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Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*

F. Gazeau[1,2], J.-P. Gattuso[1,2], C. Dawber[3], A. E. Pronker[4], F. Peene[4], J. Peene[5], C. H. R. Heip[5,6], and J. J. Middelburg[5,7]

[1]CNRS-INSU, Laboratoire d'Océanographie de Villefranche (UMR7093), BP 28, 06234 Villefranche-sur-Mer Cedex, France
[2]Univ. Pierre et Marie Curie-Paris 6, Observatoire Océanologique de Villefranche, 06230 Villefranche-sur-Mer Cedex, France
[3]Univ. of Cambridge, Department of Earth Sciences, Cambridge CB2 3EQ, UK
[4]Roem van Yerseke BV, Gr. van Zoelenstraat 35, Postbus 25, 4400AA Yerseke, The Netherlands
[5]Netherlands Institute of Ecology (NIOO-KNAW), Centre for Estuarine and Marine Ecology, Postbus 140, 4400 AC Yerseke, The Netherlands
[6]NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, P.O. Box 59, 1790 AB Den Burg (Texel), The Netherlands
[7]Faculty of Geosciences, Utrecht Univ., P.O. Box 80021, 3508 TA Utrecht, The Netherlands

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Abstract. Several experiments have shown a decrease of growth and calcification of organisms at decreased pH levels. There is a growing interest to focus on early life stages that are believed to be more sensitive to environmental disturbances such as hypercapnia. Here, we present experimental data, acquired in a commercial hatchery, demonstrating that the growth of planktonic mussel (*Mytilus edulis*) larvae is significantly affected by a decrease of pH to a level expected for the end of the century. Even though there was no significant effect of a 0.25–0.34 pH unit decrease on hatching and mortality rates during the first 2 days of development nor during the following 13-day period prior to settlement, final shells were respectively 4.5 ± 1.3 and 6.0 ± 2.3% smaller at pH_{NBS} ~7.8 ($pCO_2 \sim$ 1100–1200 µatm) than at a control pH_{NBS} of ~8.1 ($pCO_2 \sim$ 460–640 µatm). Moreover, a decrease of 12.0 ± 5.4% of shell thickness was observed after 15d of development. More severe impacts were found with a decrease of ~0.5 pH_{NBS} unit during the first 2 days of development which could be attributed to a decrease of calcification due to a slight undersaturation of seawater with respect to aragonite. Indeed, important effects on both hatching and D-veliger shell growth were found. Hatching rates were 24 ± 4% lower while D-veliger shells were 12.7 ± 0.9% smaller at $pH_{NBS} \sim 7.6$ ($pCO_2 \sim 1900$ µatm) than at a control pH_{NBS} of ~8.1 (pCO_2 ~540 µatm). Although these results show that blue mussel larvae are still able to develop a shell in seawater undersaturated with respect to aragonite, the observed decreases of hatching rates and shell growth could lead to a significant decrease of the settlement success. As the environmental conditions considered in this study do not necessarily reflect the natural conditions experienced by this species at the time of spawning, future studies will need to consider the whole larval cycle (from fertilization to settlement) under environmentally relevant conditions in order to investigate the potential ecological and economical losses of a decrease of this species fitness in the field.

Correspondence to: F. Gazeau
(f.gazeau@obs-vlfr.fr)

1 Introduction

The atmospheric partial pressure of CO_2 (pCO_2) will continue to increase with projected values for the end of this century ranging from 500 to 1000 µatm, depending on the considered CO_2 emission scenario (IPCC, 2007). Because about one third of anthropogenic CO_2 emissions (from fossil fuel, cement production and land-use changes) has been stored in the oceans since the industrial revolution (Sabine et al., 2004), seawater pH has already declined by 0.1 unit compared with pre-industrial values (Orr et al., 2005) and it is projected to decrease by another 0.35 unit by the end of the century (Caldeira and Wickett, 2003). Ocean

acidification may have profound impacts on marine biota. Beside the direct effect of decreasing pH on the physiology and metabolism of marine organisms through a disruption of inter-cellular transport mechanisms (see Pörtner et al., 2004 for a comprehensive review), calcareous organisms are particularly sensitive due to the decreasing availability of carbonate ions (CO_3^{2-}) driven by increasing pCO_2. Indeed, this generates a decrease of the calcium carbonate saturation state (Ω):

$$\Omega = \frac{[CO_3^{2-}][Ca^{2+}]}{K'_{sp}}, \quad (1)$$

where K'_{sp} is the stoichiometric solubility product, which is a function of temperature, salinity, pressure and the mineral phase considered (calcite, aragonite or high-magnesian calcite). Cold waters will become undersaturated with respect to aragonite ($\Omega_{aragonite} < 1$) in a few decades (Orr et al., 2005). Since the seminal paper of Broecker and Takahashi (1966) reporting a dependency of calcification rates on $CaCO_3$ saturation state, several experimental studies have investigated the effect of a pCO_2 increase on the growth of calcifying organisms. Most studies have investigated primary producers (corals, coralline algae and coccolithophores) and have shown a very large range of responses (Feely et al., 2004; Kleypas et al., 2006; Doney et al., 2009).

Among calcifying species, molluscans are very important both in ecological and economical terms. Shellfish are ecosystem engineers governing energy and nutrient flows in coastal ecosystems, providing habitats for many benthic organisms and constituting an important food source for, for instance, birds, crabs, starfishes and fishes (Gutiérrez et al., 2003; Norling and Kautsky, 2007). Moreover, with an average annual increase of 7.7% over the last 30 years, global shellfish aquaculture production reached 13.1 million tons in 2008, corresponding to a commercial value of US $ 13.1 billion (FISHSTAT Plus vers. 2.3[1]). The Pacific oyster (*Crassostrea gigas*) was the most cultivated species in 2008 with a volume of 6.5 million tons or 9.5% of the total world aquaculture production while mussel production represented 1.9 million tons (US $ 390 million). A negative impact of ocean acidification on the growth of these species would, therefore, not only have major consequences for coastal biodiversity and ecosystem functioning and services, but will also cause a significant economic loss (Gazeau et al., 2007; Cooley and Doney, 2009).

The bivalve *Mytilus edulis* is a benthic invertebrate typical of the North Atlantic coast of North America, Europe, and other temperate and polar waters around the world. They live in intertidal areas attached to rocks and other solid substrates. This species reproduces by means of a planktonic larval stage (meroplanktonic species). Eggs are fertilized in the water column and, thanks to their internal energetic resources (lecithotrophic phase), develop to the ciliated trochophore stage and to the D-shaped veliger (shelled) stage within few days depending on the temperature conditions (Pechenik et al., 1990). These veliger larvae start to feed in the water column and gain weight until they reach the pediveliger phase (after few weeks) during which they try to find a place to settle. Larvae become competent to settle at a shell length of $\sim$260 µm but can delay metamorphosis and remain in the planktonic compartment until they reach $\sim$350 µm (Sprung, 1984). Once the settling conditions are favourable, metamorphosis occurs, plantigrade larvae attach to the substrate thanks to the secretion of the byssus and start to secrete the adult (dissoconch) shell.

Several studies have focused on the effect of projected pH levels on the growth of benthic (e.g. Gazeau et al., 2007; Ries et al., 2009) and planktonic (Comeau et al., 2009, 2010a, b) molluscs. Most of these studies have demonstrated a negative effect of ocean acidification on the growth of these organisms although recent experiments (Ries et al., 2009) have suggested a more complicated story with species-specific sensitivities to decreasing pH levels and positive effects on calcification rates in some cases. Early life stages of calcifying organisms are generally considered to be more sensitive to environmental disturbances (Raven et al., 2005). Moreover, amorphous calcium carbonate and aragonite have been identified as the main $CaCO_3$ mineralization form in molluscs larval stages (Medaković, 2000). Therefore, as aragonite is 50% more soluble than calcite, these aragonitic larval stages are expected to be more sensitive to ocean acidification than calcitic organisms. Indeed, several recent studies have focused on the effect of ocean acidification on the early development of mollusc species (Kurihara et al., 2007; Ellis et al., 2009; Kurihara et al., 2008; Miller et al., 2009; Parker et al., 2009; Talmage and Gobler, 2009; Watson et al., 2009) and most of them have reported negative impacts of decreasing pH levels on the growth and development of these organisms. So far, there have been no studies on the effect of ocean acidification on the larval development of the blue mussel (*Mytilus edulis*), the second most cultivated bivalve species in the world after *Crassostrea gigas*. Blue mussel aquaculture is very important in The Netherlands and consists almost entirely of bottom-culture, carried out on leased sites in the Wadden Sea and in the Oosterschelde estuary (Smaal, 2002). In the Oosterschelde estuary, mussel beds (both wild and from aquaculture) play a major role in the cycling of nutrients and are able to filter the entire volume of the basin in 4–5 days (Prins and Smaal, 1994). In the last two decades, there has been an overall decline in available mussel seed due to intense fishing strategies that has forced local farmers to initiate the production of spats through hatchery techniques (Pronker et al., 2008). The present study aims to investigate the effects of future ocean pH levels on the development of *Mytilus edulis* early larval stages in a commercial hatchery.

[1]FAO: Fisheries Department, Fisherie Information, Data and Statistics Unit. FISHSTAT Plus: Universal software for fishery statistical time series, Version 2.3, 2000.

2 Material and methods

2.1 Test animals and experimental conditions

To investigate the effect of rising atmospheric CO_2 on mussel (*Mytilus edulis*) larvae, experiments were carried out in mesocosms at the commercial hatchery *Roem van Yerseke* (Yerseke, The Netherlands) between 18 October and 27 November 2007. A group of approximately 150 ripe, bottom-cultured mussels from the Oosterschelde, a tidal inlet, were kept at a constant temperature (10 °C) for about 4 months. These animals originated from a same age-class and were fished in the tidal inlet and cultivated for about 2 years on commercial production plots. Before spawning, mussels (male and female) were cleaned with 1 µm filtered seawater and placed in a spawning tank. Mass spawning was initiated by rapidly raising water temperature from 10 °C to 19 °C. Fertilized eggs were retained on a submerged 30 µm sieve. During each experiment (see below), six enclosures were used, each of them containing 130 l of filtered (1 µm) seawater from the Oosterschelde. Three enclosures were continuously bubbled with ambient air ($pCO_2 \sim 380$ µatm) while the three others were bubbled with a mixture of ambient air and pure CO_2. The flow rates of CO_2 were regulated by means of digital thermal mass-flow controllers in order to reach the desired seawater pH.

2.2 Bioassay

In a first experiment (experiment #1), the effects of a pH decrease from ∼ 8.1 (control; $pCO_2 \sim 460$–640 µatm) to, successively, ∼ 7.8 ($pCO_2 \sim 1100$–1200 µatm) and ∼ 7.6 ($pCO_2 \sim 1900$ µatm) were investigated during the first two days of development (from eggs to D-shape larvae). After fertilization (see above), embryos (57.7 ± 4.9 µm of diameter) were counted, divided into 6 groups and transferred to the enclosures (3 controls, 3 low pH) at a density of approximately 10 embryos ml^{-1}. Embryos were maintained in batch conditions (no feeding, no water flowing) until the population reached the D-veliger stage (initial development of the shell, reached in about 2 days).

In a second experiment (experiment #2), larvae were exposed to pH values of ∼ 8.1 and ∼ 7.8 during the two weeks development period following the D-veliger stage. Embryos were grown at environmental pH (∼ 8.0–8.1) during 2 days, then counted and evenly transferred to the 6 enclosures (3 controls, 3 low pH) at a density of approximately 10 embryos ml^{-1}. Cultivation period lasted for 13 days (day 2 to day 15 of development) until the population reaches the pediveliger stage. Larvae were fed in a continuous flow-through system with a mixture of *Isochrysis* sp. (T-Iso, CCAP 927/14) and *Chaetoceros muellerii* (CCMP 1332) (2:1, based on cell counts) at a concentration of approximately 80 000 cells ml^{-1}. From day 4 to the end of the experiment, larvae were fed with a mixture of *Isochrysis* sp., *Pavlova lutherii* (CCAP 931/1) and *Chaetoceros muellerii* (2:1:2, based on cell counts) at a concentration of approximately 150 000 cells ml^{-1}.

2.3 Sampling and analytical measurements

At the end of experiment #1 (day 2) and three times a week during experiment #2, the enclosures were emptied, cleaned with a mixture of diluted acetic acid and HCl and rinsed with seawater. Water from the tanks was passed through a 90 µm sieve and larvae were concentrated in 2 l jars. A sub-sample of 50 ml was fixed in a 5% neutralized-formalin seawater solution to determine the larval abundance, hatching rates (% of D-veliger larvae) and size. After sampling during experiment #2, we made sure that pH was constant and at the desired pH level before reintroducing the larvae in the enclosures.

Larval abundance was estimated based on triplicate counting of 500 µl sub-samples, under a binocular microscope. Larvae shell length (measured on 100 individuals) was measured (anterior to posterior dimension of the shell parallel to the hinge) under a microscope (20×; 0.01 µm precision in length measurement). Shell thickness was estimated from scanning electron micrograph (SEM) images acquired using the JEOL JSE 820 microscope at Cambridge University. Dried larval shells were mounted onto double sided carbon tape and sectioned using a flat edge needle. Loose organic matter and residual shell were removed with a dry paintbrush. Larval shells were removed using a wet paintbrush, reoriented and remounted onto fresh tape attached to aluminium stubs and gold coated. Shell thickness was determined on 20 individuals of each replicate treatment. Hatching rates were defined as the percentage of "normal" D-shape larvae following the criteria proposed by His et al. (1997), after observation of a minimum of 500 larvae.

During the two experiments, pH, temperature and oxygen concentrations were continuously monitored in each enclosure using Metrohm and Consort electrodes, which were calibrated daily on the N. B. S. scale for pH (pH 4 and 7). Salinity was measured at the beginning of each incubation period (3) in each enclosure. Daily measurements of total alkalinity (TA) in the 6 enclosures were performed by Gran electro-titration on 50 ml samples filtered on GF/F membranes. Titrations of TA standard provided by A. G. Dickson (batch 82) were within 0.22 µeq kg^{-1} of the nominal value (2334.8 ± 3.3 µeq kg^{-1}; $n = 5$). pCO_2 was computed from pH_{NBS} and TA using the software package CO_2 1.1 (M. Frankignoulle) and the thermodynamic constants of Mehrbach et al. (1973). The solubility products for calcite and aragonite were from Morse et al. (1980).

2.4 Statistical analysis

For final shell length (experiment #1 and #2) and shell thickness (only experiment #2), differences between replicates of each treatment as well as between control and low pH_{NBS}

Table 1. Environmental parameters and carbon chemistry of experimental seawater during the course of the different experiments. pCO_2 and related carbonate parameters were computed from mean pH_{NBS} and total alkalinity during each incubation.

	Experiment #1: day 0 to day 2				Experiment #2: day 2 to day 15	
	Control-1	Low pH-1	Control-2	Low pH-2	Control	Low pH
Measured parameters						
Temperature (°C)	16.6 ± 1	16.7 ± 0.8	16.5 ± 1	16.2 ± 1.2	19.5 ± 0.3	19.2 ± 0.4
Salinity	32.1 ± 0.1	31.9 ± 0.2	32.0 ± 0.1	32.1 ± 0.1	31.4 ± 0.3	31.5 ± 0.1
Oxygen (% of saturation)	100 ± 3	94.7 ± 2.6	96.4 ± 2.5	97.6 ± 0.6	91.3 ± 6.9	91.8 ± 4.4
pH_{NBS}	8.15 ± 0.01	7.81 ± 0.01	8.09 ± 0.01	7.58 ± 0.01	8.03 ± 0.03	7.78 ± 0.05
Chlorophyll-*a* ($\mu g\,l^{-1}$)	–	–	–	–	29.12 ± 8.87	31.12 ± 12.55
Total alkalinity ($meq\,kg^{-1}$)	2.486 ± 0.006	2.483 ± 0.006	2.436 ± 0.006	2.437 ± 0.004	2.402 ± 0.02	2.403 ± 0.02
Calculated parameters						
pCO_2 (µatm)	468	1124	537	1929	642	1213
DIC ($mmol\,kg^{-1}$)	2.254	2.397	2.237	2.432	2.218	2.321
(HCO_3^-) ($mmol\,kg^{-1}$)	2.071	2.273	2.073	2.312	2.062	2.200
(CO_3^{2-}) ($mmol\,kg^{-1}$)	0.166	0.084	0.144	0.049	0.135	0.080
$\Omega_{aragonite}$	2.75	1.38	2.39	0.81	2.30	1.37
$\Omega_{calcite}$	5.12	2.58	4.45	1.52	4.27	2.54

treatments were tested using one-way ANOVA after testing for normality (Kolmogorov-Smirnov test). No significant differences were found in any of these parameters between the replicate tanks within each experimental condition. For hatching rates and abundances, as only one value was estimated per replicate, this latter was used to obtain grand means and standard deviations (SD) values for each treatment. Since normality tests could not be applied due to the small sample size, differences of hatching rates between control and low pH_{NBS} conditions were tested by means of unpaired Student's t-tests using a Welch correction that does not assume equal variance between the two groups (Graphpad Instat software). For all tests, differences were considered significant at $p < 0.05$. In the following section, data are presented as means ± SD.

3 Results

The environmental parameters as well as parameters of the carbonate chemistry are shown in Table 1. During experiment #1, pH_{NBS} was maintained, during the first set of incubations, at 8.15 ± 0.01 (Control-1) and 7.81 ± 0.01 (Low pH-1) corresponding to pCO_2 values of 468 and 1124 µatm, while during the second set of incubations, larvae were kept at pH_{NBS} 8.09 ± 0.01 (Control-2) and 7.58 ± 0.01 (Low pH −2), corresponding to pCO_2 values of 537 and 1929 µatm. During experiment #2, seawater pH_{NBS} was maintained at 8.03 ± 0.03 (Control, $pCO_2 = 642$ µatm) and 7.78 ± 0.05 (Low pH, $pCO_2 = 1213$ µatm). Results obtained during these 2 experiments are presented in Figs. 1, 2 and 3. Data used for these figures as well as experimental abiotic parameters (pH_{NBS}, TA and temperature) measured at the time of sampling are presented in the Supplement Table 1. In the Oosterschelde tidal inlet (1998–2006, monthly measurements, 5 stations), surface pH_{NBS} varied annually between 8.00 and 8.24, while TA varied between 2.334 and 2.567 $meq\,kg^{-1}$ (data not shown). In the fall (that is the time of the experimental period), pH_{NBS} and TA in the tidal inlet were, on average, 8.04 and 2.436 $meq\,kg^{-1}$, respectively. Significantly higher pH_{NBS} values have been obtained for the control tanks in our experiments due to the continuous bubbling with external air (∼380 µatm), especially during experiment #1 that was performed in batch conditions. During experiment #2, the continuous flow-through system did not allow such an efficient equilibration with air, leading to significantly lower pH_{NBS} values (8.03 ± 0.03), closer to environmental levels. It must be stressed that the pCO_2 levels for low-pH_{NBS} treatments were slightly outside the range projected for the end of the century (500–1000 µatm) and must be considered as extreme conditions. The objective of the experiment was to test the effect of a ∼0.3 pH_{NBS} unit decrease on these organisms and, in that sense, experimental conditions were successfully set up and controlled.

During experiment #1, a seawater pH_{NBS} decrease of 0.34 unit ($pH_{NBS} = 7.81$; $\Omega_{aragonite} = 1.38$) had a significant effect on mussel larvae development (Fig. 1). Although no significant effect was found on hatching rates (unpaired Student's t-test, $p > 0.05$), the average shell lengths at the end of the 2-day incubation period at pH_{NBS} 7.81 were significantly lower (ANOVA, $n = 100$, $p < 0.001$) than at higher

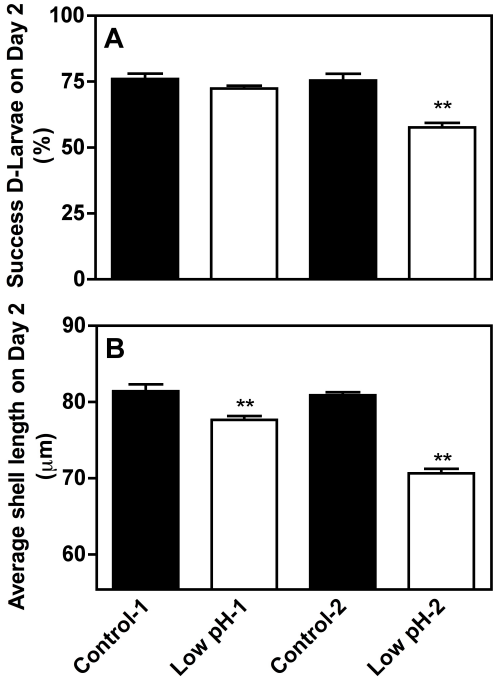

Fig. 1. Proportion of embryos that developed to D-shape larvae **(A)** and average length of D-shape shell **(B)** at the end of the two incubation periods during experiment #1, in control (black bars) and low pH (white bars) seawater. During the first incubation, seawater pH_{NBS} was maintained at 8.15 ± 0.01 (Control-1) and at 7.81 ± 0.01 (Low pH-1). During the second incubation, pH_{NBS} levels of 8.09 ± 0.01 and 7.58 ± 0.01 were used (Control-2 and Low pH-2 respectively). Errors bars represent standard deviations of the triplicate enclosures. **Significant difference between control and low-pH groups.

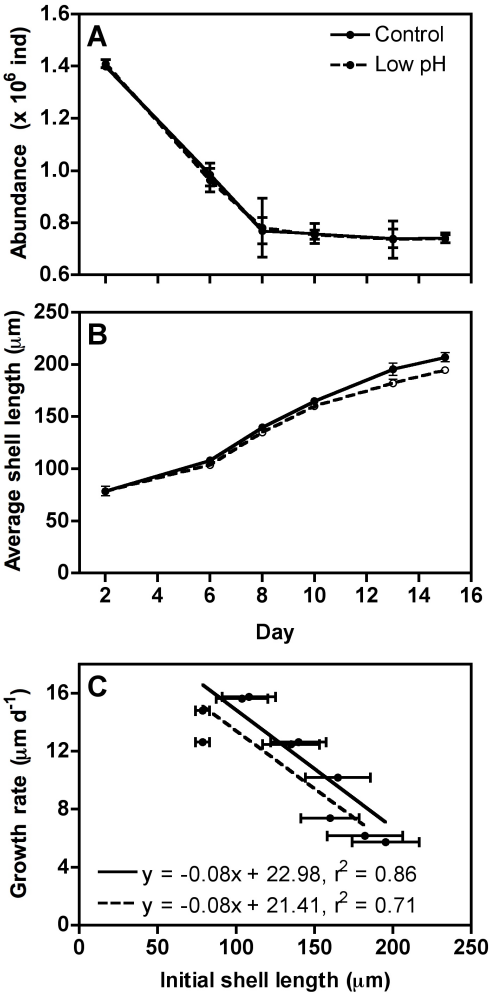

Fig. 2. Abundance **(A)**, average shell length **(B)** and shell growth rate as a function of initial shell length **(C)**, during experiment #2 (13 days, from D-shape to pediveliger larvae), in control (solid line, black dots) and CO_2 (dotted line, white dots) seawater. Seawater pH_{NBS} was maintained at 8.03 ± 0.03 and 7.78 ± 0.05 in control and low pH enclosures, respectively. Errors bars represent standard deviations of the triplicate enclosures.

pH_{NBS}. The relative decrease in shell length after 2 days has been estimated to $4.5 \pm 1.3\%$. In contrast, a decrease of 0.51 unit ($pH_{NBS} = 7.58$; $\Omega_{aragonite} = 0.81$) had large effects on both the hatching and growth rates. The hatching rates decreased by $24 \pm 4\%$ (unpaired Student's t-test, $p < 0.001$), while D-veliger shells were $12.7 \pm 0.9\%$ smaller (ANOVA, $n = 100$, $p < 0.001$). From day 2 to day 15 (experiment #2, Figs. 2 and 3), a decrease of seawater pH_{NBS} by 0.25 ($pH_{NBS} = 7.78$; $\Omega_{aragonite} = 1.37$) also did not have significant effects on larvae survivorship (unpaired Student's t-test, $n = 3$, $p > 0.05$) while a significant effect was found for final shell length (ANOVA, $n = 100$, $p < 0.001$), corresponding to a relative decrease of $6.0 \pm 2.3\%$. This relative decrease of shell length was statistically significant after day-13 of development. Growth rates, calculated as the difference in shell length between 2 sampling times divided by the time elapsed (d), decreased with increasing shell length (Fig. 2c) under both control and low-pH conditions. Statistically significant linear relationships between growth rates and initial shell length showed a shift to lower growth rates under low-pH conditions which was maintained throughout the experimental period. Effects on final shell thickness (Fig. 3) were more important with a significant (ANOVA, $n = 20$, $p < 0.001$) relative decrease of $12.0 \pm 5.4\%$.

4 Discussion

In the past few years, several papers have reported on the impacts of seawater acidification on the growth and development of shellfish early life stages. Kurihara et al. (2007, 2008) have demonstrated that a pH_{NBS} decrease to ~ 7.4 (-0.7 as compared to control values) caused a significant alteration of *Crassostrea gigas* and *Mytilus galloprovincialis* early (up to 6 d) larval development, with significant

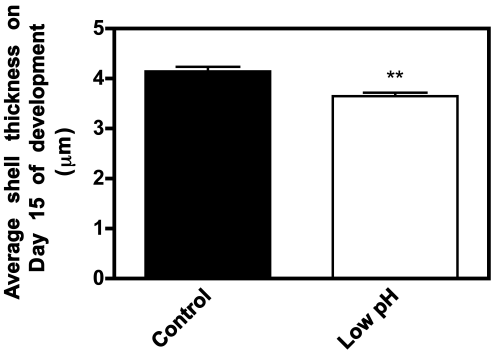

Fig. 3. Average shell thickness at the end of experiment #2 for larvae on Day 15 of development, in control ($pH_{NBS} = 8.03 \pm 0.03$; black bars) and low pH ($pH_{NBS} = 7.78 \pm 0.05$; white bars) seawater. Errors bars represent standard deviations of the triplicate enclosures. **Significant difference between control and low-pH groups.

decreases in hatching rates and shell growth. It has to be noted that at this pH level, which is much lower than the levels projected for the end of the century, the seawater was clearly undersaturated with respect to aragonite ($\Omega_{aragonite} \sim 0.68$). Parker et al. (2009) studied the synergistic effects of ocean acidification and temperature on the fertilization and early (up to 48 h) embryonic development of the Sydney rock oyster (*Saccostrea glomerata*). These authors found that both fertilization and embryonic development success were diminished at lowered pH values in the range of projected levels for 2100 (pCO_2 levels of 600, 750 and 1000 µatm) while temperature revealed an optimal level (26 °C) below and above (i.e. 18, 22 and 30 °C) which embryonic developmental rates decreased. Talmage and Gobler (2009) performed a multi-species comparison of the effects of ocean acidification on growth and metamorphosis (from 4 to 18 days of development). They actually showed that, although the growth of the 3 studied species (*Mercenaria mercenaria*, *Argopecten irradians*, and *Crassostrea virginica*) was negatively affected, they did not exhibit the same sensitivity to a decrease of up to 0.6 pH unit. This species-specific sensitivity to ocean acidification has also been observed by Miller et al. (2009) who showed that the development (from 96 h to ~ 30 d) and growth of the Eastern oyster (*Crassostrea virginica*) was significantly reduced at lowered pH levels (up to a 0.4 pH_{NBS} unit decrease), while the Suminoe oyster (*Crassostrea ariakensis*) did not appear to be sensitive to the same acidified conditions.

In this study, we show that ocean acidification has a significant effect on the blue mussel larval development although the observed decrease in growth rates both in terms of length and thickness was not accompanied by a decrease of hatching rates and an increase in mortality rates as long as seawater remained oversaturated with respect to aragonite. Although no effect on hatching and mortality rates have been observed after 2 d and after 15 d of development, the consequences, in the field with the presence of predators, of a potential decrease of shell resistance and/or an augmentation of the time spent in the water column (delay in settlement) due to a reduction in growth as observed for a 0.25–0.34 pH unit decrease, are still unknown. Since the experimental period did not extend to the settlement and metamorphosis of the organisms, it is impossible to know if the observed decrease in growth rates would translate in a miniaturization of the spats and/or an increase of the time spent in the planktonic compartment. Nevertheless, both effects could have major consequences for the survival of the populations. Suspension-feeding benthic invertebrates can be important predators of pelagic larvae. In the Oosterschelde estuary, it has been shown that larviphagy from adult bivalves is a major source of mortality for bivalve larvae (Troost et al., 2009). However, several studies showed that, thanks to their shell, larvae could be rejected unharmed with the feces (Mackenzie, 1981). A reduction of the shell both in terms of length and thickness has therefore the potential to increase mortality rates during the planktonic larval stage. Finally, decreases in size during the early developmental stages of marine organisms have been shown to effect juvenile fitness by reducing competitive ability and increasing postsettlement mortality (Anil et al., 2001).

The conditions at which the larvae were exposed in our experiment must be regarded as optimal. In the field, mussels usually spawn in spring when the water temperature is ~ 8–18 °C and chlorophyll-*a* concentrations vary between 0.5 and 19 µg l^{-1} (April–June, 5 stations, monthly measurements; see Table 1 for experimental levels). Therefore, as both experimental parameters were significantly higher than the ones encountered in situ at the time of spawning, the extrapolation, to the field, of our laboratory-based observations on the effects of decreasing pH on the blue mussel larval development, must be performed with caution. Indeed, Parker et al. (2009) have shown that the effects of ocean acidification on the growth of Sydney rock oyster larvae were greater at sub-optimal temperatures. Moreover, as food availability is a very critical parameter in limiting larval development, the fact that, in the present experiment, food concentrations were optimal could have led to a high resistance of mussel larvae to decreasing pH levels. The experimental pH level used for the control incubations in this study also does not reflect the conditions experienced by larvae in situ. Indeed, at the time of spawning, the spring bloom occurring in the Oosterschelde estuary, drives seawater pCO_2 to values below atmospheric equilibrium corresponding to an average pH level of 8.27 ± 0.09 (April–June, 5 stations, monthly measurements), a value much higher than the one used as a control during the incubations. In order to evaluate the potential effect of ocean acidification on this species fitness, there is a great need to conduct future experiments under conditions similar to the ones experienced by the organisms in the field.

At $pH_{NBS} \sim 7.6$, both hatching success and growth rates (as estimated by shell length decrease after 2 d of development) exhibited an important decrease coinciding with a

slight undersaturation of seawater with respect to aragonite. At this point, it can only be speculated that the observed decrease in larval developmental success for a $\sim 0.5\,pH_{NBS}$ unit decrease is due to the seawater undersaturation with respect to aragonite. It must be stressed that our data do not allow discriminating between the physiological impact of pH decrease alone via a disruption of inter-cellular transport mechanisms and the impact on calcification resulting from aragonite undersaturation, on the larval development of this species. More studies are needed to disentangle these different aspects of the potential effect of ocean acidification on marine organisms.

As the different pressures exerted by the environment and predators in the field result in a considerable mortality, approaching 99% (Bayne, 1976) during the free-swimming larval period, an additional 24% decrease in hatching rates as observed at a pH_{NBS} of 7.6 can therefore compromise the survival of the population. Indeed, relatively small fluctuations in the abundance of these larval stages are known to regulate the size of the population (Green et al., 2004). Shellfish predominantly inhabit coastal regions, which usually exhibit lower pH values than the open ocean because of permanent or episodic low pH water inputs from rivers (Salisbury et al., 2008), from upwellings (Feely et al., 2008) and due to intense rates of organic matter degradation and/or nitrification (Hofmann et al., 2009). While many estuaries already have high and variable pCO_2, atmospheric CO_2 enrichment will shift the baseline toward even higher values (Miller et al., 2009) that could lead to extended periods of undersaturation with respect to aragonite, although it has recently been suggested that, in some areas, eutrophication can counter the effects of ocean acidification (Borges and Gypens, 2010). Therefore, these species will most likely be exposed to suboptimal growth conditions in the coming years. In order to assess socio-economic and ecological effects of ocean acidification on shellfish, it is therefore crucial to predict accurately the evolution of pH as well as the saturation state of the ocean and its coastal waters with respect to aragonite in the near future.

Our observation of no significant effect of a $\sim 0.3\,pH_{NBS}$ unit decrease on both hatching rates and survivorship stands in contrast with results obtained by Parker et al. (2009) on *Saccostrea glomerata* during the first 48 h of development and by Talmage and Gobler (2009) on *Mercenaria mercenaria* and *Argopecten irradians* between the veliger and metamorphosed stages (18–20 days). Our results are consistent with those from Talmage and Gobler (2009) on *Crassostrea virginica* who observed significant effects on growth rates but no significant effects on mortality for a ~ 0.3 pH unit decrease. Altogether, these different studies reveal, similar to what is observed for adult stages, that the effects of ocean acidification of molluscans early life development are species-specific (Kurihara, 2008) and that the sensitivity of the organisms might depend on the pH variability at which they are naturally exposed in the field.

It is important to notice that even under aragonite undersaturated conditions, mussel larvae were able to produce a shell, highlighting that molluscs exert a control over calcification (McConnaughey and Gillikin, 2008) and are therefore not completely dependent on environmental conditions. This does not appear as a surprise since most freshwater molluscs are clearly well adapted to such conditions and bivalve growth has been showed by Tunnicliffe et al. (2009) under the extremely undersaturated conditions of deep hydrothermal sites. Most calcifying species, including molluscs, are able to concentrate Ca^{2+} and CO_3^{2-} ions at the site of calcification. Adult molluscs appear to use conventional calcification physiology by pumping protons from the calcification site (extrapallial fluid), largely through $Ca^{2+}/2H^+$ exchange catalyzed by Ca^{2+} ATPase (McConnaughey and Gillikin, 2008). The elevation of pH in the extrapallial fluid (Misogianes and Chasteen, 1979) allows an elevation of the concentration of CO_3^{2-} that favours calcification. However, as this mechanism requires energy, this can lead to substantial energy shifts from other processes and to important costs for the growth of the organism as observed by Wood et al. (2008) for the brittlestar *Amphiura filiformis*. Although the regulation of calcification by this mechanism is well documented for adults, few studies have focused on the mechanisms of larval calcification and on the capacity of bivalve larvae to regulate calcification rates by controlling the carbonate chemistry at the site of calcification. There is, however, some indication that biomineralization of *Mytilus edulis* larvae is physiologically controlled, as the activity of the carbonic anhydrase, an enzyme that catalyses the reversible hydration of CO_2 to HCO_3^- and H^+, reaches a maximum at the end of each developmental stage connected with biomineralization (Medaković, 2000). This study also reported that these larvae, as showed for other molluscs and echinoderms larvae (Weiss et al., 2002), produce mainly amorphous calcium carbonate during the first 2–3 days of development and aragonite in the following days. As the solubility of amorphous calcium carbonate is 30 greater than that of aragonite (Brečević and Nielsen, 1989), early larval stages should be much more vulnerable than older larval stages and adults that precipitate aragonite and/or calcite. Again, the fact that 2-days old larvae were able to produce a shell under aragonite undersaturation highlights the strong regulation capacity of these organisms under sub-optimal growth conditions.

As mentioned previously, in the Oosterschelde estuary, adults are exposed to a relatively narrow range of pH with winter pH levels never falling below ~ 7.9 and high pH levels in springtime (~ 8.3) when spawning and larval development occur. There is, therefore, a great need to evaluate the adaptive capacity of this species to low pH conditions. This could be achieved by comparing the responses, to a decrease in seawater pH, of populations originating from areas with contrasting environmental conditions with respect to the carbonate chemistry and/or by performing such experiments over several generations.

Finally, in the present study, we show that shell increase, by linear extension, which is the most commonly measured parameter in ocean acidification related studies for molluscs larvae, should not be the only measured parameter if one wants to investigate the effects of acidification on shell growth. Indeed, shell thickness appeared to be affected as well with a relative decrease twice the relative decrease observed in shell length. This is consistent with Miller et al. (2009) findings who estimated a much higher decrease of shell weight (estimated as the amount of calcium in the shells) than shell area (respectively −42 and −16% between pre-industrial and end of 21st century projected pH level for *C. virginica*). However, it must be stressed that, in our study, shell thickness measurements could not be performed on D-veliger (2 days old) larvae and appeared to be limited to large pediveliger larvae using our protocol. Calcium content in the shells, as an estimator of shell weight, is also an interesting parameter to follow and has been successfully applied by Miller et al. (2009). However, again, this technique has been applied to large larvae, and there is still a need to test its validity for smaller veliger larvae. Finally, more precise techniques such as ^{45}Ca labelling, recently used on pteropods (planktonic molluscs; Comeau et al., 2009) are promising and might be valuable tools to accurately evaluate the effect of ocean acidification within the range of expected levels for 2100 on calcification rates of mollusc early life stages.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/7/2051/2010/bg-7-2051-2010-supplement.pdf.

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