



# Effects of CO<sub>2</sub>-induced seawater acidification on the health of *Mytilus edulis*

A. Beesley\*, D. M. Lowe, C. K. Pascoe, S. Widdicombe

Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth, Devon PL1 3DH, UK

**ABSTRACT:** The impact of CO<sub>2</sub>-acidified seawater (pH 7.8, 7.6, or 6.5, control = pH 8) on the health of *Mytilus edulis* was investigated during a 60 d mesocosm experiment. Mussel health was determined using the neutral red retention (NRR) assay for lysosomal membrane stability and from histopathological analysis of reproductive, digestive and respiratory tissues. Seawater acidification was shown to significantly reduce mussel health as measured by the NRR assay, and it is suggested that this impact is due to elevated levels of calcium ions (Ca<sup>2+</sup>) in the haemolymph, generated by the dissolution of the mussels' calcium carbonate shells. No impact on tissue structures was observed, and it is concluded that *M. edulis* possess strong physiological mechanisms by which they are able to protect body tissues against short-term exposure to highly acidified seawater. However, these mechanisms come at an energetic cost, which can result in reduced growth during long-term exposures. Consequently, the predicted long-term changes to seawater chemistry associated with ocean acidification are likely to have a more significant effect on the health and survival of *M. edulis* populations than the short-lived effects envisaged from CO<sub>2</sub> leakage from sub-seabed storage. Ocean acidification could reduce the general health status of this commercially and ecologically important marine species.

**KEY WORDS:** *Mytilus edulis* · Ocean acidification · Seawater pH · Lysosomes · Neutral red retention assay

Resale or republication not permitted without written consent of the publisher

## 1. INTRODUCTION

Over the past 200 yr there has been a sharp increase in atmospheric carbon dioxide (CO<sub>2</sub>) levels due primarily to the continued burning of fossil fuels. As the ocean acts as a natural 'carbon sink' it has already absorbed up to 50% of all the anthropogenic CO<sub>2</sub> that has been released (Sabine et al. 2004). Whilst this ocean uptake has slowed the rate at which atmospheric CO<sub>2</sub> levels are rising, it has also led to a decrease in the pH of the surface oceans, i.e. ocean acidification (Caldeira & Wickett 2003). This reduction in pH and the coupled lowering of carbonate levels could have severe consequences for a host of marine organisms, particularly for shell-forming animals and corals. One potential way to reduce the amount of CO<sub>2</sub> emitted is to store it in suitable geological reservoirs. Whilst this process, known as geological sequestration (Holloway

2005), could reduce the atmospheric build up of CO<sub>2</sub>, there remains the potential for these storage sites to leak (Hawkins 2004). If leakage did occur, areas of the marine environment could be exposed to very high levels of CO<sub>2</sub> for days or even weeks. It is therefore necessary to determine the impact of both small (ocean acidification) and large (CO<sub>2</sub> leakage) changes in seawater CO<sub>2</sub> levels on key marine organisms and ecosystems.

The blue mussel *Mytilus edulis* is found throughout the Atlantic, stretching from the White Sea to southern France in the NE Atlantic; from the Canadian Maritimes (south) to North Carolina in the West Atlantic. It is also found on the coasts of Chile, Argentina, the Falkland Islands, as well as on the west coast of North America (Berge et al. 2006). This organism is known to play an important role within many marine and estuarine ecosystems, acting as a source of food for humans

\*Email: abee@pml.ac.uk

(FAO 2004) and other organisms (Gutierrez et al. 2003), creating habitats and maintaining sediment stability. Mussels are also widely used as indicator species for toxicology studies (Bayne et al. 1985).

Previous studies have shown that ocean acidification can have a significant impact on mussels. Berge et al. (2006) and Michaelidis et al. (2005) have shown that seawater acidification can reduce growth rate in *Mytilus* spp. Michaelidis et al. (2005) also observed that a long-term lowering of seawater pH caused a reduction in haemolymph pH, which was buffered by the dissolution of the shell ( $\text{CaCO}_3$ ), as well as a lower metabolic rate and increased degradation of protein. As mussel shells are made of up to 83% aragonite (Hubbard et al. 1981) and aragonite is more soluble than calcite, shell dissolution is going to be a considerable problem faced by mussels in acidified seawater. In a recent study, Bibby et al. (2008) have shown that elevated levels of calcium ions in mussels' haemolymph, as a result of shell dissolution, could affect the function and physiology of haemocytes. This would indicate that exposure to elevated levels of  $\text{CO}_2$  could potentially have an impact on the general health status of *M. edulis*. To test this, we exposed *M. edulis* individuals to elevated levels of  $\text{CO}_2$  during a 60 d mesocosm experiment. The treatment levels chosen represent realistic scenarios for both ocean acidification (pH 7.8 and 7.6; as predicted by the International Governmental Panel for Climate Change (IPCC 2001) IS92a scenario and Caldeira & Wickett 2003) and  $\text{CO}_2$  leakage (pH 6.8; Blackford et al. 2008). Effects of seawater acidification on the general health status of the mussels were quantified using the neutral red retention (NRR) assay, as well as observations on reproductive, respiratory and digestive tissues.

## 2. MATERIALS AND METHODS

### 2.1. Mussel collection and experimental setup

*Mytilus edulis*, measuring between 45 and 55 mm, were collected from Trebarwith Strand, North Cornwall. Animals were cleaned of epibionts, and 32 were placed into each of 8 large (50 l) exposure tanks that were supplied with continuously flowing seawater. Tanks were supplied with seawater from 1 of 4 header tanks, adjusted using the NBS pH scale to pH 6.5, 7.6, 7.8, or 8.0, through the addition of  $\text{CO}_2$  using the system described by Widdicombe & Needham (2007). Each header tank supplied 2 replicate exposure tanks, at a rate of approximately  $60 \text{ ml min}^{-1}$ . Mussels were fed with *Isochrysis galbana*, or *Pavlova lutheri*, to provide  $30 \text{ mg}$  algal dry weight mussel $^{-1} \text{ d}^{-1}$ . The feed was prepared in seawater and pumped into the tanks at a rate of  $1 \text{ ml min}^{-1}$ .

### 2.2. Experimental conditions and monitoring

Daily measurements were recorded to monitor pH, temperature and salinity in the header tanks, exposure tanks and samples from the water feed tubes. Total  $\text{CO}_2$  concentration ( $\text{TCO}_2$ ) in header and exposure tanks was measured 3 times a week by analysing  $10 \mu\text{l}$  sub-samples of seawater using a  $\text{CO}_2$  analyser (Ciba-Corning 965). Carbonate system variables (aqueous  $\text{CO}_2$ ,  $\text{pCO}_2$ ,  $\text{HCO}_3^-$ ,  $\text{CO}_3^{2-}$ , alkalinity, and saturation states for calcite and aragonite) were calculated from the measured pH and  $\text{TCO}_2$  values using the MatLab (Version 6.1.0.451) *csys.m* programme adapted (by Helen S. Findlay) from Zeebe & Wolf-Gladrow (2001) ([www.awi-bremerhaven.de](http://www.awi-bremerhaven.de)) and using saturation state solubility constants from Mehrbach et al. (1973).

### 2.3. Sampling regime

On Day 0, 16 mussels, which had been collected at the same time as those placed in the acidified seawater exposure system, were sampled to determine the health of mussels at the start of the experiment. A sample of haemolymph was taken from these animals in order to carry out the NRR assay. The mussels were then fixed in Baker's formol calcium and processed for wax histopathology. Subsequent sampling of mussels from the exposure tanks was carried out on Days 6, 13, 31 and 60. Eight mussels were removed from each of the tanks on each of these 4 sampling days. These animals were replaced with fresh mussels on Days 6, 13 and 31, in order to keep the number of animals in each tank constant. The replacement animals were collected from Trebarwith Strand before each sampling day and separated from the exposure mussels by being held in baskets within the exposure tanks.

### 2.4. Measuring the health of *Mytilus edulis* using the NRR assay

Lysosomes are central to many key biological processes, including reproduction, digestion and immune response; thus, any deleterious changes to their function can have serious consequences for those processes. The general health status of the mussels was therefore considered in terms of changes to the lysosomal system of the blood cells. This is a well established method of assessing invertebrate health on a variety of invertebrate species (Lowe et al. 1995).

A haemolymph sample was removed from each animal as follows: the valves were carefully prised apart with a broad-backed solid scalpel that was held in position whilst  $100 \text{ ml}$  of haemolymph was withdrawn

from the anterior adductor muscle. The haemolymph was collected into a 1.0 ml hypodermic syringe, fitted with a 21 gauge needle, containing 100 µl of a mussel physiological saline (20 mM HEPES, 436 mM NaCl, 53 mM MgSO<sub>4</sub>, 10 mM KCl, 10 mM CaCl<sub>2</sub>, gassed for 10 min with 95% O<sub>2</sub>:5% CO<sub>2</sub> and adjusted to pH 7.3 with 1 N NaOH; Peek & Gabbott 1989). Laboratory studies carried out by D. M. Lowe (unpubl. data) have shown that pH 7.3 is the optimum pH for neutral red retention. In order to reduce shearing forces during ejection, the needle was then removed and the contents of the syringe ejected into a 2 ml siliconised (Sigma) Eppendorf tube, which was held in water ice until required. The neutral red dye stock solution was made by dissolving 28.8 mg of dye (C.I.50040, Sigma) in 1 ml of DMSO (dimethyl sulphoxide), and the working solution was prepared by diluting 10 µl of the stock solution with 5 ml of the mussel physiological saline. The Eppendorf tube containing the cell suspension was then carefully inverted twice to resuspend the cells, and a 50 µl aliquot of the cell suspension was dispensed onto a 76 × 26 mm microscope slide for 15 min to allow the cells to attach. Excess solution was then carefully tipped off, and 40 µl of the neutral red working solution added and a 21 × 21 mm coverslip applied. The cells were then incubated for a 15 min period to allow the uptake of the dye. The preparations were then examined under the microscope at 15, 30, 60, 90, 120 and 180 min for evidence of damage to the lysosomes through dye leakage or enlargement. When it was observed that >50% of the blood cells in a sample exhibited damaged lysosomes the test was terminated. A general linear model analysis of variance was then used to test for differences in the mean retention time of the dye between the different pH treatment levels, with sampling time as a co-variate.

## 2.5. Preparation and analysis of tissue sections

After haemolymph samples were taken, the soft tissues were removed from the shell and a transverse slice through the mid-region of the digestive gland and associated tissues was removed. The tissue samples were immediately placed in Baker's formal calcium (10% formalin, 1% calcium chloride and 2.5% sodium chloride) at 4°C and transferred to a refrigerator to complete the fixation process. After a minimum of 24 h, the fixed specimens were trimmed of excess tissue, then dehydrated through an ascending alcohol series, cleared in xylene and impregnated in paraffin wax. This procedure was undertaken using an automatic programmable tissue processor over a 16 h period. Once wax impregnation was completed, the speci-

mens were blocked up in stainless steel moulds using fresh molten wax and allowed to cool prior to cutting. Sections, 7 µm thick, were cut using a microtome and disposable steel knives, and the sections were floated out onto microscope slides coated with (3-aminopropyl) triethoxysilane (A-3468, Sigma-Aldrich) to assist adhesion. Once dry, the sections were stained with Papanicolaou's stain (Culling 1963) and coverslipped. The stained sections were analysed for evidence of structural changes in 3 systems.

## 2.6. The digestive system

The condition of digestive tissues was assessed using 6 indicators. These were the presence or absence of frontal tubular atrophy, tubule phasing, tubule necrosis, digestive cell vacuoles, basophil granulation and the digestive to basophil cell ratio (Lowe & Clarke 1989). A healthy mussel should not exhibit large vacuoles in the digestive tissues. It has been shown that exposure to a large range of environmental stressors can result in abnormal accumulations of lipids in the digestive cells. In wax sections the lipid is extracted as a result of the dehydration process by solvents. The lipid manifests itself as abnormally large vacuoles (Lowe 1988), or necrosis (death). Healthy digestive cells should have a ratio of about 5:1 with basophil cells; an increase in the ratio of basophil to digestive cells may be indicative of a reduction in rate of digestive capacity: less digestive cells = lower digestion rate. Granulation of basophil cells would indicate an impacted animal, as would tubular atrophy (wasting away of tissues).

## 2.7. The reproductive system

The condition of reproductive tissues was also assessed using 5 indicators. These were the presence or absence of regression, atretic eggs (Lowe et al. 1982) and adipogranular tissue, as well as reproductive state (Seed 1975) and sex of the animal. In impacted females, atresia can sometimes be found where the structures within the follicle break down and eggs can be reabsorbed (Lowe & Pipe 1985). This can lead to an infiltration of haemocytes, even though this can also be a natural process, generally occurring immediately after spawning. Regression of tissues can also signify an unhealthy animal. Regression generally occurs when the animals are exposed to a stressing factor and needs to reabsorb tissue in order for energy sources to be relocated to vital processes such as respiration. The sex of the animal was looked at as intersex animals have occurred when mussels have been exposed to

Table 1. Measures of seawater chemistry (mean  $\pm$  1 SD) for experimental period

Treatment	pH	TCO <sub>2</sub> (mmol kg <sup>-1</sup> )	Aqueous CO <sub>2</sub> ( $\mu$ mol kg <sup>-1</sup> )	pCO <sub>2</sub> ( $\mu$ atm)	[HCO <sub>3</sub> <sup>-</sup> ] ( $\mu$ mol kg <sup>-1</sup> )	[CO <sub>3</sub> <sup>2-</sup> ] ( $\mu$ mol kg <sup>-1</sup> )	Alkalinity ( $\mu$ mol kg <sup>-1</sup> )	Calcite saturation state	Aragonite saturation state	Temperature (°C)	Salinity (ppt)
<b>Header tanks</b>											
8.0	8.08 $\pm$ 0.09	1.88 $\pm$ 0.65	10.45 $\pm$ 4.54	308 $\pm$ 1300	1685 $\pm$ 595	189 $\pm$ 57.98	2160 $\pm$ 697	4.37 $\pm$ 1.34	2.83 $\pm$ 0.87	17.96 $\pm$ 35.13	35.13 $\pm$ 0.47
7.8	7.72 $\pm$ 0.12	1.95 $\pm$ 0.62	25.37 $\pm$ 9.27	743 $\pm$ 262	1837 $\pm$ 586	91.84 $\pm$ 27.40	2069 $\pm$ 635	2.12 $\pm$ 0.63	1.37 $\pm$ 0.41	17.83 $\pm$ 0.86	35.11 $\pm$ 0.42
7.6	7.54 $\pm$ 0.09	1.97 $\pm$ 0.70	40.81 $\pm$ 40.81	1 190 $\pm$ 466	1870 $\pm$ 671	58.88 $\pm$ 19.54	2019 $\pm$ 707	1.36 $\pm$ 0.45	0.88 $\pm$ 0.29	17.76 $\pm$ 0.88	35.21 $\pm$ 0.39
6.5	6.41 $\pm$ 0.22	2.94 $\pm$ 0.97	894 $\pm$ 626	25438 $\pm$ 17673	2047 $\pm$ 614	4.20 $\pm$ 3.47	2057 $\pm$ 620	0.10 $\pm$ 0.08	0.06 $\pm$ 0.05	17.41 $\pm$ 0.92	35.05 $\pm$ 0.52
<b>Exposure tanks</b>											
8.0	8.01 $\pm$ 0.09	1.70 $\pm$ 0.39	12.19 $\pm$ 3.30	340 $\pm$ 86.9	1555 $\pm$ 359.2	133 $\pm$ 29.61	1899 $\pm$ 410.6	3.06 $\pm$ 0.68	1.97 $\pm$ 0.44	16.11 $\pm$ 1.11	35.29 $\pm$ 0.32
7.8	7.84 $\pm$ 0.08	1.84 $\pm$ 0.56	19.48 $\pm$ 6.09	545 $\pm$ 169.7	1718 $\pm$ 523.9	102.1 $\pm$ 34.37	1979 $\pm$ 588.9	2.34 $\pm$ 0.79	1.51 $\pm$ 0.51	16.05 $\pm$ 1.07	35.37 $\pm$ 0.35
7.6	7.77 $\pm$ 0.09	1.94 $\pm$ 0.34	24.19 $\pm$ 4.44	678 $\pm$ 128.8	1827 $\pm$ 318.0	92.8 $\pm$ 20.72	2062 $\pm$ 355.8	2.13 $\pm$ 0.48	1.37 $\pm$ 0.31	16.06 $\pm$ 0.94	35.27 $\pm$ 0.39
6.5	7.36 $\pm$ 0.26	1.96 $\pm$ 0.70	66.70 $\pm$ 38.61	1875 $\pm$ 1104.4	1843 $\pm$ 655.6	51.8 $\pm$ 53.13	1972 $\pm$ 751.0	1.20 $\pm$ 1.23	0.77 $\pm$ 0.79	16.03 $\pm$ 0.83	35.13 $\pm$ 0.51

contaminants. This is where both female and male gametes can be found within the same organism. If there is a large amount of adipogranular cells, this could indicate that the animals had placed gamete development on hold because the environment was potentially unsatisfactory for larval survival.

## 2.8. The respiratory system and interstitial tissues

In addition the sections were examined for changes in the gill structure, such as the presence of gill mucous cells and an increase in haemocytes around the gill area, as well as looking at connective tissues for evidence of granulocytomas, which are granular leucocytes forming clusters; haemocyte infiltration, suggesting trauma or parasitism; evidence of inflammation to the gut interstitial tissues; and evidence of pre-neoplastic lesions.

## 3. RESULTS

### 3.1. Experimental conditions

The environmental conditions within the header and exposure tanks for each treatment level are summarised in Table 1. The most notable observation is that the values for pH and pCO<sub>2</sub> in the 6.5 treatment exposure tank are considerably different from those in the 6.5 treatment header tank. Although smaller, similar increases in pH and reductions in pCO<sub>2</sub> in the exposure tanks, when compared to the corresponding header tanks, were also observed for pH treatments 7.8 and 7.6. These differences indicate a loss of CO<sub>2</sub> from the water in the exposure tanks. However, it is unlikely this is due to loss of CO<sub>2</sub> into the atmosphere via exchange across the water–air boundary in the exposure tanks. This is primarily because pH data collected during the setting-up period, prior to the addition of the mussels (Table 2) demonstrated less change in pH between header and exposure tanks than was observed during the experimental exposure period. Also, prior to the addition of mussels the pH in the pH 6.5 exposure tanks was 6.82. Within a day of mussels

Table 2. Measures of mean pH ( $\pm$  1 SD) during setting-up period prior to start of experiment

Treatment	Header tanks	Exposure tanks
8.0	7.94 $\pm$ 0.13	7.84 $\pm$ 0.15
7.8	7.72 $\pm$ 0.11	7.70 $\pm$ 0.12
7.6	7.55 $\pm$ 0.15	7.56 $\pm$ 0.14
6.5	6.45 $\pm$ 0.60	6.82 $\pm$ 0.41

being added to the tank the pH was at 7.38—an increase of 0.56 of a pH unit. It seems likely therefore that the presence of the mussels in some way buffers the pH changes, probably through the dissolution of their carbonate shells. Dissolution of the mussel shells in the 6.5 pH treatment was not unexpected as the seawater supplied to these treatment tanks was undersaturated for both calcite and aragonite (Table 1). As the increase in pH observed in the pH 6.5 exposure tanks occurred within 1 d of the addition of the mussels, it was therefore concluded that the dissolution of mussel shells occurred rapidly and was great enough to cause a significant change in the exposure tank water even though the water was effectively replaced every 14 h. This elevated value of pH ( $\approx 6.8$ ) was consistent throughout the course of the experiment, which suggests that dissolution was occurring throughout this period. For evidence to suggest that this phenomenon of CO<sub>2</sub> loss was related to the presence of the mussels and not caused by atmospheric loss is provided by the previous exposure experiments conducted in this system, which, despite using similar, open exposure tanks showed no loss of CO<sub>2</sub> (e.g. Miles et al. 2007, Spicer et al. 2007, Bibby et al. 2008).

### 3.2. Mortality

There were only 2 mortalities during the course of the exposure: 1 from the pH 7.8 treatment on Day 6 and 1 from the control treatment on Day 13.

### 3.3. General health status as measured by the NRR assay

The ANOVA test showed that a decrease in seawater pH significantly reduced neutral red retention time ( $F = 4.39$ ,  $p = 0.005$ ). The test also showed that, if Day 0 data were removed, the number of exposure days had no effect on the patterns observed ( $F = 0.03$ ,  $p = 0.873$ ). This would indicate that the impact of reduced pH on mussel health occurred very quickly ( $<7$  d), but remained constant for the remaining exposure period.

### 3.4. Impact of acidification on mussel tissues

#### 3.4.1. Digestive system

There was no significant impact on digestive processes. One-third of the observed sections showed signs of tubular atrophy, although there was no relation to either experimental time period or treatment; how-

ever, these animals were originally collected from a population where this appears to be the norm (D. M. Lowe unpubl. data).

Fig. 1 compares normal digestive tissues from Days 0 and 60 to those of an impacted digestive gland, obtained from a separate study, which shows a parasite and tubule thinning. In comparison, the experimental animals from the current study show that  $<2\%$  suffered from parasitism. There was a healthy ratio of basophil cells to digestive cells shown in nearly all of the animals, with no differences between treatments, and the cells appeared to be in several phases, which is symptomatic of healthy digestive tubules. There did not appear to be any large vacuoles or granulation of the digestive tissues in any of the treatments.

#### 3.4.2. Reproductive tissues

There was no evidence to indicate any impact on reproductive processes, with all animals exhibiting the same reproductive state and similar levels of reserve tissues to fuel gametogenesis. The animals had spawned prior to sampling on Day 60. Therefore, the reproductive state was assessed from Day 31 instead of Day 60 as shown in Figs. 2 (male) and 3 (female). The results indicated 90% of females were in an atretic state at Day 31, although there were also a further 18 mussels for which sex was not determinable; these animals could have been 'spawned-out' females, which would alter the percentage in atresia. There is no picture of female gametes from the control on Day 31; therefore, a picture from pH 7.8 on Day 31 was used instead. Regression was present in a few animals in all treatments, which could indicate that the animals 'held back' from spawning into an antagonistic environment. Intersex animals were not present in this study. The presence of adipogranular cells was common throughout all treatments, with no significant difference between pH treatments.

#### 3.4.3. Tissue surfaces

There was no evidence of changes to gill structure that would indicate irritation resulting from exposure to reduced pH. Mucous cells were not present in any of the treatments, and inflammation of the connective tissues affected  $<7\%$  of animals, although there was no distinction between treatments or sample days. Fig. 4 illustrates the gill tissue from Days 0 and 60 and a comparative picture of impacted gills that shows large mucous cells attached to the gill.

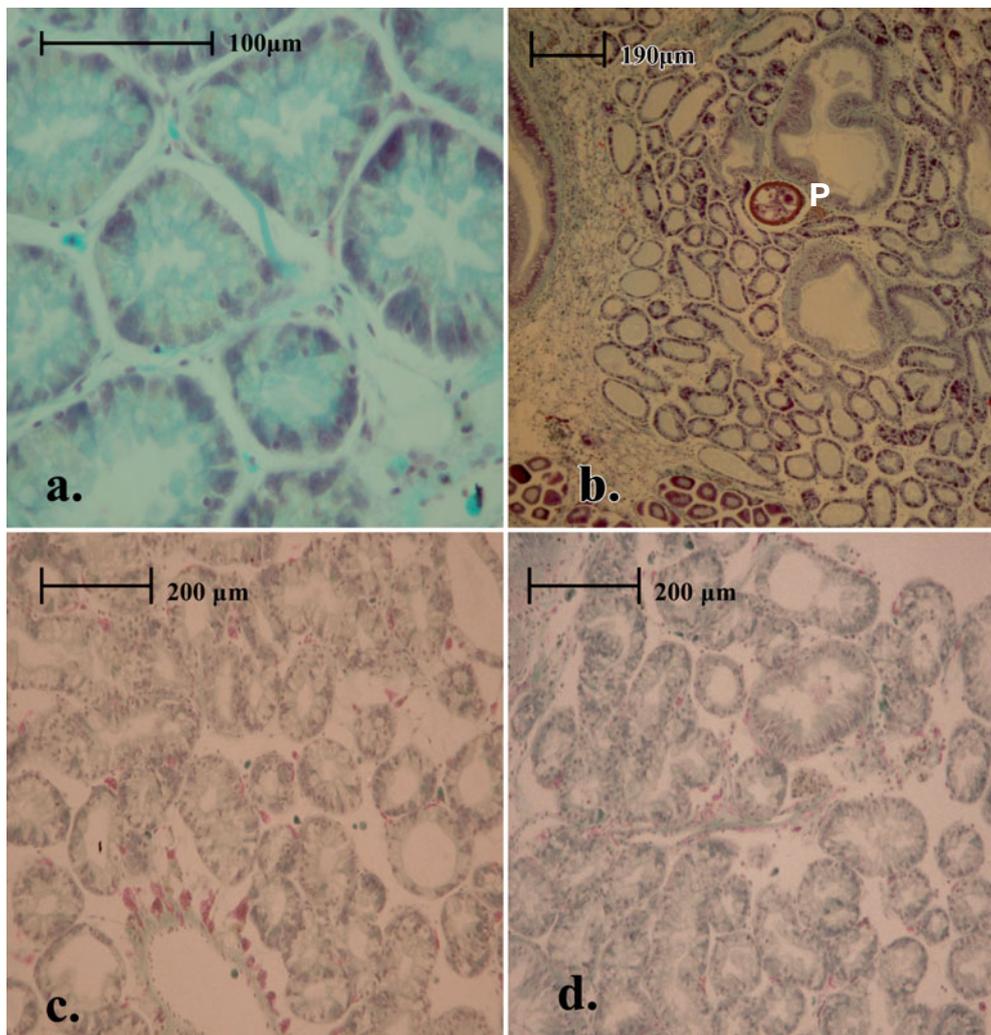


Fig. 1. Transverse sections of digestive tissue showing: (a) normal tubules at Day 0 and a natural digestive:basophil ratio; (b) typical impacted digestive cells from a separate study, illustrating thinning of tubules and the presence of a parasite (P); (c) healthy tubules and a normal digestive:basophil ratio at Day 60 in the control treatment, indicating no impact; and (d) healthy tubules and a normal digestive:basophil ratio at Day 60 in the pH 6.5 treatment, indicating no impact

#### 4. DISCUSSION

The current study has demonstrated that exposure to acidified seawater reduces the health of *Mytilus edulis* as measured by a significant reduction in the NRR time of the lysosomes. This reduction in retention time confirms that the lysosomal membrane surrounding this organelle has become porous, which allows a 'leaky lysosome'. This can lead to increased permeability to substrates, activation of previously latent hydrolytic enzymes (which can lead to cell death), disruption of normal lysosomal function and possible release of hydrolases into the cytoplasm where they could cause cytolytic damage (Bayne et al. 1985). Any damage to lysosomes could also have an impact on an animals' immune system as these organelles are used in the

digestion of macromolecules from phagocytosis (ingestion of dying cells or large extracellular material), endocytosis (during the recycling of receptor proteins) and autophagy (delivery of unwanted organelles, proteins, or microbes). Lysosomes digest the foreign bacteria or waste that invades cells and also help repair cell damage. In a recent study, Bibby et al. (2008) found that exposure to acidified seawater reduced levels of phagocytosis in *M. edulis* and concluded this would have a detrimental effect on mussel health through suppression of the immune system. These authors pointed out that, by storing the hydrolytic enzymes involved in intracellular degradation, lysosomes play a critical role in the mussel's defence system (Winston et al. 1996). The reduction in lysosome health in the present study would support the sugges-

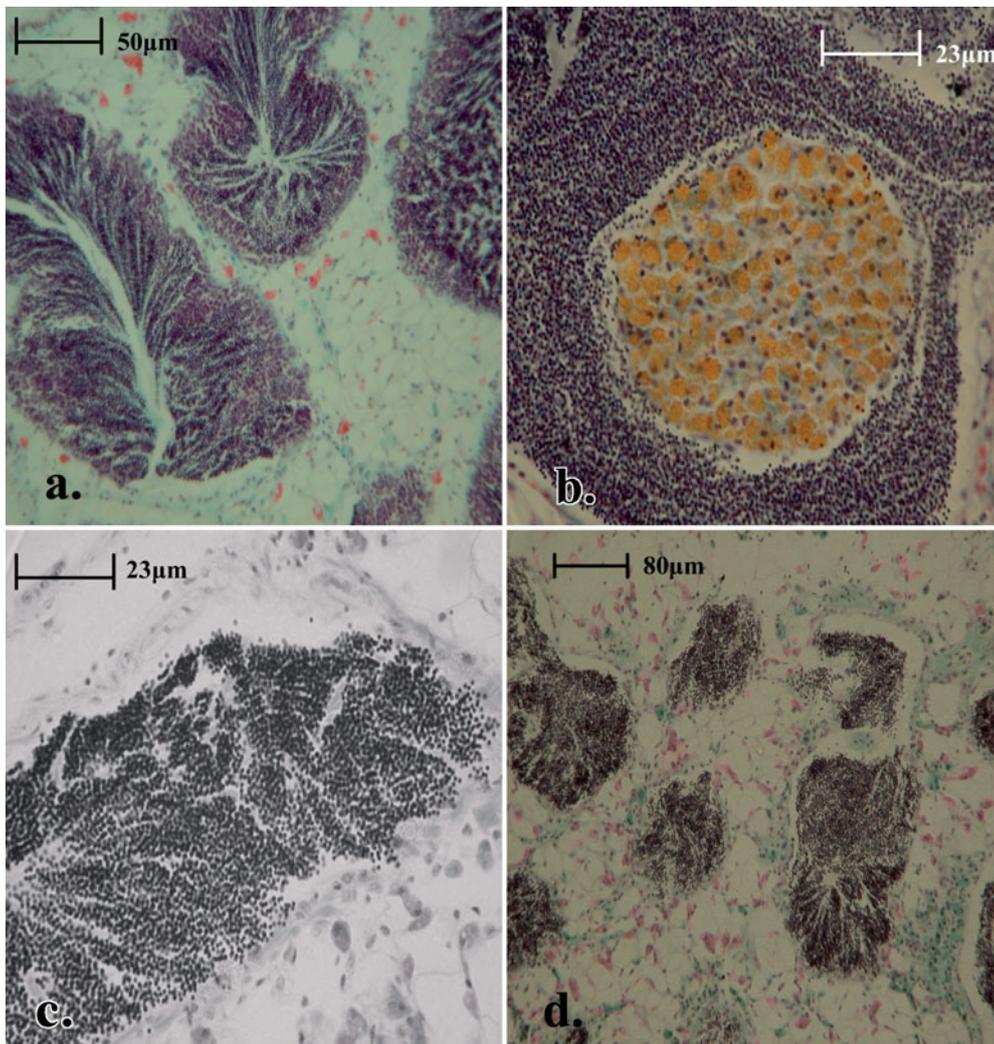


Fig. 2. Transverse sections of male reproductive tissues showing: (a) male follicles with normal sperm development; (b) example of impacted sperm follicles showing active degeneration by phagocytic brown cells (separate study); (c) healthy developed sperm at Day 60 in the control treatment, indicating no impact; and (d) healthy developed sperm at Day 60 in the pH 6.5 treatment, indicating no impact

tions of Bibby et al. (2008) that acidified seawater may disrupt cellular pathways and compromise membrane fragility. Although this study has not looked at lysosomal recovery in *M. edulis*, it is important to recognise that the mussels may have the potential to fully recover after a short exposure to high CO<sub>2</sub> conditions. Moore (1985) showed a recovery of lysosomal health in a marine snail after exposure to an external stress. This potential for recovery would have implications when considering the risks posed by CO<sub>2</sub> leakage from sub-seabed geological storage.

The reduction in lysosomal health observed in the current study may have been caused by an increase in the concentrations of calcium ions (Ca<sup>2+</sup>) in the haemolymph. Whilst no direct measurements of haemolymph Ca<sup>2+</sup> concentration were taken during the cur-

rent study, a previous study by Bibby et al. (2008) suggested that dissolution of the mussel shells had led to elevated concentrations of calcium ions (Ca<sup>2+</sup>) in the haemolymph and that, by changing cellular metabolism, function and signalling pathways, the increase in Ca<sup>2+</sup> had ultimately been responsible for the observed reduction in immune function. This seems a reasonable suggestion for 3 reasons. Firstly, shell dissolution has been proven to be a source of increased levels of Ca<sup>2+</sup> in the extracellular fluids of *Mytilus edulis* during periods of hypercapnia (Lindinger et al. 1984). Michaelidis et al. (2005) looked at a similar species, *M. galloprovincialis*, and also found elevated Ca<sup>2+</sup> levels in haemolymph. Whilst Miles et al. (2007) found a similar increase, but this time with magnesium ions, in the sea urchin *Psammechinus miliaris*. Secondly, as haemo-

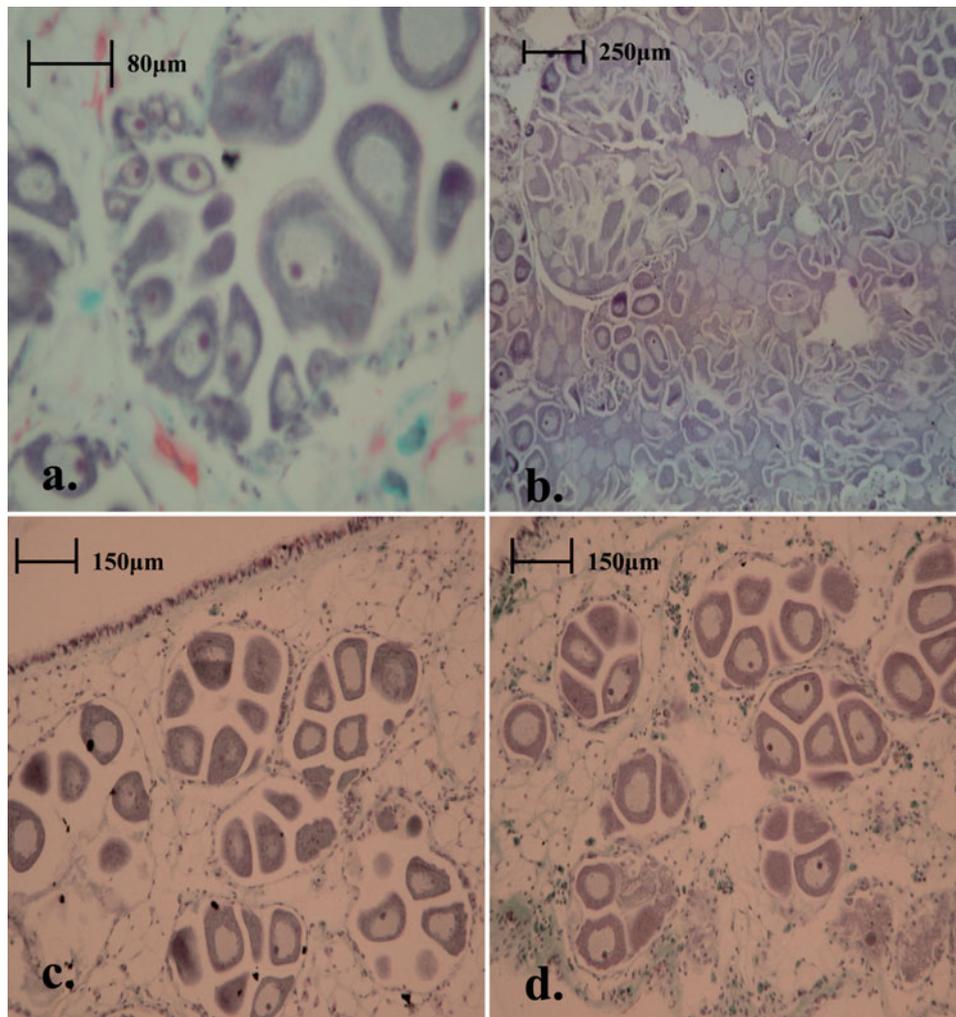


Fig. 3. Transverse sections of female reproductive tissues showing: (a) healthy developed and developing eggs at Day 0; (b) typical impacted female reproductive tissues from a separate study, illustrating atretic eggs, leading to breakdown and reabsorption of the follicle; (c) healthy developed and developing eggs at Day 60 in the pH 7.8 treatment, indicating no impact; and (d) healthy developed and developing eggs at Day 60 in the pH 6.5 treatment, indicating no impact

lymph is iso-osmotic, the movement of  $\text{Ca}^{2+}$  ions shifts between both intracellular structures and extracellular fluids. Jones & Davis (1982) demonstrated that extracellular calcium would enter epithelial cells, possibly bound to a calcium-binding protein, via pinocytosis. Finally, Marchi et al. (2004) stated that an increase in cytosolic calcium can cause lysosomal destabilisation. This is due to activation of calcium-dependant phospholipase A2. Lysosomes containing calcium are also known to aid the function in trans-cellular calcium transport during shell formation, growth and repair in some molluscs (Jones & Davis 1982).

In the absence of any direct measurements of haemolymph  $\text{Ca}^{2+}$  concentrations, it is necessary to demonstrate that mussel shell dissolution had occurred in the current study and increased  $\text{Ca}^{2+}$  concentrations were therefore possible, before this can be considered as an

explanation of the reduction in lysosomal health observed. Unfortunately, shell dissolution was not directly investigated in the current study; however, strong indirect evidence does suggest significant shell dissolution in acidified treatments. Measures of  $\text{pCO}_2$  in the acidified exposure tanks were lower than those in the corresponding header tanks. In addition, pH data from exposure tanks were higher than those in corresponding header tanks. This shows a loss of  $\text{CO}_2$  from the acidified seawater that was either due to exchange across the water–air boundary or was due to the chemical removal of  $\text{CO}_2$  via reaction with a source of carbonate with the exposure tanks (i.e. chemical buffering due to the presence of the mussel shells). By comparing the pH data collected during the setting-up period, prior to the addition of the mussels (Table 2), with the data collected during the experiment (Table 1),

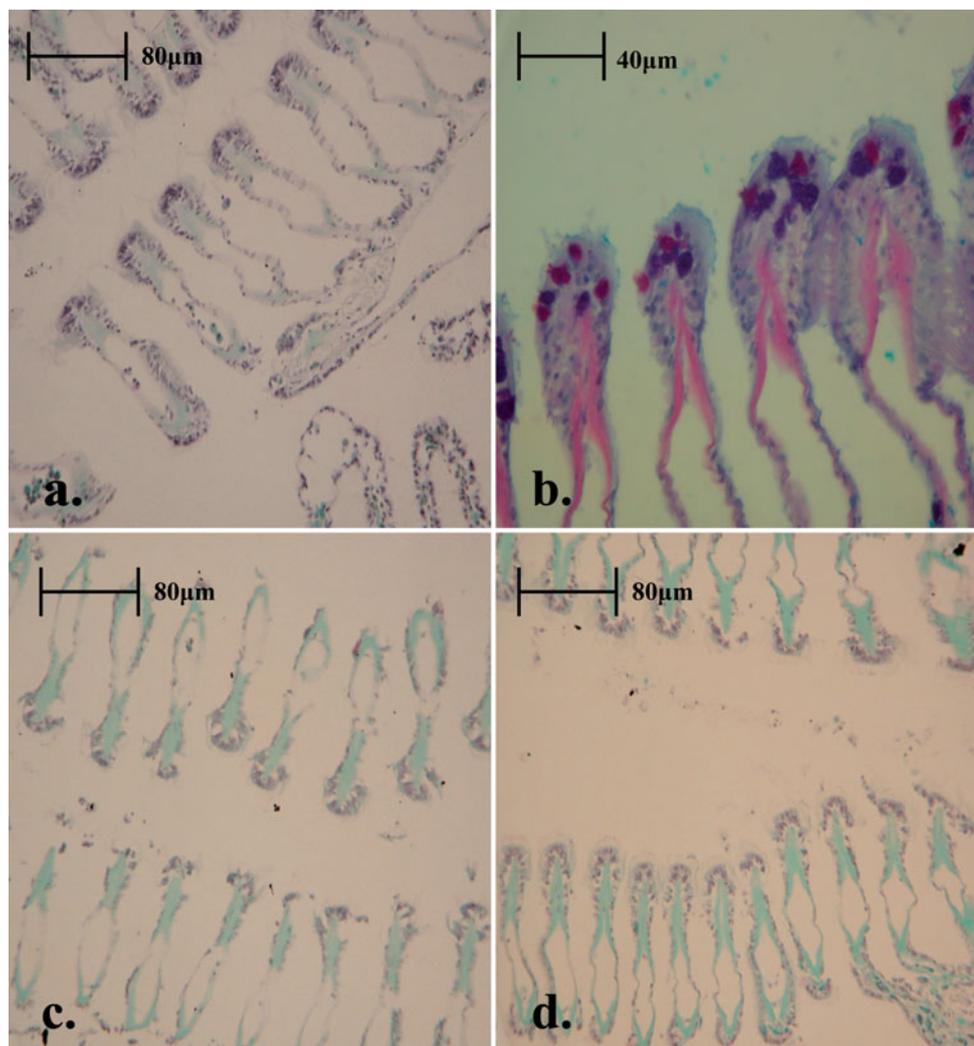


Fig. 4. Transverse sections of respiratory tissues showing: (a) normal gill tissues at Day 0; (b) typical impacted gill tissues from a separate study, illustrating large mucous cells on tips of gills (purple dots); (c) healthy gill tissues at Day 60 in the control treatment, indicating no impact; and (d) healthy gill tissues at Day 60 in the pH 6.5 treatment, indicating no impact

it is clear that, whilst some CO<sub>2</sub> loss may be due to atmospheric exchange, the majority was due to chemical buffering by the mussel shells. This conclusion is supported by previous experiments (Miles et al. 2007, Spicer et al. 2007, Widdicombe & Needham 2007) using the same design as that in the current study, which have not shown any CO<sub>2</sub> loss through atmospheric exchange. It seems reasonable to assume therefore that during the current study dissolution of the mussel shells occurred in the acidified treatments.

The current study has shown that after 60 d of exposure to acidified seawater there were no visible effects on the structure of reproductive, digestive, or respiratory tissues. Whilst it is true that no measures were made as to the viability or function of the tissues observed, it would seem that mussels possess well-developed physiological mechanisms for dealing with

short to medium periods of acidosis and thereby protecting body tissues. This is perhaps unsurprising as, during emersion, intertidal mussels close their shells to prevent desiccation. This closure increases the pCO<sub>2</sub> and Ca<sup>2+</sup> concentration of the haemolymph whilst decreasing the pH. However, the ability to internally compensate during periods of acidosis incurs physiological and energetic costs such as decreased growth, metabolic rate, ion exchange and protein synthesis (Pörtner & Langenbuch 2005). Berge et al. (2006) showed that a lowering of pH reduced the growth of *Mytilus edulis*. In pH 6.7, small mussels (11 mm mean length) showed a low growth rate, compared to control treatments, and large mussels (21 mm mean length) did not grow at all. Michaelidis et al. (2005) suggested that environmental hypercapnia was associated with a reduction in metabolic rate measured by the rate of O<sub>2</sub>

consumption and increased N<sub>2</sub> excretion, indicating net degradation of protein. Bamber (1990) also showed that a reduction in seawater pH was responsible for growth suppression, shell dissolution, tissue weight loss and feeding activity suppression.

The negative impacts of seawater acidification on the health and growth of mussels reported in the current study and by other authors (Bamber 1990, Michaelidis et al. 2005, Berge et al. 2006, Gazeau et al. 2007, Bibby et al. 2008,) could have significant ecosystem, economic and societal implications. At 1.4 million t, the global production of cultivated marine mussels in 2002 was 3.6% of the total aquaculture production, and this percentage has increased every year since (FAO 2004). Mussels are also an important food source for other marine organisms, as well as a variety of sea birds (Gutierrez et al. 2003). Mussel beds act as a habitat for a multitude of small benthic invertebrates (called cryptic fauna) that reside amongst the shells and within the byssus threads that hold the mussels onto a diverse array of substrate types. As such, mussels can be considered as ecosystem engineers for the maintenance of both biodiversity and sediment stability in coastal and estuarine environments. Finally, *Mytilus edulis* are widely used as an indicator species for toxicology studies, partly owing to the fact that the species is sessile, plentiful, inexpensive and relatively easy to maintain in laboratory conditions (Bayne et al. 1985).

Michaelidis et al. (2005), Berge et al. (2006), Bibby et al. (2008) and the present study have all shown that ocean acidification could potentially have an effect on *Mytilus* spp. Berge et al. (2006) noted reduced growth rates, Michaelidis et al. (2005) noted reduced metabolic rates, Bibby et al. (2008) noted a reduction in immune response and the current experiment showed a loss in lysosomal stability. However, ocean acidification from atmospheric CO<sub>2</sub> may not be the only potential source of high CO<sub>2</sub> that mussels may experience in the future. Leakage from sub-seabed stores of CO<sub>2</sub> could result in short-term exposure to extremely high levels of CO<sub>2</sub>. The current study has shown that adult *M. edulis* are likely to be able to survive short-term exposure (60 d or less) to extreme changes (up to 1.5 pH units) in seawater acidity, with little or no irreversible impact on general health or damage to body tissues. However, further studies are necessary to ascertain the effects on long-term acidification, including recovery rates following exposure to either chronic or acute exposure of CO<sub>2</sub> seepages (West et al. unpubl. data). It is also possible that the presence of *M. edulis* may act to buffer the effects of seawater acidification due to the partial dissolution of their shells over short exposure periods (<60 d). This may act to ameliorate the impacts of a leakage event for any cryptic fauna associated with *M. edulis*.

**Acknowledgements.** We thank H. Findlay, K. Perrett, A. Bettison, M. Liddicoat and C. Gallienne for their time and help in many aspects of this research. This study used the seawater acidification facility funded by a joint DEFRA/DTI funded project IMCO<sub>2</sub> and the NERC standard grant (NE/C510016/1). This paper is a contribution to the NERC funded program Oceans 2025 (Theme 3—Coastal and shelf processes).

#### LITERATURE CITED

- Bamber RN (1990) The effects of acidic seawater on 3 species of lamellibranch mollusc. *J Exp Mar Biol Ecol* 143:181–191
- Bayne BL, Brown DA, Burns K, Dixon DR and others (1985) The effects of stress and pollution on marine animals, Chap 2. In: *Cytological and cytochemical measurements*. Praeger Publishers, New York, p 46–74
- Berge JA, Bjerkgeng B, Pettersen O, Schanning MT, Oxnevad S (2006) Effects of increased sea water concentrations of CO<sub>2</sub> on growth of the bivalve *Mytilus edulis* L. *Chemosphere* 62:681–687
- Bibby R, Widdicombe S, Parry H, Spicer JI, Pipe R (2008) Impact of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. *Aquat Biol* 2:67–74
- Blackford JC, Jones N, Proctor R, Holt J (2008) Regional scale impacts of distinct CO<sub>2</sub> additions in the North Sea. *Mar Pollut Bull* 56:1461–1468
- Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. *Nature* 425:365
- Culling CFA (1963) *Handbook of histopathological techniques*. Butterworths, London
- FAO (Food and Agriculture Organisation) (2004) *The state of world fisheries and aquaculture*. FAO, Rome
- Gazeau F, Quiblier C, Jansen J, Gattuso JP, Middleburg J, Heip C (2007) Impact of elevated CO<sub>2</sub> on shellfish calcification. *Geophys Res Lett* 34:L07603
- Gutierrez JL, Jones CG, Strayer DL, Iribane OO (2003) Molluscs as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos* 101:79–90
- Hawkins DG (2004) No exit: thinking about leakage from geologic carbon storage sites. *Energy* 29:1571–1578
- Holloway S (2005) Underground sequestration of carbon dioxide—a viable greenhouse gas mitigation option. *Energy* 30:2318–2333
- Hubbard F, McManus J, Al-Dabbas M (1981) Environmental influences on the shell mineralogy of *Mytilus edulis*. *Geo-Mar Lett* 1:267–269
- IPCC (Intergovernmental Panel on Climate Change) (2001) *Climate change 2001: the scientific basis*. In: Houghton JT, Ding Y, Griggs DJ, Noguer M and others (eds) *Contribution of Working Group 1 to the 3rd assessment report of the IPCC*. Cambridge University Press, Cambridge
- Jones RG, Davis WL (1982) Calcium-containing lysosomes in the outer mantle epithelial cells of *Ambela*, a fresh water mollusc. *Anat Rec* 203:337–343
- Lindinger M, Lauren D, McDonald D (1984) Acid–base balance in the sea mussel, *Mytilus edulis*. Effects of environmental hypercapnia on intra- and extracellular acid–base balance. *Mar Biol Lett* 5:371–381
- Lowe DM (1988) Alterations in cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. *Mar Ecol Prog Ser* 46:91–100
- Lowe DM, Clarke KR (1989) Contaminant-induced changes in the structure of the digestive epithelium of *Mytilus edulis*. *Aquat Toxicol* 15:345–358
- Lowe DM, Pipe RK (1985) Cellular responses in the mussel *Mytilus edulis* following exposure to diesel oil emulsions:

- reproductive and nutrient storage cells. *Mar Environ Res* 17:234–237
- Lowe DM, Moore MN, Bayne BL (1982) Aspects of gametogenesis in the marine mussel *Mytilus edulis* L. *J Mar Biol Assoc UK* 62:133–145
- Lowe DM, Soverchia C, Moore MN (1995) Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquat Toxicol* 33:105–112
- Marchi B, Burlando B, Moore MN, Viarengo A (2004) Mercury- and copper-induced lysosomal membrane destabilisation depends on [Ca<sup>2+</sup>] dependent phospholipase A2 activation. *Aquat Toxicol* 66:197–204
- Mehrbach C, Culbertson CH, Hawley JE, Pytkowicz RM (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol Oceanogr* 18(6):897–907
- Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar Ecol Prog Ser* 293:109–118
- Miles H, Widdicombe S, Spicer JI, Hall-Spencer J (2007) Effects of anthropogenic seawater acidification on acid–base balance in the sea urchin *Psammechinus miliaris*. *Mar Pollut Bull* 54:89–96
- Moore MN (1985) Lysosomal responses to a polynuclear aromatic hydrocarbon in a marine snail: effects of exposure to phenanthrene and recovery. *Mar Environ Res* 17: 230–233
- Peek K, Gabbott PA (1989) Adipogranular cells from the mantle tissue of *Mytilus edulis* L. I. Isolation, purification and biochemical characteristics of dispersed cells. *J Exp Mar Biol Ecol* 126:203–216
- Pörtner HO, Langenbuch M (2005) Synergistic effects of temperature extremes, hypoxia, and increases in CO<sub>2</sub> on marine animals; from earth history to global change. *J Geophys Res* 110:C09510
- Sabine CL, Feely RA, Gruber N, Key RM and others (2004) The oceanic sink for anthropogenic CO<sub>2</sub>. *Science* 305: 367–371
- Seed R (1975) Reproduction in *Mytilus* (Mollusca: Bivalvia) in European waters. *PSZNI: Mar Ecol* 39:317–334
- Spicer JI, Raffo A, Widdicombe S (2007) Influence of CO<sub>2</sub>-related seawater acidification on extracellular acid–base balance in the velvet swimming crab *Necora puber*. *Mar Biol* 151:1117–1125
- Widdicombe S, Needham HR (2007) Impact of CO<sub>2</sub>-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. *Mar Ecol Prog Ser* 341: 111–122
- Winston GW, Moore MN, Kirchin MA, Soverchia C (1996) Production of reactive oxygen species by haemocytes from the marine mussel, *Mytilus edulis*: lysosomal localisation and the effect of xenobiotics. *Comp Biochem Physiol* 113C:221–229
- Zeebe RE, Wolf-Gladrow M (2001) CO<sub>2</sub> in seawater: equilibrium, kinetics, isotopes. Elsevier Oceanography Series 65, Amsterdam

Submitted: November 29, 2007; Accepted: April 16, 2008

Proofs received from author(s): September 8, 2008