Pathogenic challenge reveals immune trade-off in mussels exposed to reduced seawater pH and increased temperature

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Ocean acidification (OA) and warming pose a considerable threat to marine ecosystems. Previous studies show these environmental co-stressors significantly impact upon a number of key physiological functions, including calcification, metabolism and growth, in many marine organisms. Yet despite the importance of the immune system, to date only a handful of studies have investigated the impact of reduced seawater pH on an organism's immune response. Furthermore, whilst temperature has received far greater attention with respect to host defence, there is a dearth of information concerning the possible synergism of these two stressors on immune defence. Here we show that a 90 day exposure to reduced seawater pH led to a reduction in the antibacterial activity of cell-free haemolymph in the blue mussel *Mytilus edulis*, whilst temperature led to an increase in this immune parameter. However in contrast to previous research, following this initial 90 day exposure, mussels in the current study were then exposed to the pathogenic bacterium, *Vibrio tubiashii*. Crucially, whilst reduced seawater pH initially appeared to impair immunological functioning, as has been interpreted previously, mussels demonstrated the ability to restore haemolymph bactericidal activity when required. This indicated that the initial reduction in antibacterial activity was in fact a reversible physiological trade-off, rather than an irreversible impairment of immune function. By demonstrating this plasticity, the current study illustrates the need to measure organism responses within a realistic natural context (i.e. measuring the immune response of an organism in the presence of a pathogen). Failure to do so may result in a misleading interpretation of the ecological relevance of experimental data, and thus the sensitivity of different species in a rapidly changing environment.

Keywords: environmental stress, ocean acidification, pathogenic challenge, immune response, physiological trade-off, *Mytilus edulis*

1. Introduction

Ocean acidification (OA) is one of the greatest threats currently faced by marine ecosystems (Halpern, et al., 2008; Harley, et al., 2006). Caused by anthropogenic CO_2 emissions, OA has already led to measureable changes in ocean carbonate chemistry (Olafsson, et al., 2009; Santana-Casiano, et al., 2007) and it is projected to reduce surface seawater pH in the open ocean by 0.4 units before the end of this century (Caldeira and Wickett, 2003; 2005). In some coastal habitats, where the seasonal upwelling of hypoxic and hypercapnic seawater naturally produces carbonate chemistry conditions analogous to the current worst case acidification models (Hofmann, et al., 2011; Melzner, et al., 2012; Thomsen, et al., 2010), OA is likely to exacerbate such seasonal events, creating conditions that far exceed any current OA projections (Dorey, et al., 2013; IPCC, 2007; Melzner, et al., 2012). Furthermore, such alterations in seawater carbonate chemistry are occurring against a background of warming (IPCC, 2007), pollution (Rodgers and Laffoley, 2011) and disease outbreak (Elston, et al., 2008), posing a significant challenge to the many marine organisms that inhabit these coastal regions. It is therefore vital to characterise species sensitivity to multiple stressors (Dupont and Pörtner, 2013a; Dupont and Pörtner, 2013b).

Whilst reduction in seawater pH impacts a number of important physiological processes including calcification (e.g. Gazeau, et al., 2007; Wood, et al., 2010), photosynthesis (e.g. Langdon and Atkinson, 2005; Schneider and Erez, 2006), acid-base balance (e.g. Miles, et al., 2007; Spicer, et al., 2007), metabolism (e.g. Michaelidis, et al., 2005; Thomsen and Melzner, 2010), growth (Berge, et al., 2006; Thomsen, et al., 2010) and behaviour (e.g. Bibby, et al., 2007; Nilsson, et al., 2012) in a wide range of marine organisms, the investigation of host defence and its interaction with climate change stressors is still in its infancy.

The immune response is vital to all animals, controlling or fighting any pathogenic challenge (Ellis, et al., 2011). It is therefore a key determinant of host survival under changing environmental conditions. Despite this importance only a handful of studies have, to date, investigated the impact of OA on the immune system of a marine organism (e.g. Bibby, et al., 2008; Hernroth, et al., 2011; Matozzo, et al., 2012). Similarly, despite there being a significant body of literature that has investigated the impact of environmental temperature on host defence in marine invertebrates (see Ellis, et al., 2011), presently only one study has measured the combined effects of reduced seawater pH and temperature on the immune response of a marine organism (Matozzo, et al., 2012). Thus there is a pressing need to characterise the synergistic effects of reduced seawater pH and increased seawater temperature on the host defence of marine organisms to fully understand the impacts of changing environmental conditions on this important physiological process. Measuring an organism's immune response in the presence of a realised pathogenic threat arguably offers the best assessment of any altered immune functionality or immune suppression (Ellis, et al., 2011; Viney, et al., 2005), yet to date only one study has investigated the impact of reduced seawater pH in a marine organism in the presence of a pathogen (Asplund, et al., 2013). Therefore it is also a need to undertake studies that investigate the combined effects of climate change stressors on host defence, in the context of a pathogenic challenge.

As sessile filter feeders, marine bivalves are perhaps more predisposed to encountering marine microbes (Hernroth, et al., 2002), and an elevated threat of a pathogenic insult. Yet, effective defence mechanisms ensure mussels may tolerate this pathogen exposure without getting infected (Asplund, et al., 2013; Venier, et al., 2011). As calcifying marine invertebrates, bivalves may also be particularly vulnerable to reduced seawater pH (Hendriks, et al., 2010; Kroeker, et al., 2010; Kroeker, et al., 2013), which impacts on acid-base balance, metabolism and calcification in this group (e.g. Gazeau, et al., 2013; Hüning, et al., 2013;

Melzner, et al., 2011). Furthermore, bivalves are ectothermic, meaning environmental temperature also plays an important role in determining body temperature, directly impacting all physiological processes in this group (Pörtner, et al., 2006; Young, et al., 2011). Metabolic rate, for example, generally increases with increasing environmental temperature (Ede and Krogh, 1914; Pörtner, et al., 2006), whilst the rate of chemical and enzyme reactions, the fluidity of membranes and the structure of proteins are all temperature dependant (Rayssac, et al., 2010). The need to balance the allocation of energy between competing physiological processes under conditions of environmental stress, and under the combined challenge of reduced seawater pH, increased temperature and pathogen exposures in particular, means marine bivalves are thus an ideal model system for investigating the impact of climate change stressors on the invertebrate immune response.

As energy is a key limiting resource, it has been hypothesised that the increased energetic burden of an exposure to reduced seawater pH, increased temperature or the possible synergistic effects of these stressors will divert resources from processes such as host defence or growth, in favour of higher priority processes such as maintaining physiological homeostasis, metabolism or calcification (Bibby, et al., 2008; Sokolva, et al., 2012; Wood, et al., 2008). If this is the case then an OA/temperature induced trade-off in host defence should reduce immunocompetence, and increase the sensitivity of a host organism to a pathogenic challenge. In support of an OA induced reduction in immunocompetence, Bibby, et al. (2008) showed that exposure to reduced seawater pH reduced phagocytic activity, whilst Matozzo, et al. (2012) showed it reduced lysosome-like activity of cell free haemolymph, in marine mussels. However, these reductions were demonstrated to occur in the absence of a pathogen in both studies. Therefore it is possible that mussels trade-off the cost of maintaining a maximal immune response under OA conditions, with no actual impairment of immune functionality or increased disease susceptibility.

To test this hypothesis we investigated how a combined exposure to reduced seawater pH (ranging from 8.05 to 6.50) and increased temperature impacted the antibacterial activity of cell-free haemolymph in the blue mussel, *Mytilus edulis* and how a subsequent challenge with the pathogenic bacterium, *Vibrio tubiashii* (Elston, et al., 2008), altered the initial response of this immune parameter to environmental stress.

2. Materials and Methods

2.1. Organism collection, exposure and sampling protocol

The mussels used in this study were collected and maintained as described by Ellis, et al. (2014). Briefly, adult *Mytilus edulis* (shell length = 50 – 70 mm) were collected from Exmouth, UK, and transported to Plymouth Marine Laboratory, in December 2009. Mussels were divided between 60 experimental chambers, continuously supplied with sea water from 1 of 10 header tanks. Header tank pH_{NBS} was adjusted using the computerised feedback mechanism described by Widdicombe and Needham (2007), maintaining sea water at one of five nominal pH levels: 8.05 (present day ambient seawater pH), 7.80 (2100, IS92 emissions scenario; IPCC, 2007), 7.60 (2100, A2 scenario; Caldeira and Wickett, 2005), 7.35 (2300, IS92 emissions scenario; IPCC, 2007) and 6.50 (CCS leak; Blackford, et al., 2009). Chambers were distributed between 10 water baths, set to 12.5°C or 17.0 °C, creating a fully crossed experimental design. Mussels were fed *Isochrysis galbana* (algal concentration maintained between 100,000 and 150,000 cells ml^{-1}). pH_{NBS}, temperature and salinity were measured 3 times per week. Total alkalinity (A_T) was measured weekly using an open-cell potentiometric titration technique. All other carbonate system variables were calculated using CO_2SYS (Pierrot, et al., 2006), according to Findlay, et al. (2013). A sub-set of the experimental seawater carbonate chemistry parameters from header tanks are presented in Table 1. Full carbonate chemistry data for both header tanks and experimental chambers for the experimental exposure are presented in Ellis, et al. (2014).

Mussels were maintained as above for 90d, following which antibacterial activity of cell-free haemolymph was measured in one mussel per chamber. After 91d the remaining mussels were challenged with *Vibrio tubiashii* (2×10^6 bacterial cells, injected into posterior adductor muscle), before being returned to the system. The immune response was then

measured again in one mussel per chamber after 92d (1d post-exposure) and again after 98d (7d post-exposure). Mortality was assessed daily throughout the duration of the experiment, with any dead mussels removed from the set-up upon discovery.

2.2. Antibacterial activity of cell-free haemolymph

To quantify the immune response haemolymph (vol. = 500 µl) was extracted using a syringe (vol. = 1.0 ml), the needle (21G) of which was inserted into the large sinus within the posterior adductor muscle. Antibacterial activity of cell-free haemolymph was estimated by measuring the growth inhibition of a log phase bacterial culture by turbidometry, according to Wootton and Pipe (2003). Briefly, an aliquot of haemolymph (250 µl) was added to an equal volume of sterile 3% NaCl solution and centrifuged (400 x g, 150 sec). The supernatant was carefully removed and transferred to a microcentrifuge tube (Eppendorf® vol. = 1.5 ml) and stored at T = 20°C until analysis could be carried out.

Antibacterial activity of the cell-free haemolymph was measured by pipetting a 100 µl aliquot of cell-free haemolymph into 4 replicate wells of a 96-well microplate (Nunc Microwell) with an equal volume of *Vibrio tubiashii* suspension (2 x 10^6 bacterial cells per ml^{-1} suspended in sterile 3% NaCl solution). 50 µl of sterile 3% NaCl solution and 50 µl of marine broth (sterile 3% NaCl solution, 5 gl^{-1} peptone, 1 gl^{-1} yeast extract) with 100 µl of *Vibrio tubiashii* suspension were added to 4 replicate wells as controls. 150 µl of sterile 3% NaCl solution added to 50 µl of marine broth was used as the blank. Plates were read using a microplate reader (Molecular Devices VersaMax Microplate Reader) at λ = 340 nm over 22 h. Activities are expressed as percentage bacterial growth inhibition in haemolymph exposed samples compared to control bacterial growth.

2.3. Data analysis

Carbonate chemistry and haemolymph antibacterial activity data were analysed using the PERMANOVA+ add in (Anderson, et al., 2008), in PRIMER 6.1 (Clarke and Gorley, 2006). Data were first tested for homogeneity of variance using PERMDISP, a dissimilarity-based multivariate extension of Levene's test (Anderson, et al., 2008). An appropriate transformation was performed if required. Euclidean distance similarity matrices were constructed, and p-values were calculated using 9999 permutations of the residuals under a reduced model. Pair-wise comparisons were then made where significant p-values were present for a factor with more than two levels, or where two factors were shown to significantly interact. Survival data were tested using a Log-rank survival analysis of Kaplan-Meier curves in Sigmaplot 12.0 (Systat Software, California, US). Pair-wise comparisons were then made using the Holm-Sidak multiple comparison test when Log-rank survival analysis indicated a significant difference in the survival between different treatment groups.

3. Results

3.1. Antibacterial activity of cell-free haemolymph

Reduced seawater pH (Pseudo-F_4 = 7.79, p = <0.01) and increased temperature (Pseudo-F_1 = 13.50, p = <0.01) were both shown to significantly affect the antibacterial activity of cell-free haemolymph in mussels. Pair-wise analyses indicated that mussels maintained at pH 6.50 had a significantly lower antibacterial activity compared to mussels maintained at all other pH levels, whilst mussels maintained at pH 7.80 were also significantly different to those maintained at pH 7.35 (Fig. 1a). Additionally, organisms maintained at 17.0 °C had a higher antibacterial activity compared to those maintained at 12.5 °C (Fig. 1b).

As well as showing separate effects of both reduced seawater pH and increased temperature on the bactericidal activity of mussel haemolymph, these two factors were shown to interact (Pseudo-F_4 = 3.08, p = 0.02). As with the main pH effect, pair-wise analyses showed that at 12.5 °C organisms maintained at pH 6.50 were significantly different to those exposed to all other pH treatments, whilst mussels maintained at pH 7.80 were also different from those maintained at pH 7.60 and 7.35 (Fig. 2). Conversely, at 17.0 °C there was no significant reduction in the antibacterial activity of mussels maintained at pH 6.50, whilst those maintained at pH 7.35 were significantly different to those at pH 7.80 and 7.60 (Fig. 2).

As with temperature, seawater pH was also shown to interact with pathogen exposure (Pseudo-F_8 = 3.29, p = <0.01) in the current study. Pairwise analysis demonstrated that the antibacterial activity of cell-free haemolymph was significantly lower in mussels exposed to pH 6.50, compared to all other pH treatments, prior to a bacterial exposure (Fig. 3a). Furthermore, the bactericidal activity of mussel haemolymph was significantly lower in mussels maintained at pH 7.80 compared to pH 7.35 pre-challenge (Fig. 3a). Conversely, whilst mussels maintained at pH 7.35 were significantly different to those maintained at pH

7.80, 7.60 and 6.50 when measured 1 day post exposure, there was no difference in the antibacterial activity in any other treatment group (Fig. 3b). By 7 days post bacterial exposure there was no significant difference in the bactericidal activity of organisms maintained at any pH exposure level (Fig. 3c). There was no significant three-way interaction detected between reduced seawater pH, increased temperature and bacterial exposure in the current study.

3.2. Mortality

Mortalities occurred in all treatments during the experiment; however as reported by Ellis, et al. (2014), cumulative mortality was higher at low pH. Log-rank survival analysis of Kaplan Meier curves highlighted that there was a significant effect of reduced seawater pH on survival in organisms maintained at both 12.5 °C (Z_4 = 44.70, p = <0.001) and 17.0 °C (Z_4 = 54.40, p = <0.001). Pairwise comparisons revealed that at 12.5 °C the probability of survival was significantly lower in organisms maintained at pH 6.50 compared to all other treatments. Cumulative survival did not fall below 90 % at pH 8.05, pH 7.80, pH 7.60 and pH 7.35 (Fig. 4a) and was not significantly different between these groups, however at pH 6.50 survival fell below 50 %, with the mean survival time of organisms in this group being 67 days.

As at 12.5 °C, pairwise analyses revealed the probability of survival was significantly lower in organisms maintained at pH 6.50 and 17.0 °C, compared to all other pH treatments at this temperature. Whilst mussel survival was maintained above 90 % at pH 8.05, at pH 7.80 and pH 7.60 survival was 87%. At pH 7.35 survival fell to 67%. At pH 6.50, in mussels maintained at 17.0 °C, survival dropped to just 21% during the experimental exposure (Fig 4b), with the mean survival time of organisms in this group being 59 days. No additional mortality was noted following *V. tubiashii* exposure.

Discussion

In agreement with previous research (Beesley, et al., 2008; Ellis, et al., 2014; Ries, et al., 2009; Thomsen, et al., 2010; Thomsen and Melzner, 2010), mussels in the present study were tolerant of a level of seawater acidification projected to occur in the open ocean over the next 300 years (pH 7.35; Caldeira and Wickett, 2005). Under these experimental conditions the antibacterial activity of cell-free haemolymph remained unaffected and survival was maintained above 90% at 12.5 °C. Conversely, when exposed to an extreme level of CO_2 enrichment (pH 6.50), the bactericidal activity of mussel haemolymph was significantly reduced. Furthermore, the survival at this pH fell below 50%.

In showing immune system maintenance to be compromised by exposure to a reduced seawater pH of 6.50, this study would appear to support previous research on the impact of elevated seawater pCO_2 on the invertebrate immune response (Bibby, et al., 2008; Matozzo, et al., 2012). Bibby et al. (2008) found that a 32 day exposure to OA reduced the ability of impacted mussels to increase phagocytic activity when compared to controls, and they suggested that an increased haemolymph calcium concentration interfered with cellular signalling pathways, and thus immunocompetence. Similarly, Matozzo, et al. (2012) demonstrated that a 7 day exposure to pH 7.7 or 7.4 reduced lysozyme-like activity of cell-free haemolymph in *Mytilus galloprovincialis*. These authors, and subsequent reviewers, have therefore interpreted this reduced immune activity as a reduction in both organismal health and disease resistance. However, Bibby, et al. (2008) and Matozzo, et al. (2012)'s studies were undertaken in the absence of a pathogenic challenge, and therefore interpreting a reduction in immune defence as reduced immunocompetence may not be correct as it assumes a maximal immune response always maximises fitness, irrespective of the cost of immune system maintenance (Viney, et al., 2005).

Our understanding of the marine invertebrate immune response has significantly advanced over the past decade following the development and employment of novel genetic techniques (Philipp, et al., 2012). However, the observation that immune defences are often induced by infection rather than being constitutively active suggests immune activity is costly (Lazzaro and Little, 2009). These costs are central to our understanding of ecological immunology (Rolff and Siva-Jothy, 2003; Sheldon and Verhulst, 1996). Measuring an organism's susceptibility to a realised pathogenic threat therefore offers a better assessment of impaired immune function (Ellis, et al., 2011; Viney, et al., 2005).

Crucially, whilst exposure to reduced seawater pH significantly reduced the antibacterial activity of cell-free haemolymph, a pathogenic challenge was key in the response of the invertebrate immune system exposed to elevated seawater pCO_2. Despite the antibacterial activity of mussels maintained at pH 6.50 being 40 % lower than the control group prior to a bacterial exposure, when this immune parameter was measured again following a bacterial challenge there was no significant difference in the bactericidal activity of mussels maintained at this pH compared to the control group. From this it might be suggested that the initial reduction in host defence noted in these mussels was in fact a reversible, physiological trade-off, rather than an irreversible impairment of immunological functioning, as concluded previously (Bibby, et al., 2008; Matozzo, et al., 2012). Furthermore, by maintaining immune plasticity, and an ability to up-regulate the immune response when required, mussels in the current study were able to respond to a bacterial infection when the need arose.

In the current study elevated temperature was also shown to significantly impact the mussel immune response, significantly increasing the antibacterial activity of cell-free haemolymph. This is consistent with Monari, et al. (2007)'s finding of a temperature-related increase in antibacterial activity in the striped venus clam, *Chamelea gallina.* It is widely

accepted that temperature affects enzymatic activity and metabolism in ectotherms (Somero, 2002). Therefore, the increase in antibacterial activity measured in the current study, and the increase in lysozyme-like activity measured by Monari, et al. (2007), could represent an increased activity of hydrolytic enzymes at elevated temperature. Such an increase in antimicrobial activity with increased temperature has been demonstrated in the shore crab, *Carcinus maenas*, where the activity of antimicrobial proteins was higher at elevated temperatures (Chrisholm and Smith, 1994). The sensitivity of antimicrobial activity to temperature means it is possible that the rise in seawater temperature predicted to occur within the next 100-300 years (IPCC, 2007) may counteract any reduction in immune system maintenance caused by a concomitant reduction in seawater pH. This is further supported by the significant interaction between temperature and seawater pH, with the increased antibacterial activity of cell-free haemolyph at 17.0 °C counteracting the impact of reduced seawater pH on this immune parameter, even under extreme enrichment of sea water pCO_2 (pH 6.50).

Whilst seawater temperature increased the bactericidal activity of mussel haemolymph, it also led to an apparent increase in the sensitivity of mussels to OA, as measured by the higher cumulative mortality in mussels maintained at both pH 7.35 and 6.50 at 17.0 °C. This study is therefore consistent with previous research that highlighted the importance of measuring the impact of environmental stressors in combination (Harvey, et al., 2013; Kroeker, et al., 2013; Wood, et al., 2010), as doing so indicates that organisms may be more vulnerable to climate change than suggested by single stressor studies.

Conclusions

Mussels can trade-off immune system maintenance, while maintaining an ability to up regulate their immune response when a pathogen is encountered, even under extreme environmental conditions. We conclude that by overlooking, or not accounting for, physiological trade-offs, investigators are liable to misinterpret experimental results. By comparing our results with previous research (Bibby, et al., 2008; Matozzo, et al., 2012), we illustrate that it is possible a natural mechanism for managing environmental stress may be interpreted and reported as a negative result, possibly incorrectly. This study therefore cautions any assumption that physiological responses encountered in laboratory experiments are solely driven by direct effects of environmental stress. As environmental stress increases so too will the energy demands on marine organisms. Individuals will increasingly be required to adjust and balance their energy budget to meet the costs of a variety of physiological challenges as and when they arrive. Understanding and measuring physiological parameters functionally (i.e. measuring the immune response in the presence of a pathogen), will therefore allow the successful differentiation between pathological responses and physiological trade-offs. Ultimately this should allow the OA and climate change research community to better predict population and ecosystem level impacts with greater confidence.

Acknowledgements

This work was supported by the UK Natural Environmental Research Council (NERC), who funded a PhD studentship awarded to R.P.E. This study was undertaken at PML using OA exposure facilities partly funded by a joint DEFRA/DTI funded project (IMCO2) and the NERC standard grant (NE/C510016/1) awarded to S.W.

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Table 1 Carbonate chemistry of sea water in header tanks for each pH exposure level for each pH exposure level. Data are expressed as mean (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters based on pair-wise tests. †Calculated using CO_2SYS software.

Parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	8.09 ± 0.01[a]	7.77 ± 0.01[b]	7.60 ± 0.01[c]	7.33 ± 0.01[d]	6.46 ± 0.02[e]
Temperature (°C)	13.83 ± 0.11	13.94 ± 0.10	13.85 ± 0.10	14.01 ± 0.10	13.87 ± 0.10
Salinity	34.17 ± 0.07	34.15 ± 0.07	34.17 ± 0.07	34.16 ± 0.07	34.15 ± 0.07
A_T (μmol kg^{-1} SW)	2402.32 ± 30.27	2396.70 ± 27.89	2412.67 ± 30.79	2392.42 ± 31.71	2420.65 ± 30.62
pCO_2 (μatm)†	518.02 ± 22.25[a]	1177.85 ± 64.12[b]	1940.40 ± 240.51[c]	5075.37 ± 1402.88[d]	25242.14 ± 1938.82[e]

Figure Legends

Figure 1. (a) The effect of seawater pH on the antibacterial activity of mussel cell-free haemolymph. **(b)** The effect of water temperature on the antibacterial activity of mussel cell-free haemolymph. Values are expressed as means (± SEM). Data are pooled across temperature and bacterial exposure **(a)** or bacterial exposure and seawater pH **(b)**. Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

Figure 2. The effect of temperature and seawater pH on the antibacterial activity of cell-free haemolymph in mussels maintained at 12.5 °C (light grey bars) or 17.0 °C (dark grey bars). Values are means (± SEM) for data pooled across bacterial exposure. Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

Figure 3. The effect of seawater pH and a bacterial exposure on the antibacterial activity of cell-free haemolymph in mussels measured **(a)** prior to a bacterial exposure, **(b)** 1 d post-inoculation or **(c)** 7 d post-inoculation. Values are means (± SEM) for data pooled across temperature. Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

Figure 4. *Mytilus edulis* survivorship in organisms maintained under control and acidified seawater conditions at **(a)** 12.5 °C and **(b)** 17.0 °C, over the duration of the experimental exposure. Step-wise curves were calculated and generated using Kaplan Meier Log-rank survival analysis.

Fig. 1

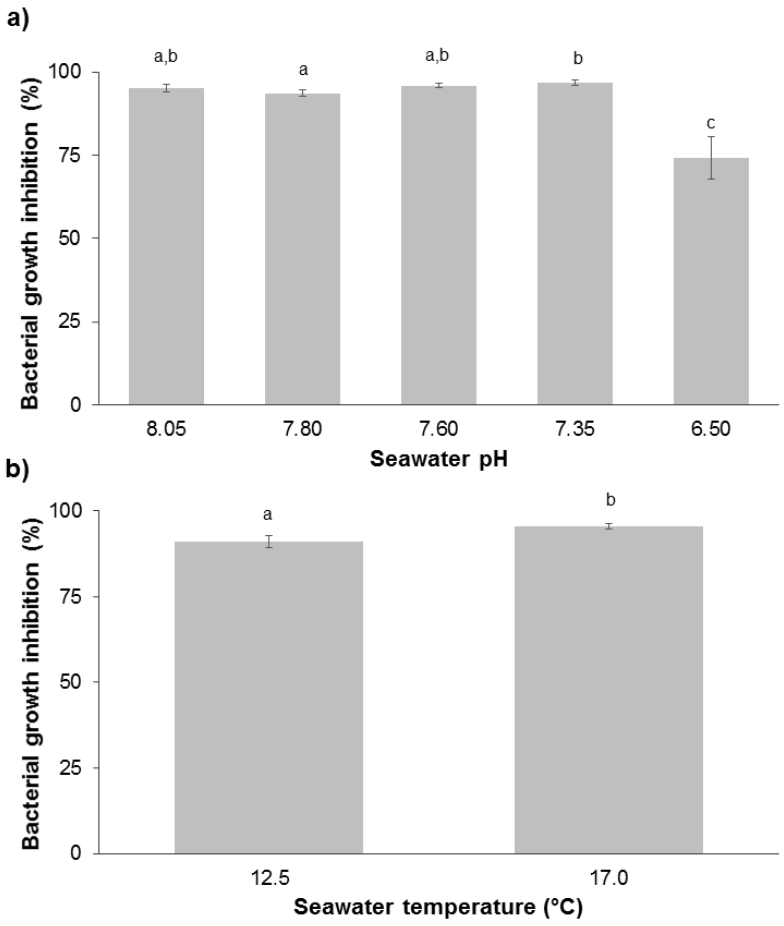

a)
a,b
a
a,b
b
c
100
75
50
25
0
Bacterial growth inhibition (%)
8.05
7.80
7.60
7.35
6.50
Seawater pH
b)
a
b
100
75
50
25
0
Bacterial growth inhibition (%)
12.5
17.0
Seawater temperature (°C)

Fig. 2

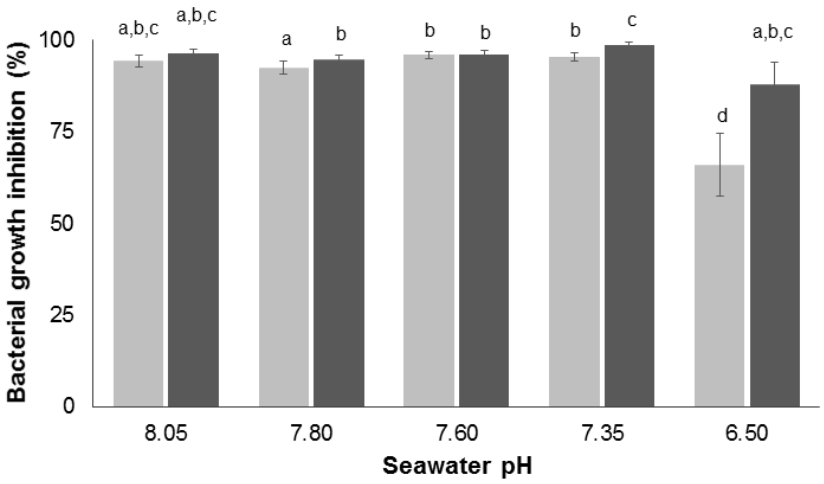

Bacterial growth inhibition (%)
100
75
50
25
0
a,b,c
a,b,c
a
b
b
b
b
c
d
a,b,c
8.05
7.80
7.60
7.35
6.50
Seawater pH

Fig. 3

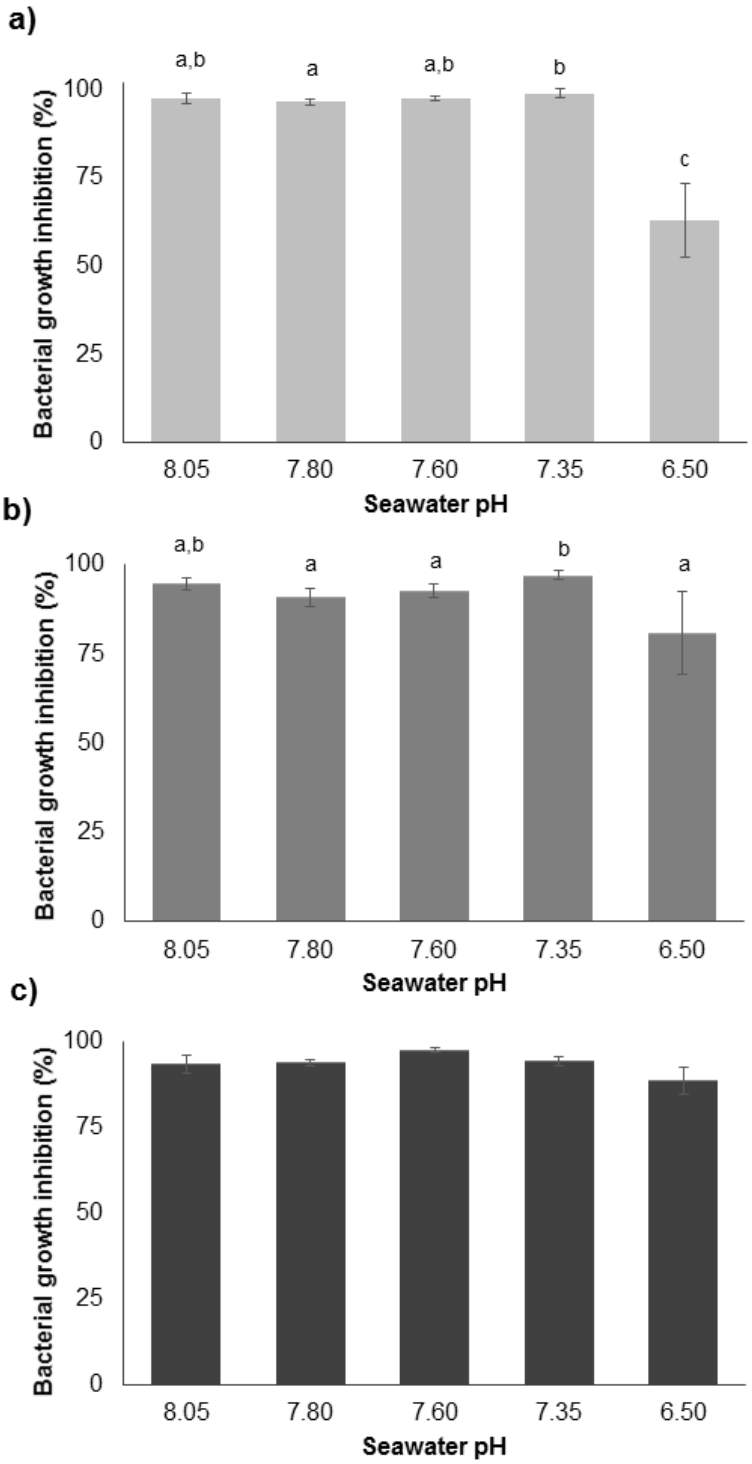
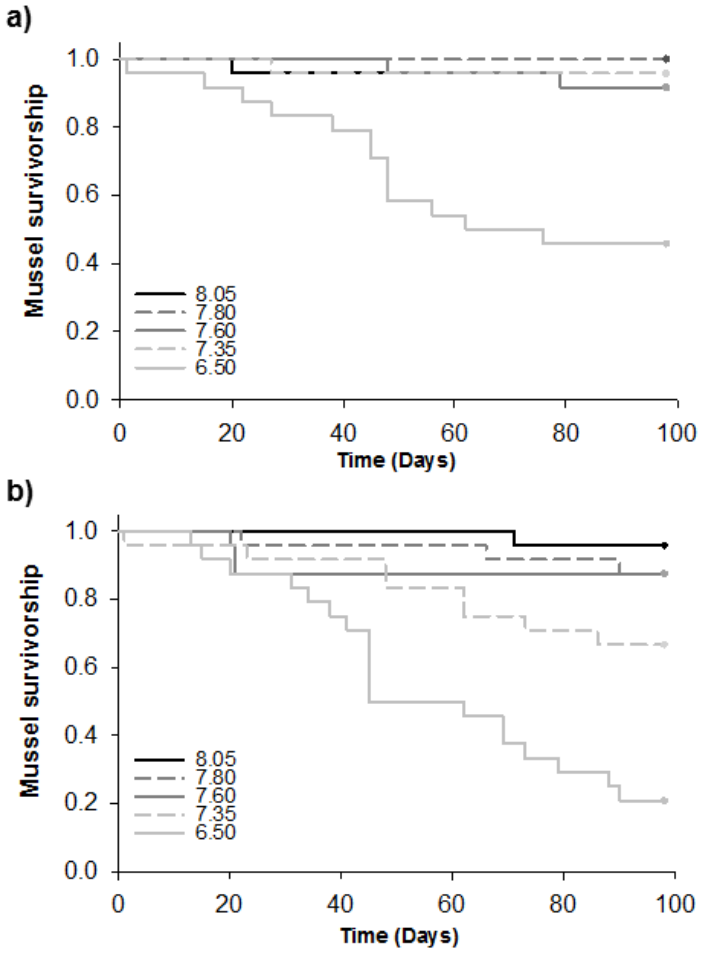

a)
a,b
a
a,b
b
c
100
75
50
25
0
Bacterial growth inhibition (%)
8.05
7.80
7.60
7.35
6.50
Seawater pH
b)
a,b
a
a
b
a
100
75
50
25
0
Bacterial growth inhibition (%)
8.05
7.80
7.60
7.35
6.50
Seawater pH
c)
100
75
50
25
0
Bacterial growth inhibition (%)
8.05
7.80
7.60
7.35
6.50
Seawater pH

Fig. 4


a)
Mussel survivorship
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (Days)
8.05
7.80
7.60
7.35
6.50
b)
Mussel survivorship
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (Days)
8.05
7.80
7.60
7.35
6.50