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Conditions of *Mytilus edulis* extracellular body fluids and shell composition in a pH-treatment experiment: Acid-base status, trace elements and $\delta^{11}B$

Agnes Heinemann, Jan Fietzke, Frank Melzner, Florian Böhm, and Jörn Thomsen
Leibniz Institute of Marine Sciences (IFM-GEOMAR), D-24148 Kiel, Germany (jfietzke@ifm-geomar.de)

Dieter Garbe-Schönberg
Institute of Geosciences, University of Kiel, D-24118 Kiel, Germany

Anton Eisenhauer
Leibniz Institute of Marine Sciences (IFM-GEOMAR), D-24148 Kiel, Germany

[1] *Mytilus edulis* were cultured for 3 months under six different seawater pCO_2 levels ranging from 380 to 4000 μatm. Specimen were taken from Kiel Fjord (Western Baltic Sea, Germany) which is a habitat with high and variable seawater pCO_2 and related shifts in carbonate system speciation (e.g., low pH and low $CaCO_3$ saturation state). Hemolymph (HL) and extrapallial fluid (EPF) samples were analyzed for pH and total dissolved inorganic carbon (C_T) to calculate pCO_2 and $[HCO_3^-]$. A second experiment was conducted for 2 months with three different pCO_2 levels (380, 1400 and 4000 μatm). Boron isotopes ($\delta^{11}B$) were investigated by LA-MC-ICP-MS (Laser Ablation–Multicollector–Inductively Coupled Plasma–Mass Spectrometry) in shell portions precipitated during experimental treatment time. Additionally, elemental ratios (B/Ca, Mg/Ca and Sr/Ca) in the EPF of specimen from the second experiment were measured via ICP-OES (Inductively Coupled Plasma–Optical Emission Spectrometry). Extracellular pH was not significantly different in HL and EPF but systematically lower than ambient water pH. This is due to high extracellular pCO_2 values, a prerequisite for metabolic CO_2 excretion. No accumulation of extracellular $[HCO_3^-]$ was measured. Elemental ratios (B/Ca, Mg/Ca and Sr/Ca) in the EPF increased slightly with pH which is in accordance with increasing growth and calcification rates at higher seawater pH values. Boron isotope ratios were highly variable between different individuals but also within single shells. This corresponds to a high individual variability in fluid B/Ca ratios and may be due to high boron concentrations in the organic parts of the shell. The mean $\delta^{11}B$ value shows no trend with pH but appears to represent internal pH (EPF) rather than ambient water pH.

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1. Introduction

[2] About 50% of anthropogenic carbon dioxide (CO_2) released to the atmosphere is absorbed by the global oceans. It is predicted that the oceans approach a pH of ~7.3 within the next 300 years [e.g., *Caldeira and Wickett*, 2003; *Sabine et al.*, 2004]. This leads to a shift in the inorganic carbon equilibrium towards higher CO_2 and lower CO_3^{2-} concentrations. Therefore, the calcium carbonate ($CaCO_3$) saturation state (Ω) will decrease by about 50% (for pCO_2 of 840 μatm) [e.g., *Feely et al.*, 2004; *Fabry et al.*, 2008] and higher-latitude oceans are predicted to become undersaturated with respect to aragonite by the year 2050 [*Orr et al.*, 2009; *Cao and Caldeira*, 2008] which may have considerable consequences for marine calcifying organisms [*Orr et al.*, 2005]. Many (mostly short-term studies, days to week duration) ocean acidification experiments have been conducted and different responses to high pCO_2 were found. The majority of the investigated species showed declining rates of calcification and reproduction [see *Doney et al.*, 2009; *Ries et al.*, 2009]. Most ocean acidification perturbation experiments are not able to properly account for the genetic adaptation potential of marine species, as time limitations usually prevent multi generation experiments. Thus, calcifying marine organisms from habitats with naturally high CO_2 concentrations can serve as analogues for future ocean conditions [e.g., *Hall-Spencer et al.*, 2008].

[3] Kiel Fjord (Western Baltic Sea, Germany) provides ideal conditions for ocean acidification studies due to summer hypoxia in bottom waters and upwelling of CO_2 enriched waters [*Hansen et al.*, 1999; *Lehmann et al.*, 2002]. Kiel Fjord is already today frequently exposed to seawater pCO_2 values that are predicted for the future global ocean of the next 100–300 years [*Thomsen et. al.*, 2010; *Caldeira and Wickett*, 2003]. The habitat is characterized by low salinity (10–20), low alkalinity (1900–2150 μmol kg^{-1}), low pH (minimum value <7.5) during summer and autumn, and high pCO_2 (maximum value of 2340 μatm; 1 μatm = 0.101 Pa) events. The $CaCO_3$ saturation state in the Kiel Fjord therefore can reach minimum values of $\Omega_{arag} = 0.34$ and $\Omega_{calc} = 0.58$ [*Thomsen et. al.*, 2010]. It can be assumed that calcifying communities in this habitat have already adapted to a fluctuating carbonate system speciation with frequent high pCO_2 events for multiple generations.

[4] Predictions of future ocean pH scenarios can be improved by studies of climate shifts in the past. In this regard the elemental and isotopic composition of biogenic carbonates (e.g., bivalve shells, coral skeletons, foraminifera) serve as proxies for the reconstruction of past ocean chemistry. In particular, boron to calcium ratios (B/Ca) have been shown to decrease with declining pH in some biological and inorganic carbonates [*Hemming and Hanson*, 1992; *Sanyal et al.*, 2000]. This results from the fact that in aqueous solutions, boric acid dissociates to $B(OH)_4^-$ and H^+ (equation (1)). Consequently, the proportion of boric acid to borate is pH dependent.

$$B(OH)_3 + H_2O \leftrightarrow B(OH)_4^- + H^+ \quad (1)$$

The borate ion is suggested to be preferentially incorporated into carbonates precipitated from seawater [*Vengosh et al.*, 1991; *Hemming and Hanson*, 1992; *Hemming et al.*, 1995; *Pagani et al.*, 2005]. For this reason, the isotopic signature of $B(OH)_4^-$ will be recorded in the carbonate. *Klochko et al.* [2009] and *Rollion-Bard et al.* [2011] proposed that both species can be incorporated. In contrast, *Foster* [2008] investigated B/Ca ratio in foraminifera and reported that the partition coefficient of borate between water and shell carbonate is primarily influenced by seawater [CO_3^{2-}] and not directly by seawater pH. The results of *Yu et al.* [2007] showed the opposite trend of those shown by *Foster* [2008]. *Foster* [2008] suggested this indicates a species specific control on boron incorporation.

[5] Boron isotopes ($\delta^{11}B$) have been measured in skeletons of different organisms like corals [*Reynaud et al.*, 2004; *Hönisch et al.*, 2004] and foraminifera [*Hönisch et al.*, 2003; *Foster*, 2008; *Rollion-Bard and Erez*, 2010] grown in laboratory experiments as a pH proxy. Therefore, the combination of these two proxies (B/Ca for [CO_3^{2-}] and $\delta^{11}B$ for pH reconstruction) might offer the opportunity to reconstruct the marine paleo-carbonate system as it is necessary to know two of the six parameters (pH, [CO_2], [HCO_3^-], [CO_3^{2-}], total alkalinity (A_T) and dissolved inorganic carbon (C_T)) to constrain the system and to establish how $CaCO_3$ saturation state of the oceans changed in the past [*Foster*, 2008].

[6] Several studies showed marine calcifying organisms being sensitive to elevated pCO_2 and lower pH [e.g., see *Doney et al.*, 2009]. However, blue mussels from Kiel Fjord showed the ability to settle, survive and even calcify under similar low pH conditions, when food concentrations are high [*Thomsen et. al.*, 2010]. Therefore mussels may be good candidate species to provide high resolution records of environmental conditions (like temperature, salinity, pH) and thus contribute to the

reconstruction of past climate. Additionally, the wide distribution and adaptation to a broad range of environments [*Gosling*, 1992] suggests *M. edulis* as a model organism for such studies.

[7] Since different studies using bivalve shells as proxy archives yielded contradictory results [*Klein et al.*, 1996a, 1996b; *Vander Putten et al.*, 2000; *Lazareth et al.*, 2003; *Immenhauser et al.*, 2005; *Freitas et al.*, 2008; *Wanamaker et al.*, 2008]. It is necessary to understand fundamental processes of bivalve biomineralization in order to understand differences between studies [e.g., *Carré et al.*, 2006; *Heinemann et al.*, 2008]. Extracellular body fluids (hemolymph and extrapallial fluid) are the connection between tissues and shell. Especially the extrapallial fluid (EPF) may influence calcification processes as it fills the extrapallial cavity enclosed by the shell, the periostracum and the outer mantle margin [*Wilbur and Saleuddin*, 1983].

[8] Thus, to contribute to the understanding of the ability of calcifying organisms to live under acidified conditions and of biomineralisation mechanisms of *M. edulis* we sampled hemolymph (HL) and extrapallial fluid (EPF) of mussels at the end of two CO_2 perturbation experiments. Fluids were analyzed for pH, C_T and elemental ratios. To consider the suitability of *M. edulis* shell as a proxy archive for ocean pH, boron isotopes ($\delta^{11}B$) were investigated by LA-MC-ICP-MS (method see *Fietzke et al.* [2010]) in shell portions precipitated during the experimental incubation (shells from the long-term growth study described by *Thomsen et al.* [2010]).

2. Material and Methods

2.1. Culture and Samples

2.1.1. General Setup

[9] Two experiments were conducted to investigate how increased water pCO_2 influences different parameters (pH, pCO_2, HCO_3^-, CO_3^{2-} and B/Ca, Mg/Ca and Sr/Ca ratios) of *Mytilus edulis* extracellular fluids and to test whether shell boron isotopes can be used as a pH proxy of the surrounding seawater.

[10] Atmospheric pCO_2 averaged 386 μatm in 2009 and concentrations are predicted to reach values between 700 and 1000 μatm by the year 2100 [*Intergovernmental Panel on Climate Change*, 2007]. Particular marine habitats like Kiel Fjord (Western Baltic Sea, Germany) are already exposed to values above 2000 μatm and might occasionally encounter pCO_2 values of >4000 μatm if water surface pCO_2 doubles [see *Thomsen et al.*, 2010]. Thus, pCO_2 levels of 380–4000 μatm were chosen for the experimental incubations. All specimen used in the experiments were collected from subtidal populations in Kiel Fjord (54° 19.8′ N; 10° 9.0′ E) and cultured at the culturing facilities of the Leibniz Institute of Marine Sciences (IFM-GEOMAR) in Kiel. Animals were acclimated under control conditions for 2 weeks prior to experimentation.

[11] The experiments were conducted in a flow through system (200 ml min^{-1} per experimental unit) using water from Kiel Fjord which was cleaned (50–5 μm filters, UV-sterilized) and pumped into a storage tank, where it was aerated. The air saturated water was pumped to a header tank and then supplied to the aquaria via gravity feed. Different CO_2-air-mixtures were used to equilibrate the experimental seawater (for a more detailed description see the auxiliary material).[1]

[12] Water pH was measured daily between Mondays and Fridays with a WTW pH meter (pH 340i, electrode: Sen Tix 81 (calibrated with Radiometric IUPAC precision pH buffer 7 and 10), WTW GmbH, Weilheim, Germany; ΔpH ± 0.01). All pH values measured with the pH meter were corrected with respect to the pH values calculated from weekly A_T and C_T measurements (n = 223, R^2 = 0.949) as the latter provide more accurate results:

$$pH_{corrected} = 0.9781 * pH_{measured} + 0.2123 \quad (2)$$

For all pH values reported in this paper, the NBS scale was used.

[13] Hemolymph (HL) and extrapallial fluid (EPF) samples were taken directly after removing the bivalves from the aquaria. Valves were opened carefully to not injure the mantle and held open with a 1000 μl pipette tip. Hemolymph was drawn anaerobically with a syringe from the posterior adductor muscle and EPF was taken from the extrapallial space by inserting a flexible syringe needle (Ø 0.6 × 80 mm) between shell and the pallial line.

[14] From all aquaria water temperature, salinity (with WTW cond 315i salinometer and WTW TETRACON 325 probe) and pH were measured daily. A_T and C_T samples were taken for carbonate system calculations (Table 1; for more details of exp. 2 see *Thomsen et al.* [2010]).

[1] Auxiliary materials are available in the HTML. doi:10.1029/2011GC003790.

Table 1. Water Conditions During Experimental Trials[a]

Treatment	pH_{NBS}	A_T (μmol kg^{-1})	C_T (μmol kg^{-1})	pCO_2 (μatm)	Ω_{calc}	Ω_{arag}
			First Experiment[b]			
387 μatm	8.02 ± 0.08	2031 ± 74	1965 ± 63	577 ± 95	1.8 ± 0.3	1.1 ± 0.2
560 μatm	7.93 ± 0.07	2031 ± 74	1988 ± 62	722 ± 97	1.5 ± 0.2	0.9 ± 0.1
840 μatm	7.86 ± 0.05	2032 ± 72	2004 ± 65	839 ± 80	1.3 ± 0.2	0.8 ± 0.1
1120 μatm	7.77 ± 0.05	2033 ± 73	2026 ± 66	1041 ± 93	1.1 ± 0.1	0.5 ± 0.1
1400 μatm	7.71 ± 0.05	2033 ± 73	2040 ± 66	1195 ± 98	0.9 ± 0.1	0.6 ± 0.1
4000 μatm	7.28 ± 0.08	2040 ± 72	2172 ± 66	3352 ± 566	0.4 ± 0.1	0.2 ± 0.04
			Second Experiment[c]			
387 μatm	8.13 ± 0.02	1966 ± 3	1891 ± 5	500 ± 30	1.94 ± 0.04	1.14 ± 0.04
1400 μatm	7.72 ± 0.06	1968 ± 5	1984 ± 12	1350 ± 200	0.81 ± 0.09	0.48 ± 0.06
4000 μatm	7.26 ± 0.04	1970 ± 4	2126 ± 13	3950 ± 220	0.28 ± 0.02	0.17 ± 0.01

[a]Data for experiment 2 are from *Thomsen et al.* [2010]. Errors are given as SD. Values represent mean over whole experiment.
[b]Temperature in °C, 12.1 ± 0.6; salinity,18.8 ± 1.59.

2.1.2. Experiment 1

[15] The first experiment was conducted from September, 3rd to December, 18th 2008. We cultured *Mytilus edulis* (~140 individuals per tank) ranging in size from 12.4 mm to 46.7 mm at six different pCO_2 levels (380, 560, 840, 1120, 1400 and 4000 μatm). Shell length was measured at the longest axis (from umbo to edge at opposite site of the shell) using calliper with an accuracy of 0.1 mm. Fluids were sampled from the five largest bivalves (45.6 ± 0.6 mm) in each treatment. The experimental aquaria had a volume of 15 l and were exposed to a 14:10 hour light:dark cycle. Water pH ranged from 8.02 ± 0.08 to 7.28 ± 0.08 in the different treatments. Temperature was 12.2 ± 0.6 °C and salinity 18.8 ± 1.6.

[16] During the first 6 weeks mussels were fed with "DTs Live Marine Phytoplankton-Premium Reef Blend" (DT's Plankton Farm, Sycamore, IL, USA), a live phytoplankton mixture of three marine algae species (*Phaeodactylum tricornotum* (40%), *Nannochloropsis oculata* (40%) and *Chlorella sp.* (20%)). Since mussel growth was unsatisfactory during that period the animals were additionally fed with *Artemia salina* after that period as *Wong and Levinton* [2004] reported best growth of *M. edulis* with a mixture of phyto- and zooplankton. Food was provided on 3 days a week (~75 mg algal biomass and ~45 mg *Artemia salina* (both dry weight) day^{-1} aquarium^{-1}) by closing the flow through for 4 hours and adding food directly into the aquaria.

[17] Fluid samples were centrifuged (30 s) and pH was measured with a microelectrode (WTW Mic-D). Then total dissolved inorganic carbon (C_T) was determined immediately after sampling via a Corning 965 CO_2 analyzer, (Olympic Analytical Service Essex, UK; accuracy 0.1 mM) in two 100 μl subsamples. Carbonate parameters (pCO_2, $[HCO_3^-]$, and $[CO_3^{2-}]$) of the fluids were calculated from measured pH and C_T with using the Henderson-Hasselbalch equation:

$$pCO_2 = C_T\left(10^{pH-pK_1'}\cdot\alpha_{CO2}+\alpha_{CO2}\right)^{-1} \quad (3)$$

$$\left[HCO_3^-\right] = 10^{pH-pK_1'}\cdot\alpha_{CO2}\cdot pCO_2 \quad (4)$$

$$\left[CO_3^{2-}\right] = 10^{pH-pK_2'}\cdot\left[HCO_3^-\right] \quad (5)$$

$$pK_1' = pH - \log\left(\frac{C_T}{pCO_2\alpha_{CO2}}-1\right) \quad (6)$$

where α is the CO_2 solubility coefficient. pK_1' and pK_2' are the first and second apparent dissociation constants of carbonic acid. α_{co2} was calculated (0.045 mol/l*μatm) [*Weiss*, 1974] and pK_2' (9.321) [*Roy et al.*, 1993] was chosen according to experimental temperature and salinity. pK_1' was calculated from pH_{NBS}, C_T and pCO_2 (measured in *Mytilus edulis* body fluids by [*Thomsen et. al.*, 2010]) using equation (6) [*Albers and Pleschka*, 1967]. A linear relationship was found for pK_1' in relation to pH_{NBS}. After adjusting temperature and salinity according to this experiment the regression for pK_1' for *M. edulis* internal fluids was $pK_1' = -0.1323pH + 7.2371$ ($R^2 = 0.3027$).

2.1.3. Experiment 2

[18] From 14th of May to 13th of July a second (long-term growth) experiment was conducted using three pCO_2 levels (380, 1400 and 4000 μatm). The level of replication was four. Each 15 l aquarium contained eight small (mean 5.5 mm), eight medium sized (mean 13 mm) and two big (mean

Table 2. Instrumental Parameters Laser Ablation

Parameter	Value
AXIOM MC-ICP-MS	
Cool gas	14 l/min
Auxiliary gas	1.8 l/min
Nebulizer gas	0.9 l/min (Ar)
RF power	1250 W
Reflected power	3 W
Accelerating voltage	4972 V
Cones	R.A. Chilton RAC19/RAC705
Resolution	500res
UP193FX	
Ablation cell gas	0.6 l/min (He)
Spot size	150 μm
Fluence	2.3 J/cm^2
Fluence Preablation	0.6 J/cm^2
Repetition rate	30 Hz
Scan mode	Line scan (1000 μm @ 12 μm/s)
Scan mode Preablation	Line scan (1000 μm @ 50 μm/s)
Pause between lines	90 s

40 mm) mussels. Shells from the medium specimen (13.3 ± 1.4 mm) and fluids from the big specimen were investigated in this study. The experiment was conducted under a mean temperature of 13.8 ± 0.6 °C and a salinity of 15 ± 0.6. The light/dark cycle was 14:10 hours. Food (*Rhodomonas sp.* suspension, 2900 ± 1200 cells ml^{-1}) was provided continuously by adding it into the aquaria at a rate of 100 ml min^{-1}. Body fluids of the big specimen were frozen at −20 °C directly after sampling and used for elemental analyses by ICP-OES (SPECTRO *Ciros^{CCD} SOP*) at the Institute of Geosciences, University Kiel, Germany. The tissue of the medium sized mussels was removed and shells were dried at 20 °C for 5 days.

[19] EPF pH could not be measured directly in experiment 2 specimen as there was not enough material for sub sampling and therefore no possibility to measure pH directly without contaminating the fluid samples with respect to boron (due to the high boron content of the glass pH electrode). Therefore boron isotopes and elemental ratios (experiment 2) were compared to EPF pH values estimated from experiment 1 fluid values (n = 12) and HL pH values measured in large *M. edulis* of an additional experiment presented by *Thomsen et al.* [2010]. The regression for the fluids and the corresponding water was pH_{EPF} = 0.383(37) pH_{water} + 4.493(288) (R^2 = 0.914). Additionally the mean offset between EPF and HL (0.037; experiment 1 this study; n = 6 each) was considered. Regression results for each treatment and the mean offset between HL and EPF pH have been subtracted from water pH of experiment 2.

2.2. Analytical Methods

[20] The fluids from the second experiment (5–7 samples per treatment) were thawed and diluted 50fold with ultrapure 2% HNO_3. They were analyzed for Mg/Ca, Sr/Ca and B/Ca elemental ratios by ICP-OES (Inductively Coupled Plasma–Optical Emission Spectrometry; SPECTRO *Ciros^{CCD} SOP*) at the Institute of Geosciences, University Kiel, Germany. An intensity-ratio calibration procedure was applied using matrix-matched calibration standards and IAPSO seawater as a consistency standard. External precision of the elemental ratios was ~0.1% RSD for Mg/Ca and Sr/Ca, and 1–4% RSD for B/Ca.

[21] Ten shells from each treatment of the second experiment were broken to obtain fragments that were grown under treatment conditions (defined by length growth). Subsequently, the fragments were bleached in 10% NaOCl (1% active chlorine) for two days to remove organic components (periostracum and other shell organics). After 24 hours samples were placed into an ultra sonic bath for 10 min and the bleaching solution was replaced. Afterwards shells were rinsed three times with ultrapure water (18.2 MOhm) and then dried at 20 °C. To prevent dissolution of the carbonates the water was adjusted to a pH of ~9 by adding NH_4OH. Boron isotopes were measured by LA-MC-ICP-MS (Laser Ablation–Multicollector–Inductively Coupled Plasma–Mass Spectrometry; Thermo Fisher MC-ICP-MS *AXIOM*, originally designed and manufactured by VG) connected to an ESI New Wave Research *UP193FX* excimer laser ablation system equipped with an ESI New Wave Research *LFC* (large format cell) via a standard sample standard bracketing procedure described by *Fietzke et al.* [2010]. Soda lime glass SRM (NIST610) was used as external standard as *Fietzke et al.* [2010] showed that there is no matrix effect between carbonate and glass. First, three lines (1000 μm x 150 μm) were measured on the NIST. Then, seven lines of sample and seven lines of standard were measured in alternation. Prior to each of these measuring procedures, a preablation was conducted to remove surface contamination. All instrumental parameters are given in Table 2. The location of the sample lines on *M. edulis* shells is shown in Figure 1.

3. Results and Discussion

3.1. Water Parameters

[22] Since the experimental incubation systems used in this study were flow through designs with con-

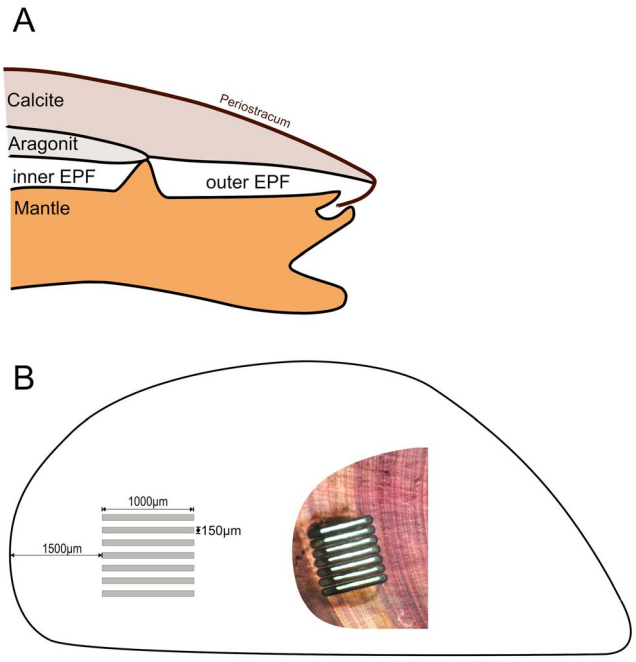

Figure 1. (a) Modified schematic anatomy of a bivalve (redrawn after *McConnaughey and Gillikin* [2008]). (b) Position of laser ablation lines on *M. edulis* shell (scheme of whole shell and picture of sampled Laser area).

tinuous supply of water from Kiel Fjord, all treatments were influenced by changes in Kiel Fjord water chemistry. Although the water was vigorously aerated prior to introduction into the experimental aquaria, mean seawater pCO_2 deviated from the nominal values. Mean seawater conditions during the two experiments are given in Table 1. Carbonate system speciation measured in the extracellular fluids are not compared to these mean values but to values from dates of sampling (Table 3).

3.2. Acid-Base Parameters of Fluid Samples (Experiment 1)

[23] Overviews of all mean extracellular acid-base parameters of the internal fluids (HL and EPF) are given in Table 3 and Figure 2 (for detailed data and regressions see the auxiliary material).

[24] High extracellular pCO_2 values between 1000 and 4000 μatm are found in all aquatic ectothermic metazoans, as diffusive excretion of metabolic CO_2 depends on a relatively steep gradient of CO_2 from the body fluids to the seawater (see *Melzner et al.* [2009] for a review). Additional increases in extracellular pCO_2 under hypercapnia [*Pörtner et al.*, 2004; *Michaelidis et al.*, 2005; *Spicer et al.*, 2007; *Thomsen et al.*, 2010] are necessary in order to maintain metabolic CO_2 flux. In this study a linear increase of fluid pCO_2 with increasing water pCO_2 was measured, with slightly lower values in the HL (1648-3122 μatm) than in the EPF (1938-3430 μatm). However, pCO_2 values in HL and EPF were not significantly different from each other. At the highest pCO_2 treatment a reduced pCO_2 diffusion gradient could be observed (only low offset between water and extracellular body fluid pCO_2), indicating reductions in metabolism in experiment 1. Similar reductions in metabolic rate have been observed in *M. galloprovincialis* under increased water pCO_2 by *Michaelidis et al.* [2005]. This was indicated by a significant decrease in oxygen consumption. However, no reductions in metabolism were witnessed in another experiment using Baltic Sea *M. edulis* at 4000 μatm under higher food concentrations, suggesting that the observed reduction in the CO_2 diffusion gradient in experiment 1 of this study might be due to food limitation [*Thomsen and Melzner*, 2010]. A recent study on the same mussel population could confirm the strong role of food supply on biomineralisation and shell corrosion intensity [*Melzner et al.*, 2011].

[25] High HL and EPF pCO_2 values are the primary driving force for comparatively low HL and EPF pH values: values range from 7.37 to 7.54 (HL) and 7.34 to 7.52 (EPF) respectively, which is significantly lower in all treatments than seawater pH with values of 8.10 to 7.39. Due to the progressive reductions in the pCO_2 gradient between extracellular fluids and seawater, the pH offset

Table 3. Acid-Base Status of Hemolymph and Extrapallial Fluid and of the Water at Date of Sampling (Experiment 1)

Treatment pCO_2 (μatm) at Day of Sampling	EPF (SD, n)	HL (SD, n)	Water at Day of Sampling
	pH		
452	7.52 (0.04; 3)	7.54 (0.05; 3)	8.10
574	7.50 (0.09; 4)	7.53 (0.05; 3)	8.01
731	7.46 (0.05; 3)	7.52 (0.04; 3)	7.91
906	7.45 (0.02; 3)	7.47 (0.05; 4)	7.82
1123	7.41 (0.04; 4)	7.47 (0.07; 4)	7.73
2724	7.34 (0.09; 4)	7.37 (0.02; 4)	7.39
	C_T *(mM)*		
452	1.79 (0.18; 3)	1.63 (0.31; 3)	1.96
574	1.41 (0.03; 4)	1.62 (0.24; 3)	1.98
731	1.71 (0.18; 3)	1.79 (0.21; 3)	2.01
906	1.55 (0.07; 3)	1.77 (0.14; 4)	2.03
1123	1.85 (0.05; 3)	1.61 (0.34; 4)	2.05
2724	1.91 (0.07; 4)	1.89 (0.31; 4)	2.15
	pCO_2 *(μatm)*		
452	1938 (93; 3)	1648 (183; 3)	452
574	1717 (427; 4)	1623 (190; 3)	574
731	2297 (284; 3)	1987 (383; 3)	731
906	2033 (169; 3)	2226 (348; 4)	906
1123	2611 (181; 4)	2199 (744; 4)	1123
2724	3430 (696; 4)	3122 (578; 4)	2724
	HCO_3^- *(mM)*		
452	1.70 (0.18; 3)	1.55 (0.30; 3)	1.85
574	1.33 (0.04; 4)	1.54 (0.23; 3)	1.89
731	1.61 (0.17; 3)	1.70 (0.19; 3)	1.92
906	1.46 (0.07; 3)	1.67 (0.13; 4)	1.94
1123	1.73 (0.06; 4)	1.52 (0.31; 4)	1.96
2724	1.77 (0.08: 4)	1.75 (0.28; 4)	2.02
	CO_3^{2-} *(mM)*		
452	0.028 (0.005; 3)	0.027 (0.009; 3)	0.085
574	0.020 (0.005; 4)	0.027 (0.005; 3)	0.070
731	0.022 (0.004; 3)	0.027 (0.003; 3)	0.056
906	0.020 (0.001; 3)	0.024 (0.003; 4)	0.047
1123	0.023 (0.003; 4)	0.021 (0.003; 4)	0.038
2724	0.019 (0.004; 4)	0.019 (0.003; 4)	0.017

between fluids and water decreased as well. At the highest seawater pCO_2 level (2724 μatm) pH values of EPF/HL and water became nearly identical. For each treatment, pH values of HL were slightly lower than EPF pH with a mean offset of 0.04 units. However, these differences were not significantly different. Fluid pH values decreased by 0.17 units in the HL and by 0.18 units in the EPF compared to a drop of 0.71 units in the water. The change of hemolymph pH relative to the change in seawater ($\Delta pH_{HL}/\Delta pH_{SW}$) in this study was consistent with that found by *Michaelidis et al.* [2005] (Table 4). The pH in *M. galloprovincialis* HL decreased from 7.55 to 7.36 (SW: 8.05 to 7.3). Although the control water pCO_2 in their study was more than double compared to the study of *Thomsen et al.* [2010] and this study (1079 μatm and 470/452 μatm, respectively) the water pH was nearly the same. This may be due to the difference in alkalinity but also to the observed accumulation of bicarbonate in that study. In general the Mediterranean Sea is characterized by a higher alkalinity (2500 μmol kg^{-1}) in comparison to the Baltic Sea (~1900 μmol kg^{-1}). The resulting higher buffer capacity leads to a smaller drop in pH in the Mediterranean Sea at comparable seawater pCO_2 values. This explains the different results with respect to hemolymph pH between the studies of *Thomsen et al.* [2010] and of *Michaelidis et al.* [2005] although the pCO_2 was nearly similar. *Thomsen et al.* [2010] measured a drop in *M. edulis* HL pH relative to ambient water pH twice as high (8.05 to 7.08 (SW) related to 7.59 to 7.16 (HL)) as measured by *Michaelidis et al.* [2005] and in this study. *Thomsen et al.* [2010] conducted experiments at pCO_2 values ranging from 470 to 4250 μatm. However, total alkalinity in the present study was 130 to 150 μmol/kg higher than in that of *Thomsen et al.* [2010], which is related to the lower salinity in the latter (11.8 compared to 18.8 in this study). This disparity in salinity/alkalinity was due to the high seasonal variability in Kiel Fjord and is likely to be responsible for the different drop in body fluid pH. These results show the high variability in extracellular pH that can be caused by the strong influence of alkalinity and of the reduced metabolism (after three months of experiment with low food conditions) on the pH decrease in response to increased pCO_2.

[26] However, no extracellular accumulation of bicarbonate (HCO_3^-) could be measured in HL or EPF, indicating that *M. edulis* does not actively buffer HL or EPF to avoid decreasing pH values. For both fluids, [HCO_3^-] was below that of the seawater and no significant differences (non-linear regressions) were found between HL and EPF. Accumulation of [HCO_3^-] (nearly equivalent to a net excretion of protons) is an efficient mechanism of extracellular pH stabilization that is primarily employed by active marine ectothermic organisms that are characterized by pH sensitive respiratory pigments (see *Melzner et al.* [2009] for a review). The magnitude of this [HCO_3^-] accumulation response varies between taxa, but highest degrees of [HCO_3^-]

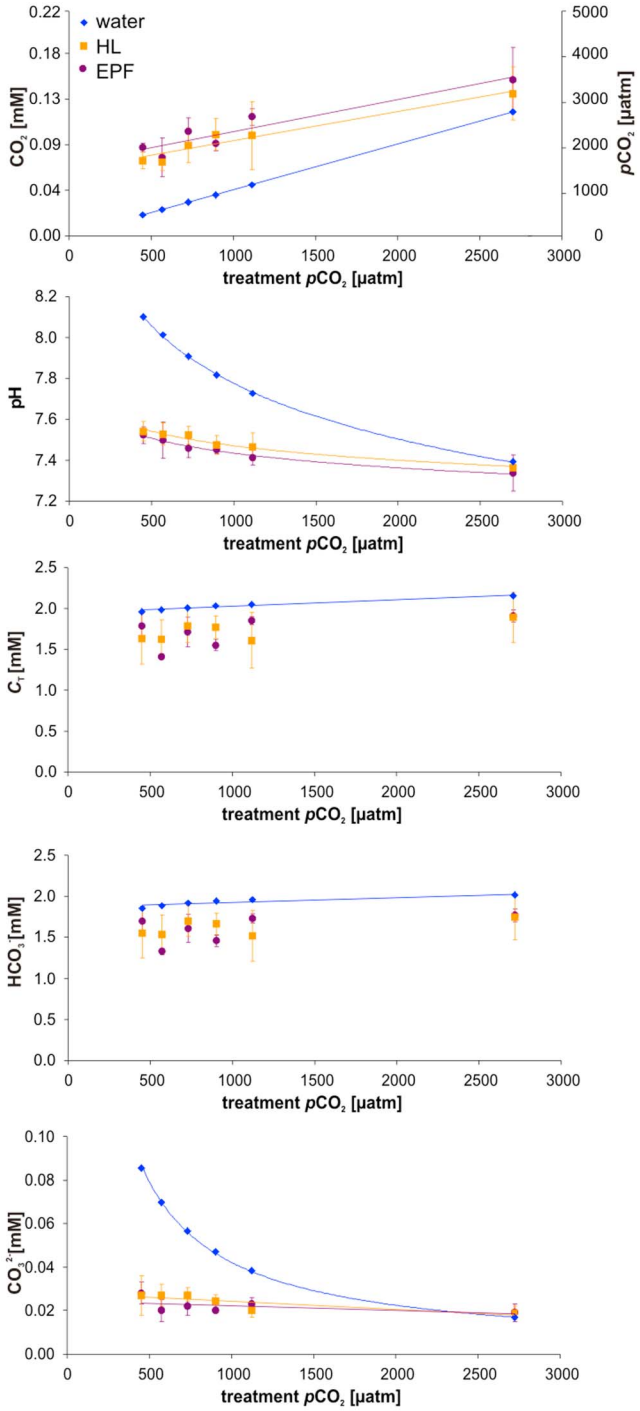

Figure 2. Acid-base status of hemolymph and extrapallial fluid and of the seawater at sampling days compared to treatment pCO_2 at the day of sampling (exp. 1).

related pH compensation are found in teleost fish, decapod crustaceans and cephalopod molluscs [*Larsen et al.*, 1997; *Pane and Barry*, 2007; *Gutowska et al.*, 2010]. Two studies also reported [HCO_3^-] accumulation in mussels to buffer extracellular pH [*Lindinger et al.*, 1984; *Michaelidis et al.*, 2005]. However, both of these were conducted in closed/recirculating systems and it is evident [*Lindinger et al.*, 1984] or very possible [*Michaelidis et al.*, 2005] that this increase in extracellular [HCO_3^-] was due to increases in ambient water [HCO_3^-] due to external/internal shell dissolution [see *Thomsen*

Table 4. Comparison Between the Hemolymph Acid-Base of This Study and the Studies of *Michaelidis et al.* [2005] and *Thomsen et al.* [2010]

	$pCO_{2\ SW}$ (μatm)	$pCO_{2\ HL}$ (μatm)	$\Delta\ pCO_2$ (HL-SW)	pH_{SW}	pH_{HL}	$\Delta pH_{HL}/\Delta pH_{SW}$	Salinity	A_T
M. edulis, control	452	1648	1196	8.10	7.54	0.239	18.8	2031
This study, treatment	2724	3122	398	7.39	7.37		18.8	2040
M. edulis, control	470	1697	1227	8.05	7.55	0.402	15.0	1901
Thomsen et al. [2010], treatment	4310	4960	650	7.08	7.16		15.0	1891
M. galloprovincialis, control	1079	1513	434	8.05	7.59	0.267	32.0	
Michaelidis et al. [2005], treatment	5026	5724	697	7.30	7.36		32.0	

et al., 2010]. Consequently, carbonate concentrations are lower in HL and EPF than in seawater, decreasing with decreasing pH (increasing pCO_2). In the highest pCO_2 treatment (2724 μatm), HL and EPF [CO_3^{2-}] were almost equal to seawater [CO_3^{2-}] within error bars.

[27] Generally, it is important to note that the acid-base conditions in the body fluids, especially in the EPF, which is in direct contact with the inner shell surface, may impact calcification and existing shell structures. Reduced shell growth under high pCO_2 (4000 μatm) was reported by *Thomsen et al.* [2010] and *Thomsen and Melzner* [2010]. Yet experiment 2 specimens increased their shell mass by >150% during the 8 week experiment, indicating great biological control and an ability to precipitate a shell in fluids that are highly undersaturated with regard to calcium carbonate. However, results from *Thomsen et al.* [2010] demonstrate that external shell dissolution at the umbo region and thinner aragonite layers may be consequences of high seawater and EPF pCO_2 values.

3.3. Elemental Ratios of Extrapallial Fluid and Water (Experiment 2)

[28] Elemental ratios (B/Ca, Mg/Ca and Sr/Ca) were measured in the EPF of *M. edulis* cultured at three different pCO_2 levels (experiment 2, Table 5). Figure 3 shows the elemental ratios compared to EPF pH. The pH values were extrapolated using a regression of HL data on water pH (see methods). An increase of all elements (B, Mg and Sr) relative to Ca could be observed with rising pH. Although a high variability of the element concentrations was found in the fluid, especially B/Ca and Mg/Ca showed a linear trend (significant at 95% confidence level), while there is no significant trend for Sr/Ca. The variability between different individuals may be due to a strong individual physiological influence on element partitioning. The magnitude of increase with pH was variable for the different elements. For B/Ca ratios (19.8%) it was slightly more than twice as high as for Mg/Ca ratios (7.9%) and lowest for Sr/Ca ratios (1.2%).

[29] While minor amounts of Mg^{2+} and Sr^{2+} substitute for Ca^{2+} during calcification boron is believed to be incorporated into carbonates as $B(OH)_4^-$ replacing the carbonate ion in the lattice [*Vengosh et al.*, 1991; *Hemming and Hanson*, 1992; *Hemming et al.*, 1995; *Pagani et al.*, 2005]. Several studies reported different abiotic factors influencing the composition of bivalve shells. The B/Ca ratio has been shown to be related to pH in different carbonates [*Hemming and Hanson*, 1992; *Hobbs and*

Table 5. pH and Me/Ca Ratios of Treatment Water and EPF[a]

Treatment in μatm	Culture pH (SD; n) Experiment 2	EPF pH Calculated	Percent Shell Mass Growth
380	8.08 (0.01; 3)	7.55	242
1400	7.68 (0.01; 4)	7.40	236
4000	7.23 (0.03; 4)	7.22	158

Treatment in μatm	EPF B/Ca (SD; n) in mmol/mol	Water B/Ca (SD; n) in mmol/mol	EPF Sr/Ca (SD; n) in mmol/mol	Water Sr/Ca (SD; n) in mmol/mol	EPF Mg/Ca (SD; n) in mmol/mol	Water Mg/Ca (SD; n) in mmol/mol
380	56.2 (1.08; 5)	54.3 (1.54; 3)	8.10 (0.09; 6)	8.09 (0.05; 3)	4947 (54; 6)	4859 (22; 3)
1400	55.0 (3.56; 5)	53.9 (3.72; 4)	8.05 (0.12; 5)	8.13 (0.04; 4)	4878 (125; 5)	4974 (124; 4)
4000	52.7 (2.71; 6)	53.1 (2.09; 4)	8.06 (0.08; 6)	8.13 (0.12; 4)	4817 (62; 6)	4898 (116; 4)

[a]Water pH values represent the day of sampling. SD represents the error of different individuals (EPF) and different aquaria, respectively. The precision based on repeated standard (IAPSO) measurements was 0.20–0.25% for Mg, Ca and Sr and 0.88% for boron. EPF pH was calculated from experiment 1 and *Thomsen et al.* [2010] (see methods).

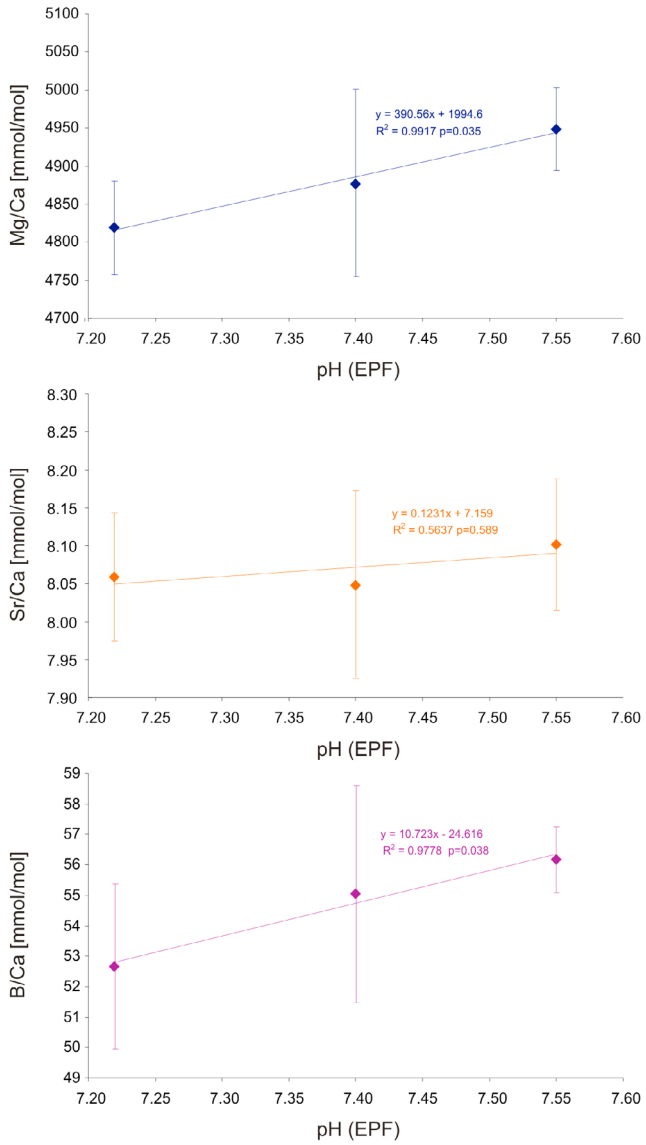

Figure 3. Elemental ratios of the EPF vs. calculated internal (EPF) pH values. Error bars show 1SD. Linear regressions show an insignificant increase (19.8% for B/Ca, 7.9% for Mg/Ca and 1.2% for Sr/Ca).

Reardon, 1999; *Sanyal et al.*, 2000; *Foster*, 2008]. *Ni et al.* [2007] found a systematic increase of B/Ca in *G. ruber* and *G. sacculifer* with increasing test size and suggested growth rate variations influencing the incorporation of boron. The results of *McCoy et al.* [2011] in *Mytilus californianus* shell did not reflect a distinct year-to-year correlation of boron concentrations with pH. However, the annual boron concentration probably reflects seasonal pH changes. The authors concluded that biological control of pH and/or boron concentrations in the EPF are the reasons for these observations. Our results show low EPF pH to be a result of high extracellular pCO_2 (Figure 2). As discussed above, high EPF pCO_2 values are necessary to excrete metabolic CO_2. Mg/Ca ratios in carbonates have been shown to be temperature-related in inorganically precipitated calcite [e.g., *Oomori et al.*, 1987; *Lopez et al.*, 2009] as well as in biogenic carbonates precipitated by different species like foraminifera [*Nürnberg et al.*, 1996; *Elderfield and Ganssen*, 2000; *Kısakürek et al.*, 2008] or bivalves [*Klein et al.*, 1996a; *Wanamaker et al.*, 2008]. *Hiebenthal* [2009] also reported a positive Mg/Ca-temperature relation in *M. edulis* shells from Kiel Fjord. Salinity was shown to likewise have an effect on Mg/Ca ratios in *M. edulis* calcite [*Dodd*, 1965].

[30] Other studies suggested different biological influences being responsible for the variations of

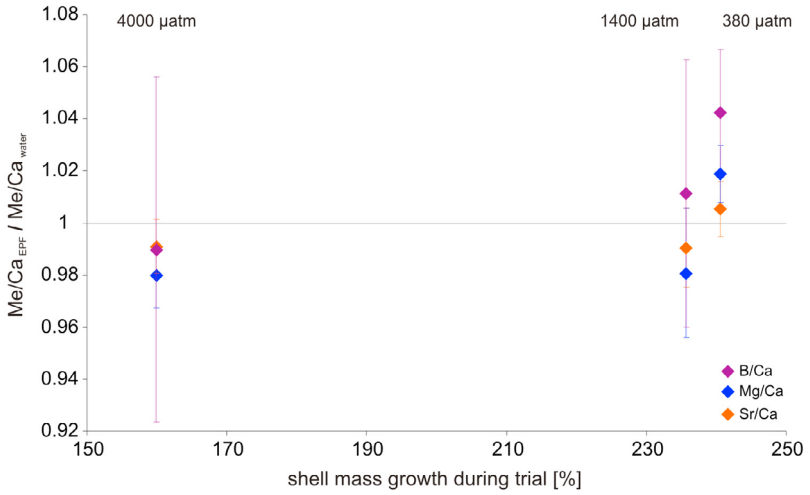

Figure 4. Distribution coefficient of EPF and water elemental ratios compared to shell mass growth of bivalves during experimental time (exp. 2). Errors are given as 1SD.

Me/Ca ratios between individuals of one species but also within single shells. In addition to their results mentioned above, *McCoy et al.* [2011] found organic-rich winter growth bands containing elevated B/Ca ratios. A comparable effect was reported by *Schöne et al.* [2010] for trace elements in *Arctica islandica* shells. They found a strong influence of organic matrix on the determination of Mg, Sr and Ca. This confirms our results from qualitative laser ablation measurements that show boron concentrations in the periostracum being about one magnitude higher than in the carbonate. *Carré et al.* [2006] observed increasing Me/Ca ratios (for Mg, Ba, Mn and especially Sr) in aragonitic bivalve shells (*Mesodesma donacium, Chione subrugosa*) when crystal growth rates increase. Their model predicts decreasing Ca^{2+}-channel selectivity when rates increase. *Klein et al.* [1996b] found significantly higher Sr/Ca ratios in a young, rapidly grown *Mytilus trossulus* than in a slowly grown adult individual. They concluded that shell precipitation along lateral margins is dominantly controlled by mantle metabolic activity at the site of carbonate formation. This suggests that also the elemental composition is dependent on the mantle activity as it seems to be influenced by growth rates. *Carré et al.* [2006] suggested the Sr/Ca measurements from different sections of *M. trossulus* shells (measured by *Klein et al.* [1996b]) can also be explained by differences in crystal growth. The results from *Ford et al.* [2010] support that of this study and show Mg/Ca ratios in shells of *Mytilus californianus* to be a function of growth rate rather than temperature related. *Takesue et al.* [2008] also found growth rate dependent alterations in Sr/Ca, B/Ca and Ba/Ca ratios in valves from *Corbula amurensis*. Sr/Ca seems to be influenced by both temperature and salinity [*Dodd*, 1965; *Wanamaker et al.*, 2008] but also by precipitation rates [*Lorens*, 1981; *Lorrain et al.*, 2005; *Freitas et al.*, 2006].

[31] Experiment 2 consisted of four replicate aquaria (two big individuals per aquarium) for each treatment, and variability in the elemental ratios of the water between different aquaria was observed (single values see auxiliary material). To eliminate this problem Figure 4 shows the distribution coefficients between EPF and water. When plotted against growth rates (shell mass growth during experiment in % from [*Thomsen et. al.*, 2010]) elemental ratios are slightly lower in the EPF than in the ambient water at lower growth rates at 4000 μatm. For this treatment external shell dissolution at the umbo region as well as thinner aragonite layers were observed [*Thomsen et. al.*, 2010]. At high growth rates (pCO_2 of 385 and 1400 μatm), elemental ratios are higher in the EPF than in the ambient water. Thus, with increasing growth rates elements like B, Mg and Sr become enriched in the EPF with respect to Ca. This effect may be due to higher growth and calcification rates. Therefore the EPF is more depleted with respect to calcium when compared to other elements and the elemental ratios of the EPF are shifted. As elemental ratios in the EPF increased with growth rate it is also likely that the elemental ratios in the shells rose when precipitated from altered fluids. Therefore our results support findings from different studies showing increased Me/Ca ratios in shells grown at high rates [e.g., *Klein et al.*, 1996b; *Carré et al.*, 2006; *Ford et al.*, 2010].

Table 6. pH and δ^{11}B of Treatment Water and EPF[a]

Culturing Water pH (2SE; n) Experiment 2	EPF pH Calculated	Shell δ^{11}B (SD; n) (‰, Experiment 2)	Shell Length (SD; n) (mm, Experiment 2)
8.11 (0.008; 53)	7.546	17.4 (3.1; 10)	22.9 (2.4; 10)
7.73 (0.020; 53)	7.396	18.3 (4.8; 11)	24.0 (1.4; 11)
7.28 (0.035; 53)	7.221	17.9 (5.5; 11)	20.7 (1.9; 11)

[32] Food availability, temperature, salinity and elevated pCO_2 (decreased pH) are influencing growth rates (and accordingly the amount of organic matter in the shell) of *M. edulis* [e.g., *Malone and Dodd*, 1967; *Kautsky*, 1982; *Kossak*, 2006; *Thomsen et al.*, 2010]. While the biological performance depends on these biotic and abiotic factors, seawater pCO_2 or pH does not seem to be directly reflected in Me/Ca ratios of bivalve carbonates, rather, they might influence them indirectly via decreased rates of growth and calcification due to a repartitioning of the energy budget of the mussel [*Thomsen and Melzner*, 2010]. Our results show that the dependence between growth and Me/Ca ratios incorporation is represented in the extrapallial fluid. Thus, elemental ratios (B/Ca, Mg/Ca, and Sr/Ca) in the EPF of *M. edulis* may primarily serve as an indicator for growth rates/shell dissolution as they become enriched with increasing length growth and depleted by dissolution. This indicates that *Mytilus edulis* shells cannot be directly used as archive for experienced environmental conditions. Extensive calibrations are necessary to see how parameters such as temperature or salinity influence growth rate. With such calibrations and one for the influence of growth rate on Me/Ca ratios in the shell it may be possible to reconstruct environmental conditions. However, the encountered variability is high due to individual physiology and a high amount of replicates must be measured.

3.4. Boron Isotope (δ^{11}B) Data of *M. edulis* Shells (Experiment 2)

[33] Shell portions precipitated during the experimental duration (additional length growth) were investigated for their δ^{11}B composition (Table 6). While the analytical precision of the carbonate boron isotope ratio determination was in the order of 0.9 ‰ the variability between individuals from the same treatment was much higher (3.1–5.5 ‰). This analytical precision results from a very low boron content in the shells, still the inter-individual variability is the dominating factor compromising the use of δ^{11}B. Using the empirical borate δ^{11}B/pH dependence [*Klochko et al.*, 2006] and the difference in the ambient water pH we expect a δ^{11}B change of 4.3 ‰ for this pH range (7.28–8.11). Considering this we would expect to find a systematic change in the mean δ^{11}B values depending on the pH. The results showed a high variability not only between different individuals but also within single shells. The high individual variability was already observed for the fluid elemental ratios. Additionally the considerable amount of boron in the periostracum (see section 3.3) suggests the organic matrix surrounding every single carbonate crystal could be a reason for this variability. Bivalve shells typically contain up to 5% of organic matter in the prismatic and nacre layer, respectively [e.g., *Wilbur and Bernhardt*, 1984]. The variability may be caused by individual and physiological influences as well as (crystal) growth rate. It is obvious that one factor inducing the variability in shell δ^{11}B is the variability in body fluid pH (EPF), not only between individuals but also over time. Extracellular pH for example was shown to decrease when bivalves close their shell valves [e.g., *Crenshaw and Neff*, 1969; *Crenshaw*, 1972]. *Crenshaw* [1972] observed a drop in EPF pH of *Mercenaria mercenaria* from 7.41 to 7.25 while valves were closed for 15 min or longer and a maximum drop in *M. edulis* to 7.2. The pattern how often and how long they close their valves for instance can be different between individuals. Average values of δ^{11}B ranged from 17.4 in the lowest pCO_2 (highest pH) to 17.9 ‰ in the highest pCO_2 (lowest pH) treatment, respectively. However, the highest value (18.3 ‰) was found at 1400 μatm but within the high variability no significant difference could be observed. Moreover no clear trend could be observed with pH but values differed from those of other biominerals when plotted against ambient water pH (Figure 5). Inorganically precipitated calcite [*Sanyal et al.*, 2000], foraminifera [*Sanyal et al.*, 2001] and corals [*Hönisch et al.*, 2004; *Reynaud et al.*, 2004] are characterized by δ^{11}B values that fit above the measured δ^{11}B curve of $B(OH)_4^-$ presented by *Klochko et al.* [2006] (Figure 5) which correlates well with ambient water pH. For the curves plotted the following parameters were used: pK_B = 8.597 [*Dickson*, 1990]; α = 1.0194 [*Kakihana et al.*, 1977]

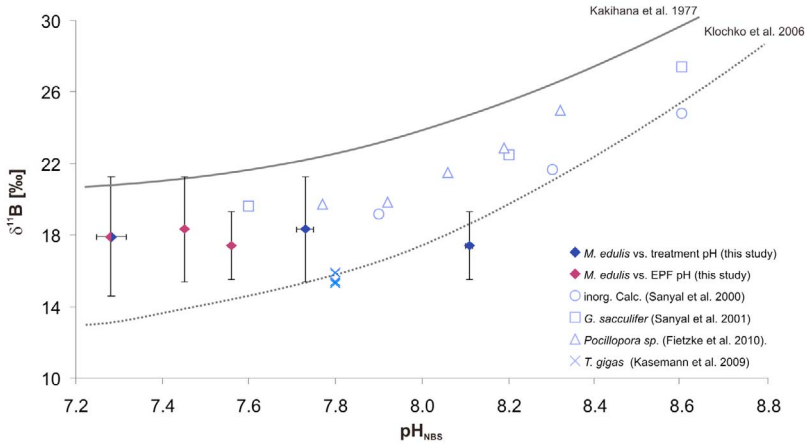

Figure 5. $\delta^{11}B$ measured in *M. edulis* shells and precipitated under different pCO_2 conditions in relation to data of other carbonates. Blue diamonds are displayed versus mean water pH during time of experiment 2, pink diamonds display the same $\delta^{11}B$ data plotted against EPF pH from experiment 1. Tridacna gigas data from *Kasemann et al.* [2009] are shown as a function of the approximated EPF pH value of Tridacna squamosa (at 28°C, SAL 31) observed by *Ip et al.* [2006] as no other data could be found. Errors are given as 1SD and display biological variability. The analytical precision of the carbonate boron isotope ratio determination was in the order of 0.9‰.

and $\alpha = 1.0272$ [*Klochko et al.*, 2006], respectively. Our data, when plotted against ambient water pH, differ from this pattern. When depicted against EPF pH, the pH at the calcification site, they also fit into this range between theoretical and measured curve. As HL and EPF pH are relatively low (calculated 7.22-7.55 for this experiment), $\delta^{11}B$ plots in a section of the curve where pH dependent changes in $\delta^{11}B$ are less pronounced. It seems likely that $\delta^{11}B$ in *M. edulis* shell reflects the internal fluid pH (EPF) and not that of ambient water. Because pH changes of internal fluids are minor in comparison to ambient water pH changes, only small changes in boron isotope fractionation of the shell can be expected. This fact and the high individual variability render boron isotopes in *M. edulis* shells a less sensitive pH-proxy for seawater pH variations.

[34] However, boron isotopes may be beneficial in providing more information on the processes of biomineralization in bivalves. The extrapallial space is described as the area where calcification takes place. Recent studies show how complex the underlying biological mechanisms probably are. Various organic components, such as a hydrophobic silk gel, acidic proteins, chitin and amorphous calcium carbonate as precursor phases are probably involved in the formation of bivalve shells [e.g., *Levi-Kalisman et al.*, 2001; *Weiss et al.*, 2009; *Suzuki et al.*, 2009; *Weiss*, 2010]. *Suzuki et al.* [2009] recently identified acidic matrix proteins (Pif97, Pif80) that bind to aragonite crystals and to chitin and might be important regulators of nacre formation. *Suzuki et al.* [2009] and *Weiss* [2010] proposed that crystals are formed in a microenvironment separated from the extrapallial fluid via a chitinous membrane. Nevertheless $\delta^{11}B$ in the shell indicate that shell formation proceeds at pH values typical of the EPF which indicates that calcification is influenced by EPF conditions independent whether calcification occurs separated in a microenvironment close to the shell.

4. Conclusions

[35] Body fluids of *Mytilus edulis* from Kiel Fjord kept under different seawater pCO_2 levels are characterized by low HL and EPF pH values. This is due to the requirements of metabolic CO_2 excretion. HL and EPF pH decreases with 0.024 units per 0.1 units in seawater pH. No significant bicarbonate accumulation to buffer internal pH could be observed. Elemental ratios (Me/Ca) in the extrapallial fluid correlate with growth rates due to the higher consumption of $[Ca^{2+}]$ during calcification compared to other elements. More data on the mechanisms of biomineralisation and the general composition of shells not only related to ambient water but also to the body fluids are needed. Also the impact of the EPF on calcification independent of the exact precipitation area (directly the EPF or a microenvironment) and the involvement of components like hydrophobic silk gel, acidic proteins, chitin and also maybe amorphous calcium carbonate should be subject of future

studies. It's necessary to better understand the underlying mechanisms of trace element partitioning and isotope fractionation in order to demonstrate a robust use of proxies in bivalve shells. Trace metal composition of the extrapallial fluid and hence of the shell seems to reflect abiotic conditions only indirectly via the impact on metabolism and growth rate. Boron isotopes in *M. edulis* shell do not reflect seawater pH, they seem to represent internal pH (EPF). In addition, boron isotopes show a high variability between individuals and also within single shells. This may reflect variable contributions of boron rich organic matrices present between single crystals. In the future, more attention should be paid to these organic shell parts also with respect to geochemistry as they often are not completely removed before measuring carbonates.

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