et al., 1979), trace elements SL10 (Widdel et al., 1983), selenite, and tungstate (Widdel and Bak, 1992) were added as supplements. Nitrate (1 mM) was added as electron acceptor and thiosulfate (1 mM) as electron donor. Although hydrogen sulfide is an important substrate in situ and was shown to be utilised by this strain (Grote et al., 2012), thiosulfate provides high growth as well and is better suitable for controlled experimental investigations (Grote et al., 2012; Bruckner et al., 2012). The substrate concentrations were added in saturation for “*S. gotlandica*” str. GD1, allowing exponential growth for several days. As carbon source bicarbonate (sterile-filtered), was provided at 25

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a concentration of 2 mM. The culture was grown in the dark at 15 °C and at a pressure of 2.5 bar. Cell growth was quantified by counting DAPI (4',6-diamidino-2-phenylindol)-stained cells by epifluorescence microscopy.

2.2 Chemical analysis

5 The pH was measured with a WTW microprocessor pH meter pH 3000 and a WTW SenTix 61 pH electrode and calibrated with standard buffer solutions (pH 4.01 and 6.87). All pH measurements are reported on the National Bureau of Standards (NBS) scale. Nitrate was quantified colorimetrically at a wavelength of 540 nm according to the spongy cadmium method, as described by Jones (1984). Sulfate was determined turbidimetrically by Ba-precipitation in a procedure modified from that of Tabatabai (1974). Here, to avoid the formation and precipitation of thiosulfate-derived zero-valent sulfur, the samples were not acidified by citric acid. Thiosulfate was analyzed with a modified method according to Zopfi et al. (2004). The samples were derivatized based on 15 3-(bromomethyl)-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2-*a*]pyrazole-1,7-dione (also known as (mono)bromobimane) and then measured by HPLC (Merck), consisting of a LiChrosphere 60RP select B column (125 × 4 mm, 5 µm). The eluents were 0.25 % acetic acid (v/v) and HPLC-grade methanol. The methanol gradient was established as follows: 0 min: 0 %, 1 min: 8 %, 4.5 min: 10 %, 7 min: 32 %, 11 min: 32 %, 18 min: 50 %, 22 min: 100 %, 24 min: 100 %, 25 min: 0 % and 30 min: 0 %. Thiosulfate was detected by 20 a fluorescence detector (excitation: 380 nm, emission: 480 nm). Standards and reagent blanks were prepared in N₂-purged deionized water and analyzed as described for the samples.

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2.3 Experimental design

2.3.1 Growth of “*Sulfurimonas gotlandica*” strain GD1 at different DIC concentrations and pH values

“*S. gotlandica*” str. GD1 was grown in batch culture (250 mL cultivation bottles including 50 mL headspace) at different pH values (between 6.7 and 7.2) and two different DIC concentrations: 2000 μM (representing current value of Baltic Sea redox zones) and 2200 μM (expected increase following ocean acidification), respectively. A buffer was not added in this experiment in order to allow DIC dependent shifts in pH. Controls were made with the buffer HEPES at a pH of 7.5 and DIC concentrations of 2000 μM and 2200 μM . After preparing the medium and adding the substrates and hydrogen carbonate, a 20 mL subsample was taken from the anoxic medium and its pH adjusted to the desired value by addition of 0.1 M hydrochloric acid. The corresponding amount of 1 M HCl was then calculated and added to the medium, which was then inoculated with the bacteria. At the end of the incubation, the pH was controlled using the same method.

Previous experiments (Grote et al., 2012, Bruckner et al., 2012) had shown that during growth in batch culture and under the conditions applied, “*S. gotlandica*” str. GD1 reaches stationary phase after 10–14 days. Thus, final cell concentrations were determined at 14 days of growth, with cell numbers quantified by DAPI staining.

2.3.2 Estimation of DIC saturation for growth (at constant pH)

“*S. gotlandica*” str. GD1 was grown in batch culture (250 mL cultivation bottles including 50 mL headspace) at bicarbonate concentrations ranging from 20 μM to 2000 μM and at a pH between 7.0 and 7.5. Bicarbonate was not measured directly but instead dissolved to yield a defined concentration and then added to the medium to obtain the desired concentration. Since there is an equilibrium between the CO_2 -compounds in the medium and those in the headspace, it was calculated that a maximum of 1.8 %

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of the DIC compounds were converted into CO₂ gas in the headspace. Bacteria were grown for 14 days, with the final cell number determined as described above.

2.3.3 Effects of different pH values on chemolithoautotrophic growth (at constant DIC concentration)

- 5 To identify the pH range allowing the chemolithoautotrophic growth of "*S. gotlandica*" str. GD1, the bacteria were cultivated in 250 mL cultivation bottles including 50 mL headspace volume within a pH range of 6–9. The pH was set up as described above. Accordingly, a pH range ± 0.05 of the target pH was established. HEPES (10 mM) was used as the buffer based on its optimum buffering capacity between pH 6.8 and 8.0.
- 10 The pH of the bacterial preculture medium was between 7.0 and 7.5. Bacteria were grown in batch culture for 14 days, with the final cell number determined as described above. At the end of the incubation, the pH was controlled using the same methods described above.

2.3.4 Substrate utilization during chemolithoautotrophic growth

- 15 After the pH range suitable for chemolithoautotrophic growth of "*S. gotlandica*" str. GD1 had been determined, substrate utilization was investigated at different pH values within this range. "*S. gotlandica*" str. GD1 was grown under the same conditions as above, but using 600 mL cultivation bottles with 100 mL headspace, and the pH was measured at the beginning and end of the experiments. The experiments were conducted at pH 20 of 7.1 (the pH of the Baltic Sea redox zones), pH 6.9 (the pH expected following ocean acidification), and pH 6.6 (the critical point at which the influence of pH on growth became visible before).

Cell numbers, nitrate and thiosulfate consumption, and sulfate production were quantified daily for 14 days, although substrate utilization per bacterium was calculated only during exponential growth. Nitrate was analyzed in 1 mL samples diluted 1 : 100, and sulfate in undiluted 1 mL samples. Thiosulfate was measured in 25 μ L samples

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centrifuged and diluted 1 : 10 prior to derivatization with 50 µL of Monobromobimane-HEPES-EDTA-buffer. The derivative was diluted again 1 : 10 to obtain a thiosulfate concentration below 20 µM, i.e. within the concentration range yielding the best linear relationship. Sulfate was measured immediately whereas nitrate and thiosulfate samples were stored at –20 °C until analysis. All vials used for the analyses were flushed with N₂ to remove oxygen and to maintain the samples as oxygen-free as possible. Negative controls without bacteria had been previously performed and revealed that purely chemical reactions can be ruled out for changes in substrate concentrations at different pH (Bruckner et al., 2012).

Statistical tests were performed using an ANOVA and an error probability of 5 %.

3 Results

3.1 Growth of “*Sulfurimonas gotlandica*” strain GD1 at different DIC concentrations and pH values

The results of this experiment (Fig. 1) showed that the bacterium grew well at the different DIC concentrations of 2000 µM and 2200 µM ($2.57 \times 10^7 \pm 4.62 \times 10^6$ cells mL^{–1}). At lower pH of ~6.8 a decrease of growth was observed and “*S. gotlandica*” str. GD1 achieved only about a three times lower maximal cell number ($9.33 \times 10^6 \pm 1.8 \times 10^6$ cells mL^{–1}) compared to the higher pH values (Fig. 1). During the incubation time the pH decreased by 0.45 ± 0.1 units. In the controls, where pH was adjusted at 7.5 and buffered with HEPES, the same cell number could be achieved ($2.33 \times 10^7 \pm 1.76 \times 10^6$ cells mL^{–1}) as in the experiment with a pH of about 7.1 at the beginning. Accordingly, a decline of pH by 0.45 during the batch culture should have had no influence on growth when the starting pH was 7.1. Based on these results we investigated the two aspects of ocean acidification, increasing DIC concentration and decreasing pH, separately.

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3.2 Estimation of DIC saturation for growth (at constant pH)

“*S. gotlandica*” str. GD1 grew well at a large range of different DIC conditions. Final cell densities increased with the supplied DIC until a saturation level was reached (Fig. 2). Maximal cell abundances of $\sim 3 \times 10^7$ cells mL^{-1} were achieved at a DIC concentration of 800 μM . The calculated half saturation concentration was 132.6 μM , with a threshold concentration of 87.5 μM .

3.3 Effects of different pH values on chemolithoautotrophic growth (at constant DIC concentration)

The optimum pH yielding maximal cell numbers of “*S. gotlandica*” str. GD1 was in the range 6.6–8.0, with no significant differences in growth (ANOVA, $p > 0.05$) (Fig. 3). At pH values above 8.0 and below 6.5 no significant bacterial growth was observed and cell numbers remained around the initial levels. Between pH 6.5 and 6.6, bacterial cell numbers increased only slightly ($3.3 \times 10^6 \pm 1.5 \times 10^6$ cells mL^{-1}). At the end of the experiment the pH was measured again. The pH between 6.5 and 8.4 remained constant (± 0.02) during the experiment, whereas above and below these points the pH decreased by about 0.18–0.25.

3.4 Substrate utilization during chemolithoautotrophic growth

For selected pH values within the pH optimum and at the lower pH limit determined above, substrate utilization paralleled the growth of “*S. gotlandica*” str. GD1. The chosen pH values were pH 7.1 (present pH in Baltic Sea redox zones), pH 6.9 (predicted due to ocean acidification), and pH 6.6 (the critical point, at which an influence of pH on cell growth was previously demonstrated). Cell growth, substrate utilization, and cell production by “*S. gotlandica*” str. GD1 showed no significant differences between the three pH values (ANOVA, $p > 0.05$) (Fig. 4) and in all trials a cell abundance of about $1.5 \times 10^7 \pm 7.8 \times 10^5$ cells mL^{-1} was reached in 9 days.

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“*S. gotlandica*” str. GD1 completely consumed 1 mM thiosulfate, transforming most of it to sulfate within 9 days ($1366.9 \pm 109.5 \mu\text{M}$) whereas the same concentration of nitrate was only partially consumed, as after 9 days $127.7 \pm 37.8 \mu\text{M}$ of nitrate was still detected. As shown in Fig. 3, growth was strongly reduced at pH values below 6.6., 5 resulting in maximal cell abundance of $3.8 \times 10^6 \pm 4.1 \times 10^5 \text{ cells ml}^{-1}$ on day 9 (Fig. 4d). Correspondingly, the bacteria used $742.5 \pm 391.8 \mu\text{M}$ of nitrate and $824.3 \pm 144.0 \mu\text{M}$ of thiosulfate and produced $903.8 \pm 373.0 \mu\text{M}$ sulfate.

The exponential phase of bacterial growth was identified by following the time-dependent development of cell numbers (Fig. 4). Exponential growth occurred between days 6 and 9, during which “*S. gotlandica*” str. GD1 used $68.4 \pm 9.8 \text{ fmol nitrate cell}^{-1}$ and $49.8 \pm 12.2 \text{ fmol thiosulfate cell}^{-1}$ while producing $109.8 \pm 25.4 \text{ fmol sulfate cell}^{-1}$ (Table 1). The generation time was $14.1 \pm 0.16 \text{ h}$. At pH 6.5, bacterial growth was somewhat slower, with a generation time of $15.7 \pm 0.08 \text{ h}$ but with strongly enhanced substrate turnover. In fact, the cells used six times more nitrate, five times more thiosulfate, and produced eight times more sulfate per bacterium 15 than cultures maintained at a higher pH (Table 1). Chemical reactions of the substrates caused by different pH values can be ruled out. The measurements of nitrate and thiosulfate at the beginning and after 24 h confirmed the added concentration of 1 mM of the substrates.

20 4 Discussion

The primary aim of this study was to examine the response of the ε -proteobacterium “*Sulfurimonas gotlandica*” str. GD1 towards the predicted changes for DIC and pH as a consequence of incremental ocean acidification. Thus, we firstly examined both aspects together and then secondly we investigated both factors, DIC concentration and pH, separately, by keeping one of them constant. This was achieved sufficiently well 25 with our experimental design to achieve clear results with regard to the influence of these factors on growth of “*S. gotlandica*” str. GD1. Other studies have shown a rapid

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4.1 Estimation of DIC saturation for growth (at constant pH)

For phytoplankton, the growth-stimulating effect of increased DIC concentrations is well known (Iglesias-Rodriguez et al., 2008) whereas for chemolithoautotrophic bacteria only few data exist. According to our results, DIC concentrations of about 2 mM and 5 3.5 mM (Beldowski et al., 2010; Frey et al., 1991), present at the redox zones in the Baltic Sea and the Black Sea, respectively, are well within the broad saturation range promoting the growth of “*S. gotlandica*” str. GD1, and any additional increase in DIC would have no further effect. The balance between carbon dioxide, hydrogen carbonate, and carbonate is pH-dependent such that at pH 7.1 88 % of the DIC speciation is 10 hydrogen carbonate while at pH 6.3 the balance shifts to 50 % carbon dioxide and 50 % hydrogen carbonate (Deffeyes, 1965). However, this shift in speciation should not influence growth since a DIC concentration of 800 μ M proved to be already sufficient to support maximum growth (Fig. 2). Comparable saturation curves for increasing DIC concentrations also have been determined for other bacterial and phytoplankton species. 15 Dobrinski et al. (2005) showed that for the chemolithoautotrophic γ -proteobacterium *Thiomicrospira crunogena*, isolated from a hydrothermal vent, the half-saturation DIC concentration is 220 μ M. In that bacterial species saturation was reached at 1 mM hydrogen carbonate, which is comparable to the values estimated for “*S. gotlandica*” str. GD1. Thus, a growth-stimulating effect on chemoautotrophic bacteria is unlikely even 20 if the predicted increase in DIC at the surface extends to include the deeper hypoxic water layers.

4.2 Effects of different pH values on chemolithoautotrophic growth (at constant DIC concentration)

The pH range at which “*S. gotlandica*” str. GD1 grew well (pH 6.6–8.0) was relatively narrow compared to that of other chemolithoautotrophic γ - and ε -proteobacteria. 25 Brinkhoff et al. (1999) described several γ -proteobacterial *Thiomicrospira* species from hydrothermal vents that grew at a pH range of 5.3–8.5 or 4.0–7.5. In other

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5 Conclusions

The predicted reduction in the pH of ocean surface waters by about 0.3 units by the year 2100 (Caldeira and Wickett, 2003) will probably reach the water layers of the redox zones in a somewhat milder and delayed form (Karstensen et al., 2008). It is

5 also possible that changes in alkalinity will counteract a further decline in pH, thereby retarding any effects on the microbial communities in these hypoxic zones. Therefore, even assuming a similar decline in pH in deeper waters as at the ocean surface, our results suggest that the direct impact on “*S. gotlandica*” str. GD1 in Baltic Sea redox zones should not be very strong.

10 However, the effects of ocean acidification are transferred more rapidly in relatively shallow hypoxic coastal areas such as lagoons and fjords (Melzner et al., 2012). Chemolithoautotrophic denitrification has been also demonstrated in those habitats when a hydrogen sulfide-nitrate interface occurs (Jensen et al., 2009) and most probably ε -proteobacteria, related to “*S. gotlandica*” str. GD1 are responsible as well. It 15 remains to be investigated how the more extreme changes in pH and DIC affect the performance of chemolithoautotrophic bacteria in those zones.

As demonstrated in previous work, this bacterium is a model organism for chemolithoautotrophic ε -proteobacteria (Grote et al., 2012; Bruckner et al., 2012). Thus, our conclusion that “*S. gotlandica*” str. GD1 will likely tolerate ocean acidification 20 can probably be extrapolated to other chemolithoautotrophic ε -proteobacteria of pelagic redox zones. Caldeira and Wickett (2003) suggested that if the actions of humans either fail to decrease or even cause a further increase of CO₂ emissions, the pH at the surface of the oceans will decrease by about 0.77 units by the year 2300. Since the effects of pH on the growth of “*S. gotlandica*” str. GD1 are dramatic within 25 a rather small decrease in pH (between 6.6 and 6.5), pH might have an effect on the competitive ability of this strain.

Previous reports concluded that ocean acidification will have no direct influence on denitrifying bacteria, although a potential indirect influence was noted (Hutchins et al.,

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2009; Schmittner et al., 2008). Ocean acidification could induce a more efficient biological carbon pump, and thus an expansion of suboxic zones, which in turn would increase denitrification rates (Riesebell et al., 2007; Conley et al., 2009). Indirect effects on “*S. gotlandica*” str. GD1 might be more important than direct ones; for example, 5 there is evidence that nitrification is negatively influenced by both a decrease in pH and an increase in the $p\text{CO}_2$ (Huesemann et al., 2002; Denecke and Liebig, 2003). Thus, a reduction in nitrification will lower the availability of nitrate, which in denitrifying bacteria serves as electron acceptor (Hutchins et al., 2009). It suggests that ocean acidification is likely to have more indirect rather than direct effects on “*S. gotlandica*” 10 str. GD1 living in deeper redox zones.

Acknowledgements. We are thankful for a grant from the German Federal Ministry of Education and Research (BMBF), joint research project BIOACID, subproject 1.1.1. We also would like to thank Bernd Schneider for his help and advices with the experimental design.

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Table 1. Substrate usage and production per formed cell of “*Sulfurimonas gotlandic*” str. GD1 during exponential growth (day 6–9), as determined at pH 7.1, 6.9, 6.6, and 6.55. Data for pH 7.1, pH 6.9, and pH 6.6 and 6.55 are the means of three, four, two, and two replicates, respectively.

Substrate usage and production (fmol cell ⁻¹)	pH 7.1	pH 6.9	pH 6.6	pH 6.55
Nitrate	57.6 ± 19.5	76.4 ± 15.6	71.3 ± 3.3	414 ± 151
Thiosulfate	36.7 ± 6.3	51.9 ± 16.2	60.9 ± 6.8	247 ± 209
Sulfate	82.6 ± 45.0	132.9 ± 29.9	113.9 ± 5.0	944 ± 563

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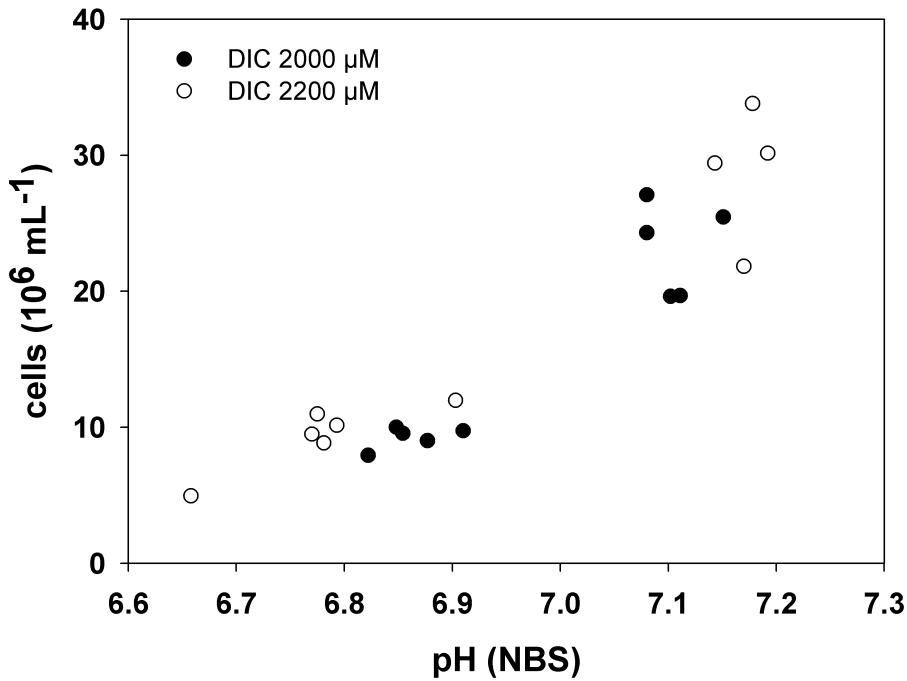


Fig. 1. Cell growth of “*Sulfuriomas gotlandica*” strain GD1 at two different dissolved inorganic carbon (DIC) concentrations (2000 μM and 2200 μM) and at different pH values (here the pH values at the beginning of the experiment are shown). The replicates are shown separately. The bacterium was grown for 14 days in batch culture. The pH declined by 0.45 ± 0.1 units during the incubation time.

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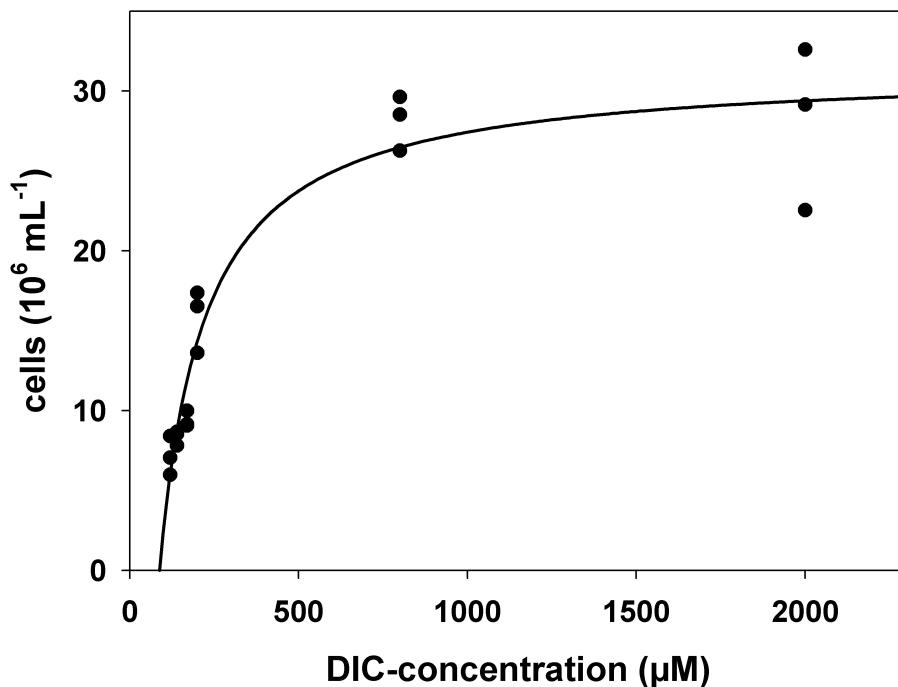


Fig. 2. Cell growth of “*Sulfuriomas gotlandica*” strain GD1 under different DIC conditions. The three replicates at each DIC concentration are shown separately. The bacterium was grown for 14 days in batch culture. Data are shown as a rectangular curve ($r^2 = 0.96$), corresponding to a half-saturation concentration of 132.6 μM and a threshold concentration of 87.5 μM DIC.

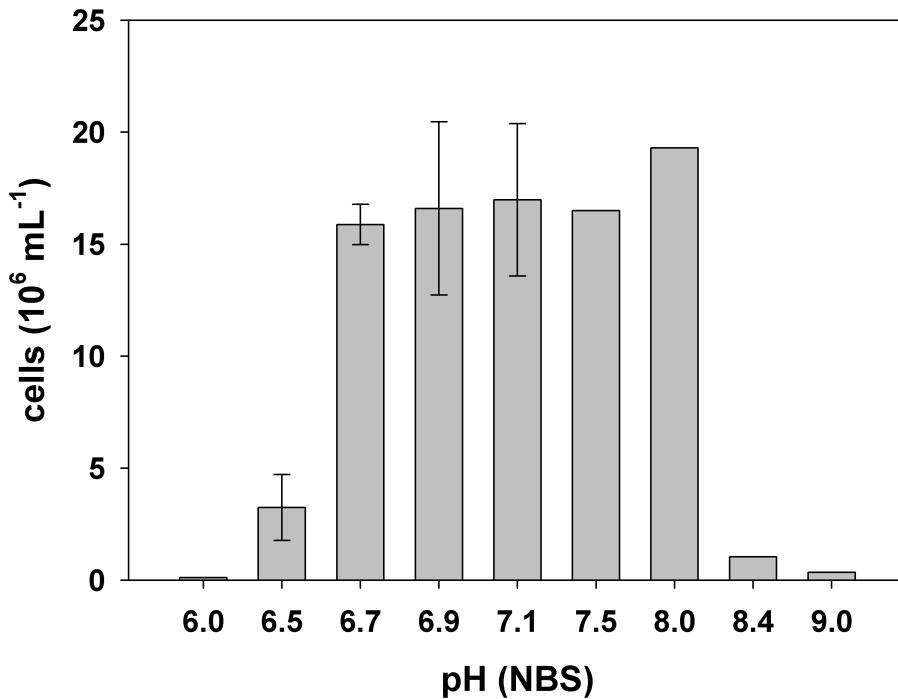


Fig. 3. Influence of pH on the growth of “*Sulfurimonas gotlandica*” str. GD1. The bacterium was grown in batch culture at different pH values for 14 days. Values between pH 6.5 and 7.1 are the means (\pm sd) of three replicates. Values below and above this pH range are single data.

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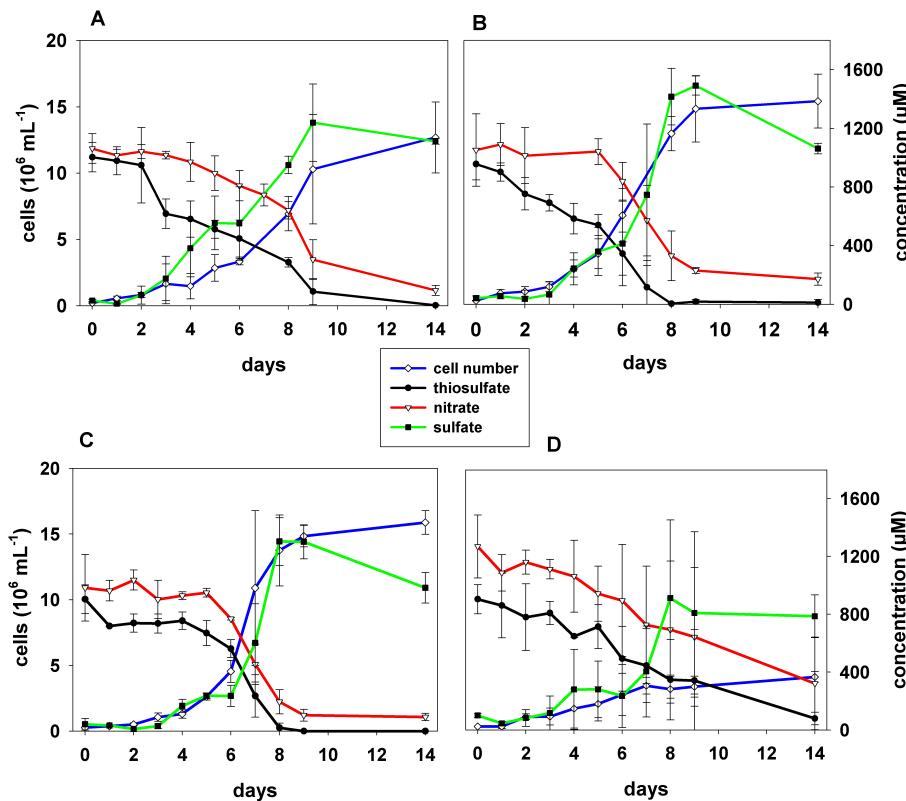


Fig. 4. Anaerobic chemoautotrophic growth of *"Sulfurimonas gotlandica"* str. GD1 in batch culture at pH 7.1 (A), pH 6.9 (B), pH 6.6 (C), and pH 6.55 (D). Cell growth and nitrate (electron acceptor), thiosulfate (electron donor), and sulfate (formed by thiosulfate oxidation) concentrations were quantified daily. (A), (B), (C), and (D) are the means of three, four, two, and two replicates, respectively. Error bars are standard deviations.