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Marine ammonification and carbonic anhydrase activity induce rapid calcium carbonate precipitation

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ABSTRACT

During Earth’s history, precipitation of calcium carbonate by heterotrophic microbes has substantially contributed to the genesis of copious amounts of carbonate sediment and its subsequent lithification. Previous work identified the microbial sulfur and nitrogen cycle as principal pathways involved in the formation of marine calcium carbonate deposits. While substantial knowledge exists for the importance of the sulfur cycle, specifically sulfate reduction, with regard to carbonate formation, information about carbonate genesis connected to the microbial nitrogen cycle is dissatisfactory. In addition to the established pathways for carbonate mineral formation, also the potential of microbial carbonic anhydrase, a carbonate-relevant, zinc-containing enzyme, is receiving currently increased attention. However, also in this field knowledge is scarce and fragmentary. Here we demonstrate microbial carbonate precipitation as a direct result of the interplay between the microbial nitrogen cycle and a microbially produced enzyme. Using Alcanivorax borkumensis as a model organism, our experiments depict precipitation of a peloidal carbonate matrix within days to weeks, induced by simultaneous ammonification and extracellular carbonic anhydrase activity. The precipitates show similar morphology, mineralogy, δ¹⁸⁴/⁴⁰Ca, and δ⁸⁸/⁸⁶Sr to analogs of modern carbonate peloids. The obtained Sr/Ca partition coefficient D_{Sr} showed no clear
deviation from inorganic carbonate phases, indicating that microbially mediated carbonate precipitation, indeed, follows the principles of physico-chemical precipitation. The observed relative enrichment of the precipitates in zinc might help to constrain zinc variations in natural carbonate archives. Our study demonstrates that ammonification, due to intense microbial organic matter degradation, and carbonic anhydrase may play a substantial role for calcium carbonate precipitation in paleo- and recent shallow marine environments.

1. INTRODUCTION

The formation and burial of carbonate-bearing rocks is by far the most important mechanism for carbon removal and storage on Earth (Sun and Turchyn, 2014). Carbonate deposits account for about one-sixth of the global sedimentary rocks (Wedepohl, 1995), representing a major fraction of the global carbon storage. A significant fraction of this carbonate is of microbial origin (Gadd, 2010). To date, a number of microbial metabolic processes, such as photosynthesis, and redox reactions using nitrogen (Castanier et al., 1999) and sulfur (Sun and Turchyn, 2014) compounds, have been identified as potentially controlling the formation of microbial carbonate minerals. Microbial sulfate reduction is suspected to be largely responsible for the formation of authigenic carbonate minerals in marine sediments (Braissant et al., 2007) and stromatolites (Visscher et al., 2000), acting as an alkalinity driver (Gallagher et al., 2012). The production of carbonate minerals due to sulfate reduction is dependent on the fate of the produced hydrogen sulfide, acting as a kinetic inhibitor for the sulfate reduction in case of excess concentrations in the vicinity of the cell (Castanier et al., 2000). Under diffusive conditions, representing the vast majority of the global marine sediments, the equilibrium pH resulting from sulfate reduction ranges between 6.5-7, rather hampering carbonate mineral precipitation (Meister, 2013). Another factor significantly impacting sulfate reduction influence on the carbonate system is the iron cycle, as hydrogen sulfide readily reacts with Fe$^{2+}$ ions producing FeS$_2$.

$$5H^+ + 2Fe(OH)_3 \rightarrow 2Fe^{2+} + S + 6H_2O$$ \hspace{1cm} \text{(Reaction 1)}

$$Fe^{2+} + HS^- \rightarrow FeS_2 + H^+$$ \hspace{1cm} \text{(Reaction 2)}

This reaction prevents excessive build up of hydrogen sulfide lowering the kinetic barrier for microbial sulfate reduction. However, as ferric iron (Fe$^{3+}$) may be not be
reduced fast enough to maintain equilibrium conditions, the availability of Fe\(^{2+}\) might represent the kinetic bottleneck controlling iron sulfide and carbonate formation (Coleman, 1985). Therefore, the question arises, whether contributing processes to sulfate reduction can be accounted for additional authigenic carbonate precipitation in marine sediments.

One of the most important of these processes is the microbial remineralization of organic nitrogen (ammonification) (Castanier et al., 1999). While plausible theoretical concepts (Castanier et al., 1999; Riding, 2002; Zhu and Dittrich, 2016) and numerical modeling approaches (Krumins et al., 2013) exist, to date, experimental evidence for ammonification-driven carbonate precipitation is scarce (Berner, 1968). Organic nitrogen remineralization is an essential global process, occurring in the terrestrial, freshwater and marine realms (Vitousek et al., 1997). Microbial communities in shallow marine coastal sediments play a key role in driving the oceanic nitrogen cycle (Herbert, 1999). For nitrogen remineralization amino acids undergo microbial deamination, a fundamental process termed ammonification (Sylvia et al., 2005) (Reaction 3). The metabolic product of this process is ammonia (NH\(_3\)) (Gruber, 2008; Sylvia et al., 2005). When dissolved in seawater about 92% of NH\(_3\) becomes protonated forming ammonium (NH\(_4^+\)) and hydroxide (OH\(^-\)) (Johansson and Wedborg, 1980), increasing the alkalinity of the liquid phase.

\[
\text{Ammonification: org.N} \rightarrow \text{NH}_3 + H_2O \rightarrow NH_4^+ + OH^- \uparrow \text{Alkalinity} \quad \text{(Reaction 3)}
\]

As a consequence the carbonate system is shifted towards CO\(_3^{2-}\) (Zeebe and Wolf-Gladrow, 2001), facilitating carbonate mineral precipitation when supersaturation is reached.

Current research activities indicate that, in addition to metabolic processes, also extracellular enzymes are involved in the formation of carbonate precipitates under natural conditions (Li et al., 2014; Li et al., 2013). In this context, one of the key enzymes is carbonic anhydrase (CA) (Li et al., 2010; Power et al., 2016), which catalyzes the interconversion of CO\(_2\) to HCO\(_3^-\) and H\(^+\) (Reaction 4). The active site of this enzyme contains a zinc ion (Maren, 1967).

\[
\text{Carbonic anhydrase activity} \quad CO_2 + H_2O \xrightleftharpoons[C{\text{A}}]{CA} HCO_3^- + H^+ \quad \text{(Reaction 4)}
\]
CAs are ubiquitously present in pro- and eukaryotic organisms, participating in numerous physiological processes (Achal and Pan, 2011). The activity of CA largely governs the concentrations of intra- or extracellular CO2 and HCO3− (Nimer et al., 1994). Although the overall dissolved inorganic carbon (DIC) concentration is not changed, unless CO2 escapes from the liquid phase, the concentration of HCO3− is directly dependent on the CA activity (Achal and Pan, 2011). The production of CO3 2− from HCO3−, a pre-requisite for the precipitation of CaCO3, is directly dependent on pH. An increase in CO3 2− concentration takes place under alkaline conditions (Zeebe and Wolf-Gladrow, 2001), as provided by ammonification (Reaction 5).

\[
\text{HCO}_3^- + \text{NH}_3 \leftrightarrow \text{CO}_3^{2-} + \text{NH}_4^+ \quad \text{(Reaction 5)}
\]

The key objects of the present study included the evaluation of microbial ammonification as a principal driver for marine carbonate mineral precipitation in an experimental approach under laboratory conditions. In addition, the role of carbonic anhydrase within a biofilm with regard to carbonate nucleation was studied by imaging of the spatial distribution of the enzyme and conducting carbonic anhydrase inhibition experiments. In order to constrain the mode of mineral precipitation and crystal growth a combination of microscopy imaging technologies was used. Mineral properties, including carbonate phase identification, as well as element and isotope geochemistry was obtained using a combination of X-ray diffraction, ion concentration measurements by inductively coupled plasma optical emission spectrometry, and thermal ionization mass spectrometer.

We demonstrate, to our knowledge for the first time, rapid carbonate precipitation as a result of combined remineralization of organic nitrogen (ammonification) and extracellular activity of the enzyme carbonic anhydrase (CA), using the benthic marine aerobic heterotrophic γ-proteobacterium *Alcanivorax borkumensis* SK2 (Yakimov et al., 1988) as a model organism. The combined processes concurrently increased the ambient alkalinity, pH, and the concentration of CO3 2−, inducing supersaturation for calcium carbonate and subsequent carbonate precipitation.

2. MATERIAL AND METHODS

2.2 Bacteria culturing and media composition
For all laboratory experiments the bacteria strain *Alcanivorax borkumensis* SK2 was used (Yakimov et al., 1988). The strain was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, DSMZ No. 11573, Type strain). Natural North Sea water was used as the basis of maintenance and experimental growth media. After UV-light and filter sterilization (Whatman Polycap 75 AS-GMF/Nylon 0.2 µm) the seawater was amended with peptone (5 g l⁻¹), yeast extract (1 g l⁻¹), Fe(III)-citrate (0.1 g l⁻¹), and Na-pyruvate (10 g l⁻¹). Agar medium was prepared by addition of 15 g l⁻¹ agar. Medium pH (total scale) was adjusted to 7.6 with 10 molar NaOH (sodium hydroxide).

Subsequently, the medium was autoclaved for 20 min at 121°C. All incubations involving liquid medium were carried out in sterilized Duran® glass bottles, closed with a sterilized cotton plug. Agar cultures were grown in sterile Petri dishes at 20°C in the dark. Liquid cultures were kept on a rotary shaker at 60 rpm in the dark. The starting carbonate alkalinity was 2.97 mEq kg⁻¹, calculated from the initial concentration of DIC and pH (Zeebe and Wolf-Gladrow, 2001). The corresponding saturation state (Ω) for aragonite was 2.2. Under these conditions abiotic precipitation of calcium carbonate was not observed.

### 2.3 Bacteria preparation for incubation

For incubations of *A. borkumensis* with agar or liquid medium, portions of 5 ml cell suspension from running liquid cultures were transferred into sterile 15 ml centrifuge tubes, vortexed, and centrifuged at 30 x g for 5 min at 10°C to remove any present carbonate crystals. The resulting bacteria-containing supernatant was transferred into new into sterile 15 ml centrifuge tubes and centrifuged again under the same conditions. Subsequent light microscopy checks confirmed the complete absence of crystals. For cell density determination, 50 µl of the centrifuged cell suspension were transferred to 5 ml of phosphate buffered saline (PBS), vortexed and filtered onto a polycarbonate membrane filter (Whatman Nuclepore Track-Etch Membrane, 25 mm, 0.2 µm), coated with warm agarose solution and stained with 4′,6-diamidin-2-phenylindol (DAPI) for 10 min in the dark. Cell enumeration was carried out microscopically using a DAPI filter set.

### 2.4 Agar incubation for crystal growth, morphology, and mineralogy analysis

For microscopic analyses of crystal growth and morphology agar cultures were grown directly on sterile microscopy glass slides. The glass slides were immersed into the hot
liquid agar medium, and then transferred rapidly into sterile Petri dishes and immediately sealed. After 3 days of drying, the agar coated glass slides were inspected for growth of unwanted microbes before further use. On each slide 50 µl of cell suspension with approximately 1x10^5 cells ml⁻¹ was distributed. Three replicates of inoculated agar-slides were investigated microscopically after 1, 3, 7, and 21 days. Imaging was carried out using white-field and polarized-light microscopy. For carbonate mineral analysis agar medium plates were prepared in sterile Petri dishes. After cooling plates were inoculated with 100 µl cell suspension. After 10 days the A. borkumensis biofilm was scraped off the agar and transferred into 15 ml centrifuge tubes with 5 ml sterile liquid medium. The separation procedure of cells and crystals was carried out as mentioned above. The yielded crystals were washed twice in ethanol (96%), dried at 20°C, and powdered for subsequent X-ray diffraction analysis. The spectra analysis was carried out with the XPowder software, version 2010.01.34 PRO.

2.5 Agar incubations for visualization of carbonic anhydrase
The sulfonamide DNSA (5-(dimethylamino)-1-naphthalenesulfonamide) was used for imaging the enzyme carbonic anhydrase in A. borkumensis biofilms. A 10 mM stock solution of DNSA in DMF (dimethylformamide) was produced (Estes et al., 2003). A 0.2 M Sorensen’s phosphate buffer was used for stock dilution to working concentrations. Herefore, two solutions were produced: Solution A: 35.61 gl⁻¹ Na₂HPO₄•2H₂O, solution B: 31.2 gl⁻¹ NaH₂PO₄•2H₂O. After combining 40.5 ml of solution A and 9.5 ml of solution B the pH was adjusted to pH 7.5 with 1 M NaOH. Carbonic anhydrase staining was carried out with a 500 µM DNSA working solution. Crystal-free inoculates of A. borkumensis were grown for two days on agar medium-coated glass slides before 100 µl of DNSA working solution was applied to the biofilm. After staining for 20 minutes in the dark the biofilm was sealed with a cover slip. After agar medium from the backside of the glass slide was removed with ethanol (96%) the sample was imaged with epi-fluorescence (DAPI filter set) and polarized-light microscopy. As unbound DNSA does not emit fluorescence sample washing after staining was unnecessary.

2.6 Inhibition of carbonic anhydrase activity
The two sulfonamides acetozolamide (AZ) and ethoxzolamide (EZ) were used for carbonic anhydrase inhibition (Maren and Sanyal, 1983). Stock solutions of 625 mM
(AZ) and 1250 mM (EZ) in DMF were produced. Seawater medium (see above) was used for stock dilution to working concentrations of 0, 10, 50, and 100 µM. To allow for spatial comparison of crystal abundance all inhibition experiments were carried out in a defined area. Herefore, sterile polyethylene rings, manufactures from slip lids, were used. The rings had an inner diameter of 10 mm and a wall height of approximately 6 mm. For the inhibition experiments the rings were placed on freshly prepared agar-coated microscopy slides. Into each ring 50 µl of running A. borkumensis culture was given and left to settle for 5 min. Subsequently, 500 µl of sterilized seawater including different concentrations of AZ and EZ was added. The prepared slides were placed into sterile petri dishes and incubated at 20°C in the dark for three days. Subsequently, the rings were removed and excess liquid was allowed to run off. Then, a drop of glycerin was applied on the location where the ring was situated and a cover slip was mounted. Crystal enumeration was carried out using polarized light microscopy counting 70 views of 96100 µm² each, for each treatment. For data fitting and statistics the non-linear model of the R software program was used (Team, 2016).

2.7 Liquid medium incubations for monitoring of chemical changes in the liquid phase
In order to constrain the change of the chemical parameters relevant for calcium carbonate precipitation, a batch experiment was conducted over 17 days. In total, three replicates and three controls were prepared in 0.5 l Duran ® glass bottles. Each bottle contained 200 ml of liquid sterilized medium. The openings were closed with sterilized cotton plugs and aluminum foil. A sterile needle (0.9 x 150 mm), closed with a sterile syringe filter (Whatman Filter device Puradisc 25AS, 0.2µm) was inserted into the volume of each bottle for the duration of the experiment to allow for easy and sterile repetitive liquid sampling. For sampling the filter was removed shortly. Each replicate was inoculated with 1 ml of crystal-free A. borkumensis suspension (2x10⁷ cells ml⁻¹) and grown for 14 days on a rotary shaker (60 RPM) at 20 °C in the dark. Samples were taken at nine time points until the cell concentration reached the stationary phase after 17 days. For each sampling 3.5 ml liquid were extracted from each bottle. The pH was measured using a WTW pH 3210 pH-meter with a WTW SenTix RJD electrode. For precise pH- electrode calibration for seawater matrix the BIS- and AMPHY buffer system was used (Dickson, 1993). Subsequently, 100 µl of solution were transferred into 100 µl 4% formalin for bacteria enumeration. The rest of the liquid was
centrifuged at 4500 x g for 10 minutes at 10°C and subdivided for different analytical procedures. Another 100 µl of the supernatant was used for total alkalinity measurement by direct titration with 0.01 N HCl in an open cell using Bruevich’s method (Ivanenkov and Lyakhin, 1978). Calibration was carried out with standard seawater (IAPSO). The titration method has a precision of 0.4%. Concentrations were expressed in mEq l⁻¹.

For DIC measurements 1 ml of supernatant from each sample was transferred into a 10 ml serum vial containing 100 µl of 6 molar HCl in a CO₂-free N₂ atmosphere. After vigorous shaking, 100 µl of the headspace were extracted with a gas-tight syringe and CO₂ concentration was measured by gas chromatography (Shimadzu GC-2014AF) after conversion of CO₂ to CH₄ in a methanizer.

The ammonium concentrations were measured photometrically (Grasshoff et al., 2009) using 1 ml of supernatant. For ion concentration measurements by inductively coupled plasma optical emission spectrometry (ICP-OES) 1 ml of supernatant was acidified with HNO₃ (65%, ultra pure). The saturation state (Ω) for aragonite was calculated from pH and DIC values (Zeebe and Wolf-Gladrow, 2001). Precipitates were scraped off the inner glass walls and transferred into 15 ml centrifuge tubes with 5 ml of sterile medium. The separation of crystals and remaining cells was carried out as described above. Sterile controls remained bacteria-free during the course of the experiment and showed no abiotic precipitation of carbonate crystals.

### 2.8 Liquid medium incubations for monitoring of $^{44}$Ca/$^{40}$Ca, $^{88}$Sr/$^{86}$Sr, and trace element ratio development

A second batch experiment was conducted with *A. borkumensis* in liquid medium over 14 days under the same conditions as for the monitoring of the chemical changes, to analyze element as well as calcium (Ca²⁺) and strontium (Sr²⁺) isotope fractionation caused by carbonate precipitation. This time, a total volume of 500 ml of medium in a 1 L Duran® glass bottle was inoculated. The determination of main and trace element concentrations was carried out using (ICP-OES). At experiment start, 15 ml medium were obtained for Sr and Ca isotope measurements. At experiment termination medium liquid was collected in 50 ml acid-washed centrifuge tubes and centrifuged at 30x g for 3 min at 4 °C to separate crystals from organic components. The resulting supernatant, was transferred into new tubes and centrifuged at 4,000x g for 10 min at 4°C. From the clear supernatant 15 ml were transferred into a Teflon beaker for Sr and Ca isotope
measurements. The remaining pellet was washed three times in ultra-purified water with a pH of 8-9 (adjusted with NH₄⁺). The supernatants of the washing steps were transferred separately into 50 ml acid washed centrifuge vials. After supernatant removal the remaining organic pellet was transferred into a Teflon beaker and dried at 60°C for 12 hours. Crystals precipitated on the inner walls of the glass bottle were scraped off using a plastic rod and transferred into a 50 ml centrifuge tube. All crystals yielded, either by separation from organics or by scraping off the walls, were washed by centrifuging three times in ultra-purified water with a pH of 8-9 (adjusted with NH₄⁺) and subsequently dried at 38°C for 24 h. The liquids from the three washing steps were saved and also measured for calcium and strontium isotope composition. From the medium liquids, aliquots of 5 ml were transferred into Teflon beakers and dried at 95°C for 24 h. Subsequently, the samples were resuspended in 1 ml HNO₃ (8N) and 20 µl of HClO₄ and left to react for 3 hours at 95°C. Sample-free procedure blanks were also produced. After drying at 180°C, 1 ml of 8N HNO₃ and 0.5 ml H₂O₂ was added and left to react at 80°C for 5 h. Subsequently, samples were dried at 80°C. The carbonate crystals were dissolved in 2.25 N HNO₃ and dried at 80°C for 12 h. All samples were then re-dissolved in 2% HNO₃ for element determination (Mg, Ca, Sr, and trace metals) by quadrupole inductively coupled plasma mass spectrometry (Agilent 7500cx, no-gas mode). Aliquots of up to 3000 ng Ca equivalent were taken for Ca-isotope preparation and mixed with a ⁴³Ca/⁴⁸Ca double spike. Spiked samples were dried at 95°C, resuspended in 2.2 N HNO₃, and Ca was separated using 600 µl columns (biorad) with MCI-Gel 75-150 µm. The calcium isotope ratios of carbonate crystals, initial and final medium, and organic component, as well as from the three crystal washing steps were measured with a Finnigan Triton TI thermal ionization mass spectrometer (TIMS) at GEOMAR, Kiel following the method described before (Krause et al., 2012). Prior to filament loading samples were dried down and transferred into chloride form by evaporation in 2.2 N HCl. After uptake with loading solution (H₃PO₄), aliquots of about 300 ng were loaded with TaCl₅ activator in a sandwich technique on a zone-refined Re filament. The calcium isotope (⁴⁴Ca/⁴₀Ca) values are expressed relative to NIST SRM 915a using the common delta notation and expressed as δ⁴⁴/⁴₀Ca. For Sr isotope analysis two fractions from the dissolved sample (2% HNO₃), each containing 1000–1500 ng Sr, were obtained for isotope composition (IC) and isotope dilution (ID) analyses (Krabbenhöft et al., 2009). A ⁸⁷Sr/⁸⁴Sr double spike was added to the ID fraction and both fractions were then dried at 90°C separately. Chromatographic
column separation was performed using 650ll BIO-RAD columns filled to one-third with Eichrom Sr-SPS resin (grain size 50–100 µm). The resin was washed three times with 4.5 ml H₂O and 4.5 ml 8N HNO₃. The resin was then conditioned three times with 1 ml 8N HNO₃. Sample were dissolved in 1 ml 8N HNO₃ and loaded onto the column. In order to remove the sample matrix the resin was washed with 6 ml of 8N HNO₃. The Sr-fraction was eluted into a Teflon beaker in three steps with 3 ml H₂O. Resin residues were removed by drying down and then heating samples to 80°C in 50 µl H₂O₂ and 200 µl 2N HNO₃ for at least 5h in closed beakers. After evaporation to dryness sample were re-dissolved in H₃PO₄. Sr isotope analysis was performed using TIMS measurement procedures, described before (Krabbenhöft et al., 2009). Briefly, the samples were loaded on common rhenium single filaments in combination with a TaCl₅-activator. As for calcium, strontium isotope measurements were carried out on a Finnigan Triton TI. Sr isotope results ([⁸⁸Sr/⁸⁶Sr]) are reported relative to NIST SRM 987 using the common delta notation and expressed as δ⁸⁸/⁸⁶Sr. Whole procedure Ca and Sr blanks were determined to be contributing less than 6% and 0.06%, respectively, to the chemically separated material.

External precision is expressed as two times the standard error of the mean (2SEM = 2σ/n⁰.⁵). The δ⁴⁴/⁴⁰Ca values were calculated with the session mean of the NIST-SRM915a standard. External reproducibility of the NIST-SRM915a was ± 0.22‰ (2SD, n = 6) during the measurement period. The external reproducibility of δ⁸⁸/⁸⁶ Sr values was determined by repeated measurements of the JCp-1 standard. Average precision of the JCp-1 standard was 0.056‰ (2SD, n=4) during the measurement period. Calcium and strontium isotope fractionation was corrected for Rayleigh fractionation was calculated using the following equation (Griffith et al., 2008):

$$\alpha = \ln (\Delta_{\text{meas}}) * \frac{f - 1}{1000} + f / \ln (f)$$

where Δ_{meas} is the measured difference between the isotope ratio of the precipitate and the reservoir at t=0. The fraction of calcium or strontium remaining in the liquid phase is denoted by f, while α represents the true fractionation factor.

2.9 Normalized precipitation rate and Sr/Ca partition coefficient

The specific surface area of the crystals was calculated from light microscopy measurements of 100 crystal diameters. As all late stage crystals had a spherical shape the volume was simply calculated from the average diameter. The normalized
precipitation rate \( R \) was calculated using the specific surface area and the duration of maximum calcium decrease during the incubation experiment. The partition coefficient \( D_{\text{Sr}} \) \( \frac{\text{Sr/Ca}_{\text{carbonate}}}{\text{Sr/Ca}_{\text{aquatic phase}}} \) was also corrected for Rayleigh fractionation according to the equation above.

2.10 Light-, polarized light-, and epifluorescence microscopy
For light-, polarized light-, and epifluorescence microscopy analysis and imaging a Zeiss AxiolImager.M2, a HXP 120 V (D) lamp, and an AxioCam MRm Rev.3 monochrome camera were used. For epifluorescence imaging an AHF F46-000 DAPI ET filter set was used. Images were obtained with the ZEN pro 2012 (Blue edition) software.

2.11 Scanning electron microscopy (SEM)
SEM observations were carried out with a Hitachi 4800s scanning electron microscope in normal mode. For imaging purified and dried crystals were mounted on aluminum stubs with double sided sticking carbon tape and gold-palladium coated. Images were obtained using a secondary electron detector (SE), operated at acceleration voltage of 3.0 kV and 12.6 mm working distance.

2.12 X-ray diffraction analysis
For XRD analyses, the bulk precipitates were powdered and placed on a silicon disc. Analyses were run from 4° to 75° 2-theta (\( \Theta \)) angle at 0.5°/min (Fig. 1) on a Philips X-ray diffractometer PW 1710 with monochromatic Co anode. Spectra analysis was carried out with the X-powder software. The (104) calcite surface peak was used to calculate the MgCO\(_3\) mol% (Goldsmith et al., 1961).

3. RESULTS
3.1 Crystal mineralogy and morphology
Inoculation of liquid and agar medium with \( A. \) borkumensis resulted in mineral precipitation within three days, while sterile controls remained crystal-free. The precipitates were composed of aragonite (40-60 wt%) and high-magnesium calcite (MgCO\(_3\) concentration 25 ± 6 mol%, SD) (Fig. 1). Systematic differences in morphology and mineralogy were not observed between crystals grown on agar or in liquid medium. During the incubation time, the nucleated crystals underwent considerable changes in morphology and size. After three to five days of biofilm growth, newly formed crystals
appeared as spindle structures of 10 to 15 µm in length showing extensive growth at both exterior ends, resulting either in dumbbell-shaped twin spheroids or hemispheres of increased diameter (20–50 µm) with irregular surface structure (Fig. 2 A-B). After seven days, individual crystals agglomerated to larger structures (Fig. 2 C), subsequently beginning to form a porous, laterally extended, carbonate layer (Fig. 2 D) during the following two weeks, which was visible on the vessel walls with the naked eye. SEM imaging revealed that the crystal surfaces were characterized by an irregular rough topography, apparently mediated by the presence of nano-crystallites, ranging approximately between 20 and 50 nm (Fig. 2 F).

This sequence of carbonate nucleation, growth, and cementation was observed during A. borkumensis growth in liquid and agar medium, demonstrating that the mode of carbonate growth and cementation was primarily caused by microbial metabolic activity.

3.2 Carbonic anhydrase imaging and inhibition

For confirming the presence of extracellular CA, biofilms of A. borkumensis were stained with 5-dimethylaminonaphthalene-1-sulfonamide (Li and Ci, 1995). Microscopy analyses of stained biofilms revealed multiple areas with extracellular CA present, enclosing micrometer-sized carbonate crystals (Fig. 3). In contrast, regions with low concentrations (low intensity) or devoid of CA showed no crystal nucleation. Growth experiments with the two sulfonamide CA-inhibitors acetazolamide and ethoxzolamide were carried out, to further eradicate the possibility of coinciding CA presence and crystal nucleation. Both inhibitors are highly specific for CA inactivation (Supuran, 2011). The addition of either acetazolamide (membrane-impermeable) or ethoxzolamide (membrane-permeable) to new A. borkumensis inoculates on agar medium evoked a marked reduction of crystal nucleation. The observed crystal abundance showed an exponential decline (acetazolamide, multiple R² =0.98, y=3.54 e ^{-0.023x}, residual standard error a= p-value a=0.001, p-value b=0.030, n=15; ethoxzolamide, multiple R²=0.95, residual standard a=0.32, residual standard b=0.32, y=0.771 e^{-0.0102x}, p-value a=0.027, n=15) with increasing inhibitor concentrations (Figure 4), clearly showing the direct involvement of extracellular CA in carbonate crystal formation.

3.3 Bulk liquid chemistry
To constrain the changes in carbonate-relevant chemical parameters, a batch experiment was conducted with *A. borkumensis* in liquid medium over 17 days. During the course of the incubation, bulk liquid pH, dissolved inorganic carbon (DIC), total alkalinity (TA), and ammonium (NH$_4^+$) concentration showed continuous increase during the first 14 days (Fig. 5) in accordance with the growth phase of the *A. borkumensis* cultures. As the cultures reached the stationary phase all parameter concentrations concordantly stabilized.

After about seven days of culture growth, a simultaneous decrease in concentration of dissolved calcium (Ca$^{2+}$), strontium (Sr$^{2+}$) and magnesium (Mg$^{2+}$) was apparent, indicating the onset of aragonite and Mg-calcite precipitation. The saturation state ($\Omega$) for aragonite also increased during the first 10 days, showing a peak value of 60. The subsequent decline of $\Omega$ to about 10 illustrates substantial removal of Ca$^{2+}$ (and CO$_3^{2-}$) ions from solution due to precipitation.

### 3.4 $\delta^{44/40}$Ca, $\delta^{88/86}$Sr, and element fractionation

A second batch experiment was conducted with *A. borkumensis* in the same liquid medium to constrain element as well as calcium (Ca$^{2+}$) and strontium (Sr$^{2+}$) isotopes fractionation caused by carbonate precipitation. Element concentrations, and calcium (Ca$^{2+}$) and strontium (Sr$^{2+}$) isotope ratios were obtained from the liquid medium, the microbial organics, and the newly formed crystals. The newly formed carbonates consisted of aragonite (60 ± 4 wt%, SD from X-powder quantitative analysis) and Mg calcite (40 ± 7.3 wt%, SD from X-powder quantitative analysis).

Between start and end of the incubation experiment bulk medium liquid exhibited a marked decline of magnesium (Mg$^{2+}$) (10 ± 0.11%, SD), Ca$^{2+}$ and Sr$^{2+}$ ions (> 87 ± 0.4%, SD) (Table 1). Total alkalinity and dissolved inorganic carbon concentrations increased four-fold to final concentrations of 41.5 ± 1.3 SD mEq l$^{-1}$ and 34.7 ± 1.1 SD mmol l$^{-1}$, respectively. Accordingly, initial and final medium liquid Mg/Ca (mol mol$^{-1}$) and Sr/Ca (mmol mol$^{-1}$) ratios changed notably from 5.08 ± 0.01 SD to 56.99 ± 0.27 SD and from 8.37 ± 0.04 SD to 11.48 ± 0.09 SD, respectively, clearly demonstrating higher relative removal of Ca$^{2+}$ and Sr$^{2+}$ from the liquid phase compared to Mg$^{2+}$ (Table 2).

At experiment termination after 14 days, seawater medium, microbial organics and crystals were separated and analyzed individually. The $\delta^{44/40}$Ca and $\delta^{88/86}$Sr isotope ratios of the seawater medium changed considerably during the incubation period,
showing strong depletion in light isotopes at the end of the incubation (Table 2). As the experiment was carried out using a finite reservoir, the carbonate Ca and Sr isotope ratios were corrected for Rayleigh distillation. A clear fractionation between the initial bulk liquid and the purified bulk carbonate crystals was apparent with $\Delta^{44/40}\text{Ca}_{\text{carbonate-seawater medium}}$ and $\Delta^{88/86}\text{Sr}_{\text{carbonate-seawater medium}}$ of $-1.2 (2\sigma \pm 0.2)$ and $-0.152\%_0 (2\sigma \pm 0.023)$, respectively. Corresponding carbonate $\delta^{44/40}\text{Ca}$ and $\delta^{88/86}\text{Sr}$ were $0.57\%_0 (2\sigma \pm 0.11, n=14)$ and $0.249\%_0 (2\sigma \pm 0.012, n=6)$, respectively. The corresponding ratios of main and trace elements (Table 2) in the liquid phase revealed a considerable increase in the Mg/Ca molar ratio by a factor of 11.2 at the end of the incubation period illustrating that more than 90% of the dissolved Ca$^{2+}$ were removed from solution. Similarly, also the liquid phase concentration of dissolved Sr$^{2+}$ was strongly reduced over time, as the corresponding Sr/Ca molar ratio exhibited only a minor change by a factor of 1.3. Among the measured trace elements zinc (Zn) showed an increased molar ratio with Ca. The molar ratios with the trace elements nickel (Ni) and aluminum (Al) showed considerable depletion of Zn at the end of the incubation.

The separated organic matter fraction, including cells and biofilm components was characterized by deviating molar ratios compared to the initial and terminal values of the liquid phase. The Mg/Ca molar ratio was less than half of the initial liquid demonstrating that Ca was preferably enriched over Mg in the biofilm. In comparison to Ca and Ni, Zn was markedly enriched in the organic fraction, compared to the initial liquid phase. Only the Zn/Al molar ratio showed an opposite trend here. In the carbonate fraction a Mg/Ca ratio of 0.29 was observed, which is consistent with the identification of high-Mg calcite in the XRD-spectra. In comparison to Ni and Al, Zn was enriched by a factor of 5.4 and 2.1, respectively, in the precipitated carbonate, with regard to the initial liquid phase.

4. Discussion

4.1 Ammonification and carbonic anhydrase

Microbial carbonate precipitation in nature occurs as a consequence of either autotrophic or heterotrophic processes (Castanier et al., 2000). Heterotrophic pathways include the dissimilatory reduction of nitrate (Tiedje, 1988), sulfate reduction (Visscher et al., 2000), anaerobic methane oxidation (Greinert et al., 2001), the degradation of urea (Ferris et al., 2003), and the ammonification of amino-acids (Castanier et al., 1999). While conclusive evidence exists for the first four processes mentioned, knowledge of
the contribution of microbial ammonification is still dissatisfactory. In fact, this process is mentioned in numerous studies as a potential pathway, while definite prove is, to our knowledge, currently lacking. Work on microbially mediated carbonate formation is in the focus of intensive research since several decades, using the classical B4-medium, e.g. (Boquet et al., 1973). While the focus of attention was mostly the observed increase in pH by individual microbial strains resulting in precipitation, the mechanism behind it remains unclear (Marvasi et al., 2012). Based on the current study it can be speculated that also microbes that are able to carry out deamination and ammonification might have been causative for the observed pH increase in the B4 medium and subsequent carbonate precipitation.

The present study demonstrates that carbonate precipitation is principally possible as the result of microbial ammonification, largely increasing TA and also pH. In our laboratory experiments, the location of crystal precipitation in a microbial biofilm was largely governed by the heterogenic presence of extracellular carbonic anhydrase, locally causing high concentrations of HCO$_3^-$; Due to the ammonification process, consuming H$^+$ ions, the conversion of HCO$_3^-$ to CO$_3^{2-}$ is facilitated, subsequently leading to carbonate precipitation. The evolution of the bulk solution chemical parameters showed a rapid increase of ammonium in the liquid phase as the direct result of ammonification (Fig. 5), which A. borkumensis is capable of (Baker, 2016). The conversion rate of ammonia to ammonium is primarily dependent on the concentration of H$^+$ ions in a solution (Whitfield, 1974). Under the prevailing temperature (20°C) and pH of the medium during incubations (pH 7.7-8.3) around 92-98% of the produced ammonia reacted to ammonium by protonation (Emerson et al., 1975), as the pK$_a$ value for ammonium is 9.46. Therefore excessive outgassing of ammonia during incubations with medium can be excluded. The genome of A. borkumensis does not include a gene or operon for the enzyme ammoniummonooxygenase (amo) (Schneiker et al., 2006), which is responsible for the conversion of ammonium to nitrate. Consequently, an oxidation of ammonium to nitrate in the pure cultures can be excluded.

TA increased by 32 mEq during the experiment, while precipitation of about 10 mmol of CaCO$_3$ indicates an alkalinity loss of 20 mEq (Table 1). Gross TA production during the experiment consequently was 52 mEq. The final ammonium concentration was by a factor of ~3.7 lower than the gross TA. Therefore, it can be assumed that approximately 73% of the produced ammonium has been consumed for glutamine production during culture growth (Sabirova et al., 2008).
Although ammonification directly controlled the TA in our experiments, this process does not affect the DIC concentration. *A. borkumensis* is a heterotrophic aerobic organism, that produces $\text{CO}_2$ as a metabolic end product, and thus increases the partial pressure (p$\text{CO}_2$) in the liquid medium incubations. The p$\text{CO}_2$ controls the concentration of dissolved $\text{CO}_2$ according to Henry's law, directly affecting the concentration of DIC. At a pH of 8 to 9 most of the dissolved fraction partly converts into bicarbonate and carbonate ions. The experiments allowed for permanent $\text{CO}_2$ equilibration with the atmosphere. During the batch experiment to constrain bulk liquid chemistry the p$\text{CO}_2$ of the liquid increased over time from 1081 to 4254 µatm (calculated according to Zeebe and Wolf-Gladrow, 2001). As the laboratory atmosphere was close to atmospheric conditions, with respect to p$\text{CO}_2$, a permanent loss of $\text{CO}_2$ from the solution due to permanent $\text{CO}_2$ production could be expected. Contrasting, the measured DIC concentrations were only between 16 and 3% lower than the calculated values for the carbonate system in a water body, using solution pH and TA. This result illustrates that the loss of $\text{CO}_2$ as a result of air-water equilibration was minimal, which seems counter intuitive at first sight. However, this phenomenon can be explained by the activity of carbonic anhydrase catalyzing the hydration of $\text{CO}_2$ to carbonic acid and subsequent dissociation to non-gaseous bicarbonate and carbonate ions, thus limiting loss of $\text{CO}_2$ to the ambient atmosphere. The visualization of CA in *A. borkumensis* biofilm substantiates this hypothesis, as carbonate precipitation exclusively occurred in regions with a relatively high extracellular concentration of this enzyme. Concordantly, the inhibition of CA was causative for a steep decline in carbonate crystal abundance precipitating in the biofilms. As $\text{CO}_2$ was constantly produced, and thus intracellular available, as a direct consequence of the heterotrophic life style of *A. borkumensis*, an enzymatically catalyzed conversion of bicarbonate to $\text{CO}_2$ is unlikely. In contrast, the presence of extracellular CA provides several obvious advantages. $\text{CO}_2$ is membrane permeable and can freely diffuse. Therefore, an energy demanding trans-membrane transport mechanism of bicarbonate can be avoided. Due to the extracellular activity of CA, a constant $\text{CO}_2$ flux out of the cell can be maintained. This in turn prevents the increase in $\text{CO}_2$ concentration and consequential pH decline in the cytoplasm. As a by-product of this cellular pH regulation mechanism the extracellular DIC concentration is increased. In combination with the elevated total alkalinity and pH, due to ammonification, the extracellular vicinity in *A. borkumensis* biofilms can become supersaturated with regard to calcium carbonate, facilitating mineral precipitation at locations, where CA catalysis and
ammonification are both carried out, as documented in this study. In fact, extracellular microbial CA has been identified to induce carbonate formation under various aqueous conditions (Silva-Castro et al., 2013). Direct nucleation of carbonate minerals on the outer cell surface and subsequent self-entombment was not observed in our experiments. A plausible explanation here fore is the production of extracellular polymeric substances (EPS), a principle component of microbial biofilms (Costerton et al., 1995). Several studies (e.g. Bontognali et al. (2008); Krause et al. (2012)) have shown that EPS play a key role in the carbonate precipitation process as they temporarily accumulate cations to high concentrations. During labile EPS degradation the release of cations creates localized supersaturation for carbonate minerals favoring precipitation (Dupraz et al., 2004). As biofilms are generally very heterogeneous (Donlan, 2002), calcium carbonate precipitation predominantly occurs in regions enriched in EPS rather than at cell surfaces.

4.2 Crystal morphology

One of the most intriguing feature of submarine carbonate cements is the presence of peloidal crystal aggregates (Macintyre, 1985). Similar to natural analogues, the crystals precipitated during the laboratory incubation of the present study also formed peloids with a fine micritic structure (Flügel, 2010). With time individual peloids aggregated to larger units. This type of peloidal matrix is typically encountered in Holocene and older (Kazmierczak et al., 1996; Riding, 2000) carbonate deposits forming in marine areas including shallow areas (Bontognali et al., 2008; Harris, 1978; Kazmierczak et al., 1996; Riding, 2000), deep-water mud mounds (e.g. Bridges and Chapman (1988)), and methane-seep limestones (e.g. Peckmann et al. (2002)). Although peloids represent a ubiquitous component of modern and fossil carbonates (Adachi et al., 2004), their origin is still subject of debate (Samankassou et al., 2005). However, various studies confirmed the capability of microbes to produce peloidal carbonates (Kamber and Webb, 2001; Krause et al., 2012; Reid et al., 2000), a component frequently observed in limestone.

One of the observed features during the analysis of crystal surfaces was the presence of prominent intra-crystal microporosity in the form of regular and irregular channels and voids (Fig. 2F). The origin and dynamics of microporosity is one of the key petrophysical properties of carbonate rocks, with regard to the characterization of hydrocarbon bearing reservoir rock (Ghous et al., 2007). In addition, microporosity is also applied
diagnostically for microbial origin of carbonate deposits (Folk and Chafetz, 2000). As the diameter of A. borkumensis cells ranges between 0.4-0.7 µm (Yakimov et al., 1988), the observed regular porosity structures appears to be simply cell imprints (Bosak et al., 2004). The encountered irregular micro-porosity features are generally too small to resemble cell shapes, and therefore advocate for a different formation mechanism. The nano-crystalline fine structure of the precipitates (Fig. 2F) indicate that amorphous calcium carbonate (ACC) may have been the initial precipitate and thus, a reactive intermediate phase that later transformed into crystalline products (Bots et al., 2012; Rodriguez-Blanco et al., 2017). During this transformation, potentially involving dissolution and re-precipitation (Weiss et al., 2002), the irregular micro-porosity features might have formed.

The potential for carbonate precipitation mediated by various marine microbial metabolic pathways (Morita, 1980; Reitner et al., 2005; Vasconcelos and McKenzie, 1997; Vasconcelos et al., 1995) and biofilm components as EPS (Dupraz et al., 2009; Kremer et al., 2008) is well described. The present study documents the formation of a laterally extended carbonate matrix in a marine bacterial biofilm, including various morphological features, which are characteristic for natural carbonate deposits in marine environments.

### 4.3 Geological implications

The δ\(^{44}/^{40}\)Ca and δ\(^{88}/^{86}\)Sr values obtained from bulk carbonate precipitation during the present laboratory study are well in agreement with modern marine biogenic carbonates (δ\(^{44}/^{40}\)Ca 0.2 to 1.1‰, (Blättler et al., 2012; Holmden et al., 2012) and δ\(^{88}/^{86}\)Sr 0.0 to 0.4‰ (Böhm et al., 2012; Krabbenhöft et al., 2010; Stevenson et al., 2014; Vollstaedt et al., 2014), resulting from biologically controlled precipitation of eukaryotes.

The genesis of microbial carbonate differs fundamentally from that of metazoan organisms. The latter facilitating mineral precipitation in a strongly controlled chemical environment, precisely meeting the required structural shapes and geometries for crystal growth. This genetically mediated carbonate formation mechanism is therefore regarded as biologically controlled (Dupraz et al., 2009). In contrast, extracellular microbial carbonate genesis is anticipated to result from the build-up of metabolic products increasing the carbonate saturation state. As this process is not genetically controlled, it is termed biologically mediated carbonate formation (Dupraz et al., 2009).
As a consequence, fractionation of isotopes and partitioning of trace elements in microbiologically mediated carbonate should, in principle, display a high degree of similarity to abiotic carbonate. Recent investigations demonstrated the strong rate-control on $\Delta^{88/86}\text{Sr}_{\text{calcite-aq}}$, $\Delta^{44/40}\text{Ca}_{\text{calcite-aq}}$, and the strontium partitioning coefficient ($D_{\text{Sr}}$) for inorganic calcite (AlKhatib and Eisenhauer, 2017b; Böhm et al., 2012; Tang et al., 2008a) and to a lesser degree for inorganic aragonite (AlKhatib and Eisenhauer, 2017a; Fruchter et al., 2016), allowing to constrain the precipitation rate of the two carbonate phases. As extracellular microbial carbonate is biologically mediated and not genetically controlled we assume that the rate- and temperature-controlled correlation of $\Delta\text{Sr}^{88/86}$ vs. $D_{\text{Sr}}$ (Böhm et al., 2012; Stevenson et al., 2014; AlKhatib and Eisenhauer, 2017a, b) of this precipitation mode resembles inorganic precipitation due to similar kinetic conditions. The observed $\Delta^{44/40}\text{Ca}$ and $\Delta^{88/86}\text{Sr}$ values of the present study are in the range of the published values for inorganic aragonite and calcite (AlKhatib and Eisenhauer, 2017a; Tang et al., 2008a) (Fig. 6). The microbiologically precipitated carbonate is a mixture of about 50% aragonite and 50% high Mg-calcite and could be expected to plot in a mixing triangle defined by the $\Delta^{88/86}\text{Sr} - \Delta^{44/40}\text{Ca}$ space of slowly precipitated calcite, fast precipitated calcite and room temperature aragonite (Fig. 6). Considering the strontium partition coefficient ($D_{\text{Sr}}$), the $\Delta^{88/86}\text{Sr}$ and the $\Delta^{44/40}\text{Ca}$ of the current study, which were all corrected for Rayleigh fractionation, clear deviations from inorganically precipitated aragonite and calcite are evident (AlKhatib and Eisenhauer, 2017a, b; Fruchter et al., 2016; Tang et al., 2008a) (Fig. 7, 8). In both cases log($D_{\text{Sr}}$) obtained during the recent study is more positive (-0.20±SD 0.1) than expected (-0.49) for the measured 1:1 ratio of aragonite and calcite. This deviation indicates relatively more sequestration of Sr from the aqueous phase than expected. As shown by (Mucci and Morse, 1983) $D_{\text{Sr}}$ of Mg-calcite increases by about 0.018 per mol-% of MgCO$_3$. Consequently, for the precipitates of this study (approximately 25 mol%) $D_{\text{Sr}}$ would be 0.46 higher than for Mg-free calcite, i.e. increase log($D_{\text{Sr}}$) by about 0.6. The normalized precipitation rate (R) of the recent study has a log (R) value of 3.13 µmol/m$^2$/h. Considering the higher $D_{\text{Sr}}$ of Mg-calcite, the $\Delta^{88/86}\text{Sr}$ and $\Delta^{44/40}\text{Ca}$ vs. the log($D_{\text{Sr}}$) lie close to (Fig. 7) and between (Fig. 8) the aragonite-calcite mixing lines for log (R) value between 2.5 and 3.5 µmol/m$^2$/h. Consequently the cation isotope fractionation and trace element partitioning during extracellular precipitation of aragonite and calcite observed during the present study appear to follow the principles for inorganic
precipitation. There is no indication of a significant biological influence on cation incorporation during crystal growth.

The vast majority of the microbial community does not lead a solitary life style but are embedded within a self-produced matrix of extracellular polymeric substances (EPS), which is referred to as biofilm (Costerton et al., 1995; Dufour et al., 2010). Biofilms represent effective diffusion barriers allowing for the establishment of chemical conditions differing from the adjacent exterior liquid. EPS contain numerous chemical functional groups with a negative charge effectively binding divalent cations (Dupraz et al., 2009) building up large inventories, e.g. for strontium (Rogerson et al., 2008). This binding might be partly explanatory for the observed minor deviation of the $D_{Sr}$ from purely inorganic precipitation, as EPS cause additional Sr sequestration from the aqueous phase. Biofilm components have the potential to induce considerable element partitioning, as demonstrated for Mg, Zn, Ni and Al in this study (Table 2), and isotope fractionation, effecting geochemical characteristics of carbonates precipitating within the biofilm (Krause et al., 2012).

The current study clearly demonstrates the critical role of the extracellular enzyme carbonic anhydrase for carbonate precipitation within the biofilm. While the process of ammonification largely increases the ambient alkalinity, the localities of crystal nucleation were characterized by the presence of the enzyme most likely inducing localized increases of DIC subsequently facilitating crystal nucleation. As the observed extracellular microbial crystal formation involves a controlled chemical environment and the activity of an enzyme it can be debated whether this process is to be termed microbially mediated or microbially controlled.

Several studies indicate that microbial carbonate precipitation in biofilms is mediated by the initial formation of an ACC precursor phase forming nano-crystallites (Benzerara et al., 2011; Dupraz et al., 2009; Littlewood et al., 2017), similar to those observed in this study (Fig. 2). Recently, it has been demonstrated that calcite, which has formed via ACC has incorporated more Sr compared to direct nucleation (Littlewood et al., 2017).

Possible reasons for this phenomenon are the high reactive surface area, the poorly ordered structure and the rapid formation rate of ACC (Rodriguez-Blanco et al., 2011). The high Sr/Ca ratio is apparently maintained in the final mineral, although a series of dissolution and precipitation may take place during the transformation from ACC to calcite (Giuffre et al., 2015; Littlewood et al., 2017). Consequently, the observed deviation of the $D_{Sr}$ from inorganic low-Mg calcite precipitation could also be the result
of carbonate formation in the biofilm via the ACC pathway. Compared to the open ocean, rates of ammonification are generally amplified in shallow coastal marine environments as a consequence of the high load of labile organic matter (Herbert, 1999). Highest rates of ammonification are reported for vegetated coastal sediments (Moriarty et al., 1985), exceeding 1100 mg N m$^{-2}$ day$^{-1}$, largely governing the availability of inorganic nitrogen. (Castanier et al., 1999) calculated that marine aerobic heterotrophic bacterial processes, of which ammonification might represent a sufficient fraction, can be responsible for an annual CaCO$_3$ layer production between 4 µm and 2 mm thickness. Consequently, after $10^6$ yr these processes may result in a limestone layer between 4 and 2000 m. Considering this result and the present study it can be assumed that microbial ammonification markedly contributed to the formation of carbonate deposits in the form of peloids during the Phanerozoic (Riding, 2000). Also for the lithification of modern microbial mats ammonification is discussed as one of the key mechanisms (Chafetz and Buczynski, 1992; Diaz et al., 2012). Our results provide a proof of concept, augmenting the importance of the N-cycle in the context of microbially mediated carbonate formation.

The carbonic anhydrase molecule requires a zinc (Zn) atom at its active site. In our study we clearly demonstrate Zn sequestration and enrichment in the organic biofilm components (Table 2). A part of Zn was further sequestered into the carbonate. The current study might also contribute to the understanding of the heterogeneous Zn distribution in mineral layers of modern microbialites (Sforna et al., 2017). According to our study results, carbonate phases with enriched Zn concentration could originate from the combination of CA and an alkalinity-producing microbial pathway as ammonification or sulfate reduction. Therefore, we can speculate that a similar mechanism, with regard to CA activity and distribution might also be active in modern natural biofilms. Consequently, it can be postulated that heterogeneous Zn distribution in modern and ancient microbialites is indicative for microbially mediated carbonate formation.

Placing the study results of geochemical, mineralogical and isotopic signatures into a geological context, we propose that microbial carbonate, formed under nutrient-rich marine conditions favoring ammonification, adds to the Earth’s limestone inventory. As microbial carbonic anhydrase is an ancient and phylogenetically wide-spread enzyme, it is very likely that also other microbes use the prosed mechanism of extracellular pH
control within a biofilm, thus also potentially facilitating authigenic carbonate precipitation and subsequent deposition in shallow marine areas.

5. Conclusions
The present study extends recent theories of microbial marine carbonate production and diagenesis by showing that (I) CaCO$_3$ matrix precipitation can be microbially mediated already close to the sediment surface; (II) extracellular enzymatic activity of microbial ammonification in combination with CA can be directly involved in peloidal carbonate formation, a substantial matrix fraction of shallow marine carbonate deposits. (III) The element and isotope uptake kinetics of the microbial carbonate are similar to inorganic precipitation, possibly involving a transient ACC phase. The precipitation rates observed during the present study using monoculture experiments under laboratory conditions may not be easily transferred to natural setting, where O$_2$- and carbon consumption by numerous microbial and eukaryotic organisms may decrease ambient pH, leading to a reduction in calcium carbonate saturation state. This fact in turn is likely to result in considerably slower precipitation rates. In addition, due to the fact that the observed microbially mediated cation isotope fractionation follows the principles of inorganic aragonite and calcite precipitation, in natural systems microbial precipitation might be indistinguishable from truly abiotic carbonate production. In case primary mineral morphology is preserved the characteristic dumbbell or spherical crystal structures might provide additional evidence for microbial origin of carbonate minerals. The activity of extracellular CA in the carbonate rock record might be particularly difficult to prove, but documented Zn enrichment in individual carbonate layers in combination with rare earth element signatures indicative for microbial activity might point to the involvement of this enzyme for carbonate nucleation.
References:


Acknowledgments

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Figure 1. XRD-spectra of bulk carbonate samples from seawater medium (pattern A) and agar-seawater medium (pattern B) experiments. Peaks marked with A are ascribed to aragonite, Peaks marked with C are ascribed to calcite. The Peak denoted as C* represents the (104) surface. The (104) surface peak shift was used to calculate the MgCO$_3$ mol% of calcite from bulk carbonates.

Figure 2. Light microscopy (A, B) and scanning electron microscopy images of carbonate crystal formation sequence in $A$. $borkumensis$ biofilm. After three days of biofilm growth, initially formed carbonate crystals exhibit spindle structures. Immediately after spindle formation prominent growth at both ends sets in (A), forming dumbbell-shaped crystals (B). With progressing biofilm age, individual crystals aggregate (C, after 7 days of biofilm growth) to large micritic porous cement units (D, after 21 days of biofilm growth). Crystal surfaces are characterized by irregular surface topography exhibiting microporosity represented by voids and irregular channels (E, F - enlarged detail of E, black arrow indicated cell imprints, white arrows denote primary irregular microporosity). Observed regular porosity structures ranging between 0.4-0.7 µm in diameter appear to be imprints of $A$. $borkumensis$ cells.

Figure 3. Composite image of polarized light and 460 nm-emission for the combined in-situ identification of extracellular carbonic anhydrase (stained with 5-dimethylaminonaphthalene-1-sulfonamide (DNSA)) and crystalline structures in $A$. $borkumensis$ biofilm grown for 5 days. Blue regions indicate carbonic anhydrase presence in biofilm, spherical bright blue locations represent carbonate crystals.

Figure 4. Decline of crystal abundance in $A$. $borkumensis$ biofilm with increasing concentrations of carbonic anhydrase inhibitors acetazolamide (AZ, membrane-impermeable) and ethoxzolamide (EZ, membrane permeable). Curves represent exponential functions fitted to the data. Values for $R^2$ and fit functions are given. Standard deviations are calculated from three replicates.

Figure 5. Change of solution chemical parameters during the incubation. Values for pH, total alkalinity (TA), NH$_4^+$, saturation state ($\Omega$) for aragonite, and dissolved inorganic carbon (DIC) increase concordantly with increasing cell abundance. The onset of carbonate precipitation is indicated by the simultaneous decline of dissolved Ca$^{2+}$, Sr$^{2+}$,
Mg$^{2+}$ and Ω. Error bars indicated standard deviation of three replicates. Most error bars within symbols.

Figure 6. This figure shows the relationship between $\Delta^{44/40}$Ca$_{\text{crystal-aq}}$ and $\Delta^{88/86}$Sr$_{\text{crystal-aq}}$. The plot includes data for inorganic aragonite and calcite from previous studies by (AlKhatib and Eisenhauer, 2017a, b) and (Tang et al., 2008b). For the data point of this study a calcite-aragonite ratio of 1 was assumed. The figure shows that the $\Delta^{88/86}$Sr of the biogenic carbonate is slightly less negative compared to the majority of inorganically precipitated aragonite and calcite.

Figure 7. Correlation of $\Delta^{88/86}$Sr and log(DSr). The plot includes data for inorganic aragonite and calcite from previous studies; (AlKhatib and Eisenhauer, 2017a, b), (Tang et al., 2008b), and (Fruchter et al., 2016) for 25°C, being close to the reaction temperature of present study (20°C). For the data point of this study a calcite-aragonite ratio of 1 was assumed. The log(DSr) value of the present study is more positive than the expected value around -0.5 for an inorganic aragonite-calcite ratio of 1, clearly lying above the aragonite-calcite mixing line for inorganic precipitates (solid). The corresponding $\Delta^{88/86}$Sr value exceeds the log(R) line between 2.5 µmol/m$^2$/h (fine dashed) and 3.5 µmol/m$^2$/h (coarse dashed) for inorganic aragonite-calcite ratios between 0 and 1.

Figure 8. Correlation of $\Delta^{44/40}$Ca and log(DSr). The plot includes data for inorganic aragonite and calcite from previous studies; (AlKhatib and Eisenhauer, 2017a, b), (Tang et al., 2008a), and (Gussone et al., 2005) for 25°C, being close to the reaction temperature of present study (20°C). For the data point of this study a calcite-aragonite ratio of 1 was assumed. Similar to aragonite the log(DSr) is more positive than the expected value around -0.5 for an inorganic aragonite-calcite ratio of 1 lying above the aragonite-calcite mixing line for inorganic precipitates (solid). Also the corresponding $\Delta^{44/40}$Ca value exceeds the log(R) line between 2.5 µmol/m$^2$/h (fine dashed) and 3.5 µmol/m$^2$/h (coarse dashed) for inorganic aragonite-calcite ratios between 0 and 1.
Figure 2
Figure 3

Carbonate peloids

Cells

10 μm
Figure 4

Crystal abundance $[x \times 10^4 \text{ cm}^{-2}]$ vs. inhibitor concentration $[\mu \text{mol L}^{-1}]$

- **AZ**: $3.54 \times e^{-0.023x}$, $R^2 = 0.98$
- **EZ**: $3.82 \times e^{-0.054x}$, $R^2 = 0.95$
Figure 7
Figure 8
### Table 1. δ¹⁸⁴⁰⁰Ca, δ⁶⁸⁰⁰⁰Sr, and element fractionation. Initial and final (after 14 days) pH, total alkalinity (TA), dissolved inorganic carbon (DIC), magnesium (Mg), calcium (Ca), and strontium (Sr) concentrations of the liquid medium.

<table>
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<tr>
<th></th>
<th>pH (mEq kg⁻¹)</th>
<th>TA (mEq l⁻¹)</th>
<th>DIC (mmol l⁻¹)</th>
<th>Mg⁺⁺ (mmol l⁻¹)</th>
<th>Ca⁺⁺ (mmol l⁻¹)</th>
<th>Sr⁺⁺ (µmol l⁻¹)</th>
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<tr>
<td><strong>Medium at start</strong></td>
<td>8.02 ± 0.01</td>
<td>8.13 ± 0.05</td>
<td>51.32 ± 0.05</td>
<td>10.10 ± 0.02</td>
<td>84.53 ± 0.39</td>
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<tr>
<td><strong>Medium at end</strong></td>
<td>8.24 ± 0.05</td>
<td>41.54 ± 1.26</td>
<td>34.67 ± 1.07</td>
<td>46.26 ± 0.11</td>
<td>0.81 ± 0.01</td>
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### Table 2. Element ratios and isotope signatures of experiment components

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<th>Seawater medium-experiment start</th>
<th>Seawater medium-experiment end</th>
<th>Organic matter</th>
<th>Carbonate</th>
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<td>9.3 ± 0.1</td>
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<td>total alkalinity (mEq kg⁻¹)</td>
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<td>41.6 ± 1.26</td>
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<td>Mg/Ca (mol mol⁻¹)</td>
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<td>56.99 ± 0.27</td>
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<tr>
<td>Sr/Ca (mmol mol⁻¹)</td>
<td>8.37 ± 0.04</td>
<td>11.48 ± 0.09</td>
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<td>Zn/Ca (mmol mol⁻¹)</td>
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<td>2.38 ± 0.01</td>
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<tr>
<td>Zn/Al (mol mol⁻¹)</td>
<td>54.83 ± 1.76</td>
<td>21.07 ± 0.73</td>
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<tr>
<td>1.76 ± 0.19 (n=2)</td>
<td>4.49 ± 0.033 (n=3)</td>
<td>2.08 ± 0.13 (n=2)</td>
<td>0.57 * ± 0.12 (n=14)</td>
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<tr>
<td>δ⁶⁸⁰⁰⁰Sr (‰ NBS987)</td>
<td>0.401 *</td>
<td>0.769 ± 0.014 (n=2)</td>
<td>0.395 ± 0.026 (n=2)</td>
<td>0.249 * ± 0.013 (n=6)</td>
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<td>δ¹⁸⁴⁰⁰⁰Ca (‰ SRM915a)</td>
<td>-1.19 ± 0.22</td>
<td>-0.152 ± 0.024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

δ¹⁸⁴⁰⁰Ca, δ⁶⁸⁰⁰⁰Sr, and Δ⁸⁴⁰⁰⁰⁰Ca uncertainties expressed as 2nd Standard Deviation (2SD)
Mg/Ca and Sr/Ca uncertainties expressed as Standard Deviation
* Within uncertainty identical to modern seawater (Krabbenhof et al. 2009), one measurement for test: typical ext. reproducibility of appl. method: 0.020 (2SD)
* isotope ratios and error propagation corrected for Rayleigh distillation.
Table 1. Initial and final (after 14 days) pH, total alkalinity (TA), dissolved inorganic carbon (DIC), magnesium (Mg), calcium (Ca), and strontium (Sr) concentrations of the liquid medium.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>TA (mEq l⁻¹)</th>
<th>DIC (mmol l⁻¹)</th>
<th>Mg²⁺ (mmol l⁻¹)</th>
<th>Ca²⁺ (mmol l⁻¹)</th>
<th>Sr²⁺ (µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium at experiment start</td>
<td>8.02 (± SD &lt; 0.01)</td>
<td>9.27 (± SD 0.11)</td>
<td>8.13 (± SD 0.1)</td>
<td>51.32 (± SD 0.05)</td>
<td>10.10 (± SD 0.02)</td>
<td>84.53 (± SD 0.39)</td>
</tr>
<tr>
<td>Medium at experiment end</td>
<td>8.24 (± SD 0.05)</td>
<td>41.54 (± SD 1.26)</td>
<td>34.67 (± SD 1.07)</td>
<td>46.26 (± SD 0.11)</td>
<td>0.81 (± SD 0.01)</td>
<td>9.32 (± SD 0.06)</td>
</tr>
</tbody>
</table>
Table 2: Element ratios and isotope signatures of experiment components

<table>
<thead>
<tr>
<th></th>
<th>Seawater medium-experiment start</th>
<th>Seawater medium-experiment end</th>
<th>Organic matter</th>
<th>Carbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.02 ± 0.1</td>
<td>9.3 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total alkalinity (mEq kg⁻¹)</td>
<td>8.24 ± 0.1</td>
<td>41.6 ± 1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg/Ca (mol mol⁻¹)</td>
<td>5.08 ± 0.01</td>
<td>56.99 ± 0.27</td>
<td>2.23 ± 0.05</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Sr/Ca (mmol mol⁻¹)</td>
<td>8.37 ± 0.04</td>
<td>11.48 ± 0.09</td>
<td>12.19 ± 0.25</td>
<td>7.23 ± 0.12</td>
</tr>
<tr>
<td>Zn/Ca (mmol mol⁻¹)</td>
<td>0.26 ± 0.01</td>
<td>2.38 ± 0.01</td>
<td>8.86 ± 0.17</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Zn/Ni (mol mol⁻¹)</td>
<td>54.83 ± 1.76</td>
<td>21.07 ± 0.73</td>
<td>1451.12 ± 18.89</td>
<td>296.49 ± 15.44</td>
</tr>
<tr>
<td>Zn/Al (mol mol⁻¹)</td>
<td>2.14 ± 0.09</td>
<td>0.26 ± 0.01</td>
<td>1.47 ± 0.02</td>
<td>4.63 ± 0.03</td>
</tr>
<tr>
<td>δ⁴⁴⁴⁰Ca (% SRM915a)</td>
<td>1.76 ± 0.19 (n=2)</td>
<td>4.49 ± 0.33 (n=3)</td>
<td>2.08 ± 0.13 (n=2)</td>
<td>0.57# ± 0.12 (n=14)</td>
</tr>
<tr>
<td>δ⁸⁸⁸⁶Sr (% NBS987)</td>
<td>0.401 *</td>
<td>0.769 ± 0.014 (n=2)</td>
<td>0.395 ± 0.026 (n=2)</td>
<td>0.249# ± 0.013 (n=6)</td>
</tr>
<tr>
<td>Δ⁴⁴⁴⁰Ca_carbonate-seawater medium</td>
<td></td>
<td>-1.19 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ⁸⁸⁸⁶Sr_carbonate-seawater medium</td>
<td></td>
<td>-0.152 ± 0.024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Δ⁴⁴⁴⁰Ca, δ⁸⁸⁸⁶Sr, Δ⁴⁴⁴⁰, and Δ⁸⁸⁸⁶ uncertainties expressed as 2nd Standard Deviation (2SD)
Mg/Ca and Sr/Ca uncertainties expressed as Standard Deviation
* Within uncertainty identical to modern seawater (Krabbenhoft et al. 2009), one measurement for test: typical ext. reproducibility of appl. method: 0.020 (2SD)
# isotope ratios and error propagation corrected for Rayleigh distillation