Physiological Effects of Global Climate Change on Common British Marine Invertebrates

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by

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Abstract: Climate change is likely to have profound effects on marine animals due to the predicted increases in water temperature and acidity. Many studies have examined the effects of these elevated temperatures and decreased pH at the extreme temperatures expected in the summer season, but few studies have investigated how climate change may affect animals in the winter. In this study, we investigate the effects of both winter and summer temperatures on the growth rates, body composition and metabolic rate of four species of intertidal marine invertebrates: two calcified (common mussel - *Mytilus edulis* and edible periwinkle – *Littorina littorea*) and two non-calcified (beadlet anemone – *Actinia equina* and sea squirts – *Ascidiella aspersa*) species. Samples divided to two groups, one group exposed to winter temperature condition and the second group exposed to summer temperature condition. Following a period of acclimatization during which temperature was gradually increased and pH decreased, animals were exposed to the predicted climatic conditions of 2050 (TR 2050) and 2100 (TR 2100) for six weeks. During the study period, the mortality rates were monitored as well as growth rates by taking body weight, buoyant weight and body morphometrics (length and Width). At the end of experiments, body composition were measured by taking water content, dry shell and dry body weight weight, fat content and C:N ratio. In addition, metabolic rates were measured using a closed-system respirometry. During the experiments, seawater parameters such as acidity, temperature, salinity and dissolved oxygen were measured. The results of the experiments found that there was a significant increase in mortality of *A. aspersa* at the higher temperatures and water acidity in winter. Furthermore, growth rates of *A. equina* and *A. aspersa* were significantly reduced at TR 2050. On the other hand, it was observed that the C:N ratio of *L. littorea* was significantly increased at TR 2050 and that metabolic rate was significantly higher at TR 2100. However, under summer conditions, *L. littorea* there was a significant decrease in buoyant weight at TR 2050. While there was no mortality amongst *A. equina*, a significant reduction growth was found at elevated temperature and decreased pH level. The results in this study indicate that inter-species responses to environmental changes are likely to differ but also that the inter-species response will also vary depending on the season and life stage of the animal.

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Chapter 1. Introduction

1.1 Background:

The world has begun to clearly see the effects of changes in the Earth's climate. Elevated temperatures (air and ocean), floods, hurricanes, and heavy rains are the most notable effects that have been recognised reflecting this change in the global climate (IPCC, 2007). Climate change may also cause agricultural land to flood, forests to burn, sea levels to rise, and polar and glacial ice to melt (IPCC, 2007). These phenomena may cause changes in natural habitats and ecosystems (Markham, 1996; Justus and Fletcher, 2001), which may threaten the biodiversity (Cheung *et al.*, 2009) and the food security of humans (Schubert *et al.*, 2006). So far, no one can determine with any degree of accuracy the size, rate and timing of these changes (Justus and Fletcher, 2001), which increases concerns about the effects that might arise from these changes.

1.2 Greenhouse Gases:

To date, perhaps the most prominent phenomenon related to global warming is the blockage of heat generated by the sun's rays from exiting the Earth's atmosphere. This blockage occurs due to a group of gases called greenhouse gases. The most important of these gases are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (IPCC, 2007). These gases are emitted during some human activities, predominantly fossil fuel (gas, coal, and oil) burning and various agricultural activities (IPCC, 2007). The most important source of CO_2 is the burning of fossil fuels (IPCC, 2007; Doney, 2009) by human activities (Doney, 2006). It is now largely accepted by the scientific community that continuing increases in CO_2 emissions at the same levels as at present will, in the future, have strong effects on the warming and acidification of the ocean.

1.3 Global Warming and Ocean Warming:

Greenhouse gases including CO_2 contribute to rising air temperatures, which have actually risen by almost one degree Celsius (1 °C) during the past 100 years (IPCC, 2007). This rate of increase is expected to result in further temperature increases by up to 1 °C to 6.4 °C by the end of this century, due to increased CO_2 emissions (IPCC, 2007). The seawater surface (SS) will be affected by increased air temperatures more than the depths of the oceans, due to its proximity to the atmosphere (Brierley and Kingsford, 2009). Already the seawater surface temperature (SST) has increased by 0.76 °C since the 19th century (IPCC, 2007; Findlay *et al.*, 2008), which means that future warming will continue to affect the ocean (Tyrrell, 2011) as the rate of CO_2 emissions continue. The global mean temperature will be slightly higher than the SST that has been predicted for the future (Gruber, 2011). The current predictions indicate an increase in ocean temperature by 2050 of up to 2 °C (IPCC, 2007) and reaching 4 °C in 2100 (Findlay *et al.*, 2008) (see Fig. 1.1).

1.4 Increased Seawater Temperatures:

The ability of organisms to cope with changes in temperature varies from one species to another (Peck *et al*., 2004). Organisms that have a high ability to withstand temperature changes are likely to be less susceptible to the negative effects of the temperature rise (Noone *et al.,* 2013). Among organisms that may experience the greatest effects of changes in temperature are the ectothermal marine organisms (Brierly and Kingsford, 2009). Temperature is the main factor that controls the metabolic and respiratory rates in ectothermal marine organisms (Somero, 2010; Noone *et al.*, 2013), so seawater temperature is a critical environmental factor affecting their physiological processes (Hochachka and Somero, 2002; Sokolova and Portner, 2003; Dong *et al.*, 2011), growth (Dong *et al.*, 2008; Ji *et al*., 2008), seasonal timing (Portner and Knust, 2007), survival (Barange *et al*., 2010), thermal limits, and species distribution (Harley *et al*., 2006; Noone *et al.,* 2013). If increased, temperatures can have direct impacts on physiological processes and growth (Byrne *et al*., 2009). An increase in temperature may negatively influence the performance and survival of marine organisms (Harley *et al.*, 2006).

Table 1.1 The best estimates for the highest temperatures and the likely temperature range at the end of this century, according to the Special Report on Emissions Scenarios (SRES) by the Intergovernmental Panel on Climate Change (IPCC, 2007) due to greenhouse gas emissions from anthropogenic sources and a number of different expectations. Among these scenarios, the least extreme is B1, wherein the volume of emissions in the atmosphere is expected to reach 600 ppm, followed by the A1T scenario at 700 ppm, then B2 at 800 ppm, A1B at 850 ppm, and A2 at 1250 ppm. The worst expectation is under the A1F1 scenario, in which anthropogenic greenhouse gases emissions in the atmosphere are expected to reach 1550 ppm; they are currently at around 390 ppm. Adapted from (IPCC, 2007).

Case	**Temperature change (ºC from 2090-2099 relative to 1980-1999)**		**Atmospheric CO_2 levels (ppm)**
	Best estimate	**Likely range**	
Constant year 2000 concentrations	0.6	0.3 - 0.9	
B1 scenario	1.8	1.1 – 2.9	600
A1T scenario	2.4	1.4 – 3.8	700
B2 scenario	2.4	1.4 – 3.8	800
A1B scenario	2.8	1.7 – 4.4	850
A2 scenario	3.4	2.0 – 5.4	1250
A1F1 scenario	4.0	2.4 – 6.4	1550

Usually increases in seawater temperature lead to increased metabolic and behavioral activities in aquatic ectotherms, leading to a rise in their rate of oxygen consumption, and thus reduced energy reserves, which could decrease opportunities for growth (Levinton, 2009). For example, the weight of sea cucumber (*Apostichopus japonicus*) decreased gradually upon gradual increases in temperature (Ji *et al*., 2008). Also, in some calcified marine invertebrates increasing temperatures up to summer averages led to increased calcification (Marshall and Clode, 2004; Langdon and Atkinson, 2005), followed by decreased calcification when temperatures exceeded average summer temperatures (Clausen and Roth, 1975; Abramovitch-Gottlib *et al.*, 2002; Rodrigues and Grottoli, 2006). Predicting the impact of changes in temperature on thermal stress to these model organisms is central to the goal of exploring the consequences of climate change (Helmuth *et al*., 2002). Somero (2005; 2010) reported that many organisms are already living at temperatures near their thermal

tolerance limits, for example, porcelain crabs, genus *Petrolisthes* (Stillman, 2002) and turban snails, genus *Tegula* (Hellberg, 1998). Therefore, any additional increase in temperature may exacerbate the existing negative effects of climate change (Vihtakari *et al*, 2013). However, the temperature is just one of a range of climate change factors and one cannot as accurately predict future effects by relying solely on the relationship between organisms and the temperatures of their habitats (Harley et al., 2006).

1.5 Ocean Acidification:

In addition to contributing to global warming, CO_2 plays a central role in ocean acidification. The amount of CO_2 estimated to be present in the atmosphere in the middle of the 18th century was 280 ppm (IPCC, 2007; Bulling *et al.*, 2010). This value had risen to 379 ppm by 2005 (IPCC, 2007; De Bodt *et al.*, 2010) as a result of human activities (Feely *et al.*, 2004; Bibby *et al.*, 2008; Fabry *et al.*, 2008). This represents an addition of approximately 5.6×10^{11} tons of CO_2 to the atmosphere; slightly more than half of which has now been absorbed by the sea (Doney *et al.*, 2009). As a result, the pH level of the surface ocean has decreased by 0.1 unit compared to the pH levels prior to 1750 (Caldeira and Wickett, 2003; Orr *et al.*, 2005; Arnold *et al.*, 2009). The ocean's pH levels were estimated to have been 8.1 before 2007 (IPCC, 2007), and this pH value is expected to decrease by around 0.22 units from current levels by 2050 (Caldeira and Wickett, 2005; Vézina *et al.*, 2008) to reach approximately 7.88. The pH levels of the ocean are currently expected to decline by further approximately 0.4 units by 2100 to 7.7 (Findlay *et al.*, 2008), when CO_2 concentrations may rise to 1200 ppm (Caldeira and Wickett, 2003; Raven *et al.*, 2005; Arnold *et al.*, 2009). The ocean pH levels are expected to continue to decrease during the next two centuries to approximately 6.7, a decrease of 1.4 units compared to the current pH of 8.1 (Caldeira and Wickett, 2005; Harley *et al.*, 2006). Such a drop in pH may have severe biological consequences (Caldeira and Wickett, 2005).

The importance of these abiotic interactions lies in the fact that such chemical changes in seawater may affect the metabolic processes, ecosystems, and biodiversity of marine organisms (Kleypas *et al.*, 2005; Raven *et al.*, 2005; Fabry *et al.*, 2008).

Scientists expect the increased temperatures and decreased pH that are direct effects of increased [CO_2] (carbon dioxide) (Fabry *et al.*, 2008) to have measurable consequences for marine organisms (Kurihara *et al.*, 2004) and marine ecosystems (Findlay *et al.*, 2008) in the future. Increased [CO_2] and decreased pH in themselves both affect marine organisms (Wood *et al.*, 2008). According to Gutowska *et al.* (2008) low pH has negative effects on a range of marine invertebrates, such as molluscs, crustaceans, and sea urchins (Wood *et al.*, 2008) because it directly influences the physiological processes of these creatures (Pörtner *et al.*, 2004; Raven *et al.*, 2005; Arnold *et al.*, 2009). As is the case for all living organisms, survival rates generally depend on the efficiency of all physiological processes (Findlay *et al.*, 2009a). So, in the case of marine invertebrates, increases in [CO_2] can cause the animal's body fluids to acidify and deviate from the pH optima for many enzymes, thereby reducing metabolic efficiency (Reipschläger *et al.*, 1997; Pörtner *et al.*, 1998; Portner *et al.*, 2000; Langenbuch *et al.*, 2006). However, the ability to mitigate such acidification in the ocean environment differs among various species of marine organisms (Pörtner *et al.*, 2004; Wood *et al.*, 2008).

Feely *et al.* (2004), Harley *et al.* (2006), and Wood *et al.* (2008) have pointed out that the organisms most affected by acidification are those species with exoskeletons composed of calcium carbonate ($CaCO_3$), because the pH-dependent rate of metal reduction as it affects calcium saturation in these organisms affects rates of calcification (Fabry, 1990; Gattuso *et al.*, 1998; Orr *et al.*, 2005; Findlay *et al.*, 2008). Among calcareous organisms, calcification is an important process for organising the body's internal pH, growth, and calcium homeostasis (Pörtner, 2008; Findlay *et al.*, 2009a). Decreases in pH typically result in decreases in activity levels and metabolic rates of calcified marine invertebrates (Gutowska *et al.*, 2008).

1.6 CO_2 and Ocean Chemistry:

Oceans are often thought of as large reservoirs that take up CO_2 gas from the Earth's atmosphere (Feely *et al.*, 2004; Sabine *et al.*, 2004; Morse *et al.*, 2006; Arnold *et al.*, 2009) by absorbing approximately 30% of the total CO_2 emitted into the atmosphere (Sabine *et al.*, 2004; Fabry *et al.*, 2008). This represents 50% of the CO_2

output resulting from human activities during the past 100 years (Sabine *et al.*, 2004; Harley *et al.*, 2006; Fabry *et al.*, 2008). Therefore, the oceans have a role in mitigating the effects of CO_2 on the Earth's atmosphere (Orr *et al.*, 2005). As the [CO_2] increases in the atmosphere, its absorbance by seawater increases. When carbon dioxide dissolves in ocean water, it reacts with seawater (H_2O) to form carbonic acid (H_2CO_3) (Doney, 2006). Most of the carbonic acid (H_2CO_3) that is formed then decomposes rapidly to bicarbonate ions (HCO_3^{-1}) by losing hydrogen ions (H^+) (Findlay *et al.*, 2009a). This process may also produce carbonate ions (CO_3^{-2}) and more hydrogen ions (H^+) from the decomposition of bicarbonate ions (HCO_3^{-1}) (Fabry et al., 2008). Thus, abundant hydrogen ions (H^+) will be produced, which can react again with the carbonate ions (CO_3^{-2}) to form more bicarbonate ions (HCO_3^{-1}) (Fabry *et al.*, 2008). Carbonate ions (CO_3^{-2}) can interact with water (H_2O) and carbon dioxide (CO_2) dissolved in water to form still more bicarbonate ions (HCO_3^{-1}) (Orr *et al.*, 2005). This abundance of hydrogen ions (H^+) causes pH to decrease, leading seawater to become more acidic (Orr *et al.*, 2005). This change in seawater chemistry upon reaction with dissolved CO_2 (Wood *et al.*, 2008; Zeebe and Wolf-Gladrow, 2010) is known as ocean acidification (OA).

Hence, there will be an abundance of bicarbonate ions (HCO_3^{-1}) and hydrogen ions (H^+), and lower concentrations of carbonate ion (CO_3^{-2}), which are necessary for the formation of calcium carbonate ($CaCO_3$) that some marine organisms require to build structures and calcareous shells (Fabry *et al.*, 2008). The relevant chemical reactions are illustrated and summarised by the following equations:

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad (1.1)$$

$$H_2CO_3 \rightarrow HCO_3^{-1} + H^+ \quad (1.2)$$

$$HCO_3^{-1} \rightarrow CO_3^{-2} + H^+ \quad (1.3)$$

$$H^+ + CO_3^{-2} \rightarrow HCO_3^{-1} \quad (1.4)$$

$$CO_2 + CO_3^{-2} + H_2O \rightarrow 2HCO_3^{-1} \quad (1.5)$$

$$Ca^{+2} + CO_3^{-2} \rightarrow CaCO_3 \quad (1.6)$$

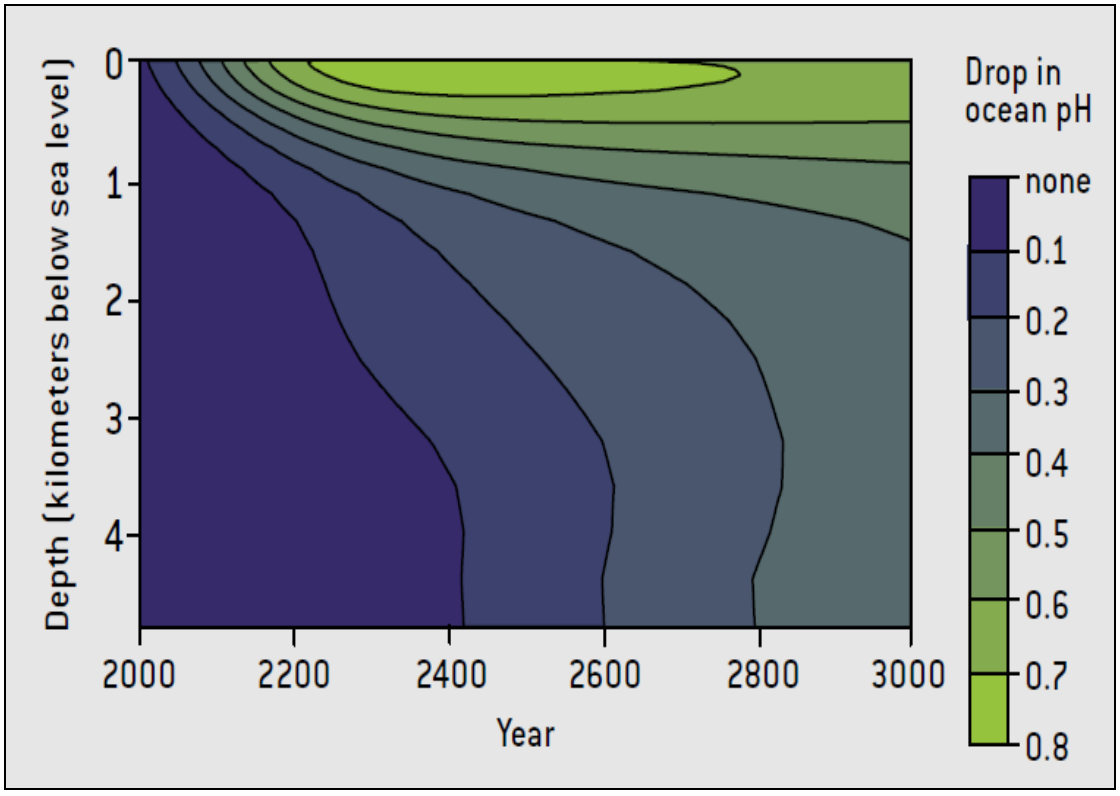

Figure 1.1 Decreases in the pH the oceans are expected to continue in the future (Caldeira and Wickett, 2003), with effects on surface water occurring faster than in deeper water. These projections indicate the possibility of decreases in pH of 0.1–0.2 units by 2050 and 0.3–0.4 units by 2100 compared to the pH values recorded in 2000 (Doney, 2006).

1.7 Methodological Comparisons:

In the 20th century, scientists became aware of the potential seriousness of OA on marine organisms, and research (e.g., Talmage and Gobler, 2009) was initiated in this area and has continued until the present. Some researchers (Byrne *et al.*, 2009; Havenhand and Schlegel, 2009; Parker *et al.*, 2009; Christensen *et al.*, 2011) recognised the need to study the combined effects of high temperature and high [CO_2] concentration, while other studies focused on the impact of only higher temperatures on marine organisms continue (e.g., Dong *et al.*, 2011). In recent years, researchers have started to take different approaches, with some studies conducted over only a few hours (Kurihara *et al.*, 2007; Comeau *et al.*, 2009), while others are conducted for several days (Kurihara *et al.*, 2004; Miles *et al.*, 2007; Dupont *et al.*, 2008; Kurihara

et al., 2008b). Other researchers have proposed that it is necessary to expose animals for several weeks to the pH and temperature conditions expected in the future, in order to give animals time to acclimatize to these conditions (Wood *et al.*, 2008; Gooding *et al.*, 2009). However, only a few studies have been conducted for extended periods (several months) (Jokiel *et al.*, 2008; Findlay *et al.*, 2009a; Findlay *et al.*, 2009b). Most research studies have been conducted in laboratories (Li and Gao, 2012), although field experiments have been conducted from time to time (Andersson *et al.*, 2009), and some researchers have performed both laboratory and field experiments (Green *et al.*, 2009). Studies have examined the effects of climate change variables on adult animals (Findlay *et al.*, 2009a; McDonald *et al.*, 2009) and effects on larval stages (Dupont *et al.*, 2008; Arnold *et al.*, 2009; Findlay *et al.*, 2010). While most of these experiments have been conducted on living marine organisms with calcified structures (Bibby *et al.*, 2008; Ellis *et al.*, 2009; Ries *et al.*, 2009), very few studies have been conducted on non-calcified marine organisms (Connell and Russell, 2010).

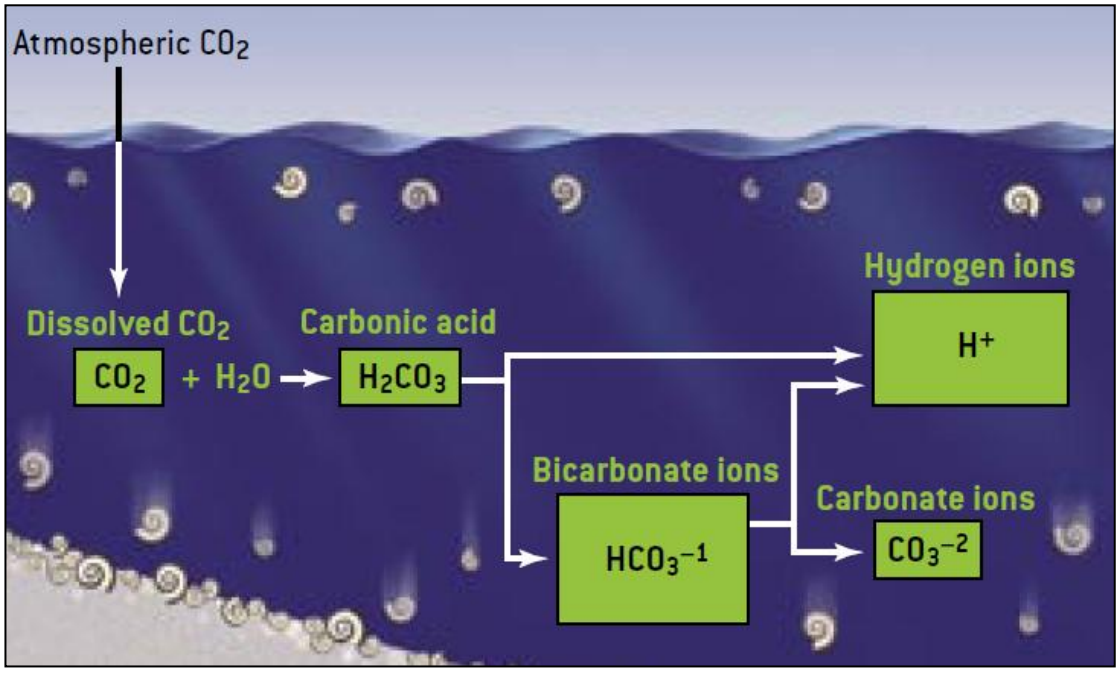

Figure 1.2 This schematic diagram depicts the process of absorption of atmospheric CO_2 by the ocean. Adapted from (Doney, 2006).

It is worth mentioning that most of these research projects used carbon dioxide gas (CO_2) to manipulate seawater pH conditions to the required acidity levels (Dashfield *et al.*, 2008; Gutowska *et al.*, 2008; Kurihara and Ishimatsu, 2008; Kurihara *et al.*, 2008b) to demonstrate the toxic effects of carbon dioxide (CO_2) in itself on marine organisms. However, some researchers have used strong acids such as HCl to control seawater pH levels (Andersson *et al.*, 2009; Kuroyanagi *et al.*, 2009).

Seasonality is a particularly important factor to account for during analysis of invertebrate marine animals because some of them breed in summer and others do so in winter (Kurihara *et al.*, 2008b). De Moel *et al.* (2009) found a difference in shell weight that depended upon the season. Also, growth rates tend to be higher in the summer than at other times of the year (Levinton, 2009). However, few researchers have investigated seasonal effects (Batten and Bamber, 1996) as they relate to OA.

Many studies have contributed to our understanding of OA, as the importance of this phenomenon to our environment becomes increasingly evident. However, more studies of the combined effects of decreased pH and increased temperature on the physiology of individual animals, as well as on broader ecosystem processes, should be undertaken (Findlay *et al.*, 2008). Byrne *et al.* (2009) and Gooding *et al.* (2009) have reported a few examples of such investigations. The former mentioned that temperature, not acidification, was the factor responsible for the negative effects observed on the animals under investigation. The latter, in contrast, found that the negative effect of decreased growth was due to reduction of pH, as was found by Comeau *et al.* (2009). Therefore, many more studies will be required to determine the future implications of combined acidification and temperature increases, and to distinguish whether only one of these factors might be responsible for any adverse effect on the physiology of marine invertebrates. While many results indicate that acidification has adverse effects on marine organisms, other experiments have shown otherwise. Widdicombe and Needham (2007), for example, did not report any significant differences in mortality or metabolic rates of *Nereis virens* at elevated temperatures and lowered pH. This difference appears more clearly in the findings of Wood *et al.*, (2008), whose results showed a greater increase in the growth and metabolism than had been expected in the organisms they studied. Havenhand and Schlegel (2009), Hauton *et al.* (2009) and Kurihara and Ishimatsu (2008) showed

clear examples of the potential negative impacts of ocean acidification, and emphasised the need to continue in this type of research, although their results did not conclusively demonstrate substantial damage to species exposed to projected acidic pH conditions. A study of sperm motility and fertilization kinetics (Havenhand and Schlegel, 2009) found no significant effects of seawater acidification on the oyster *Crassostrea gigas*. There were no significant effects on the growth and survival of the amphipod *Gammarus locusta* under high [CO_2] seawater and pH reduction of up to 0.5 units (Hauton *et al.*, 2009). Similarly, Kurihara and Ishinatsu (2008) also showed no significant effects of exposure to OA on survival, body size, or egg production in *Acartia tsuensis*. It is important to realize that some previous experiments were carried out at very high, unrealistic [CO_2] that were also not close to the expected concentrations (Findlay *et al.*, 2008). As the worst estimates predicted by IPCC (2007) indicate a future pH decrease of 0.4 units by 2100, it is not strictly relevant to conduct experiments under a pH lower than 7.5, as done by Bibby *et al.*, (2007) and Widdicombe and Needham (2007). Similarly, the exposure of animals to concentrations of pCO_2 exceeding 1500 ppm (Kurihara and Ishimatsu, 2008), and Gutowska *et al.* (2008) was also not realistic. Neither of the experiments immediately above were conducted under conditions closely approximating conditions anticipated for the middle to end of this century. Studies that exposed animals for short periods (Kurihara *et al.*, 2008a) are less relevant, as the animals were not gradually exposed to the final temperature and pH levels required. These approached would not permit the marine invertebrates to adjust to new temperatures, pH, or [CO_2] in the seawater. Therefore, experiments with medium-term exposure, ranging from five to eight weeks, with one to two weeks to allow the animals to adjust gradually to the new variables, may be informative. Not many previous experiments compared conditions and parameters in summer and winter, despite the importance of seasonal pH changes (Findlay *et al.*, 2009a).

Higher temperatures, up to the maximum levels recorded in summer, may lead to increased rates of calcification (Marshall and Clode, 2004; Rodrigues and Grottoli, 2006). However, low temperatures increase the likelihood of decomposition of calcium carbonate ($CaCO_3$) (Findlay *et al.*, 2008). It may therefore be important to include future experiments that compare the effects of acidification and rising temperatures on marine invertebrate organisms in summer and winter. Despite the

evidence that high [CO_2] has a direct impact on marine life (Kurihara *et al.,* 2004), some experiments still use strong acids such as HCl to alter the pH of seawater (Kuroyanagi *et al.,* 2009). Because it is important to observe treatment effects under the most realistic conditions possible, and because seawater can be acidified using CO_2, the use of strong acids to change the acidity of seawater for laboratory testing can no longer be justified. Thus, under the realistic conditions applied in this study I hope to provide a more realistic estimate of the physiological effects that could result from increased temperatures and increased acidity due to high concentrations of CO_2.

1.8 Hypotheses and Aims:

This study investigates the effect of elevated temperatures and decreased pH (using CO_2 to adjust pH) on both calcifying and non-calcifying coastal marine invertebrates. Laboratory experiments were conducted in winter and summer by exposing the animals under study to temperatures and acidity levels near those expected to occur in the oceans in the years 2050 and 2100.

1.8.1 Aims:

The aims of this research are:

1. To determine the combined effects of high temperature and high carbon dioxide concentrations, and consequent reduced pH, on marine invertebrates;

2. To estimate the potential of these species of marine organisms to adapt and survive despite higher temperatures and higher carbon dioxide concentrations in the future, according to parameters measured under experimental pH and temperature conditions expected in 2050 and 2100; and

3. To clarify some of the risks that may arise for marine biodiversity due to combinations of high temperature and high carbon dioxide concentrations during winter and summer seasons.

1.8.2 Hypotheses:

1. Calcified marine invertebrates are expected to be more susceptible to the adverse effects of elevated temperature and lower pH than non-calcified marine invertebrates, due to their requirement for carbonate ions during the calcification of their calcium carbonate exoskeletons.

2. Coastal marine invertebrates are expected to be more susceptible to the adverse effects of elevated temperature and lower pH during the summer than during the winter, due to the maximal temperatures reached in seawater during the summer.

Strong responses to increased temperature and decreased pH may lead to sharp declines in physiological processes and levels of calcification in most of the organisms studied here. Some organisms may respond by increasing their formation of lime, or other metabolic processes that could protect the organisms from the changes in their surrounding environment. Thus, non-calcified marine invertebrates may have a greater capacity to adapt and resist the impact of the acidification than do the calcified marine invertebrates, because the non-calcified species do not need to expend energy for the growth and maintenance of shells or other structures. Seasonal conditions might also influence physiological processes and calcification under conditions of acidification. Finally, do the seasons affect the calcification rates and metabolism of the organisms under study?

1.9 About this thesis:

This thesis has been divided into eight chapters. Chapter 1 comprises the Introduction, and presents the research background and the literature review. Chapter 2 describes the methods employed in these studies, the study sites, the species of animals studied, the parameters measured, and the devices used in this study. Chapters 3 and 4 describe results of the study of potential effects of climate conditions projected for the next century on animals under study in winter. Chapters 5 and 6 discuss results of the same kinds of studies as performed in the two previous chapters, but during the summer. Chapter 7 presents comparisons between the results from the

studies conducted during winter and summer for most of measurements obtained in the previous chapters.

Chapter 2. Methods

2.1 Choices and Collection of Animals:

2.1.1 Choices of Animals:

Four species of coastal marine invertebrates were chosen (Fig. 2.1) including two species that are calcified species (*see* 1 & 2 below), and two that are non-calcified (*see* 3 & 4 below):

1. Common Blue Mussel (*Mytilus edulis*):

M. edulis is a calcified intertidal zone invertebrate and is an important food source for other marine organisms, as well as birds and humans (Beesley *et al.*, 2008; Bibby *et al.*, 2008). These mussels are also used widely as an indicator species in toxicology studies, and they play an important role in many marine ecosystems (Beesley *et al.*, 2008). They occur in temperate coastal regions of the Atlantic Ocean and the Mediterranean Sea, along the coasts of France and Canada to the coasts of Chile and Argentina, and the west coast of the North American continent (Berge *et al.*, 2006). This species is also found around the North Sea, the Baltic Sea, and the English Channel. These mussels feed by filtering plankton from sea water, and grow to sizes of up to 100 mm (Hook, 2008). Blue mussels from the North Sea have quite a wide range of thermal tolerance from sub-zero temperatures in the winter to 35˚C in summer (Brenner and Buck, 2011). They can have extended life spans of 18 to 24 years old (Zagata *et al*., 2008) (see Fig. 2.1A).

2. Common Periwinkle (*Littorina littorea*):

L. littorea, are calcified invertebrates that are food for various seabirds that are common to the North Atlantic (Hook, 2008). They are often highly abundant in intertidal zones, where they play an important role as grazers (Bibby *et al.*, 2007). Common Periwinkles are also found in the western and eastern Atlantic and from southern Portugal to the White Sea (Cummins *et al.*, 2002). This species has a wide range of thermal tolerance from sub-zero to 30˚C (Jackson, 2008). Individuals usually grow to sizes between 15 and 40 mm. In the field, they may live for at least five years,

while in captivity their life span may extend up to nine years (Oehlmann, 2004) (see Fig. 2.1A).

3. Beadlet Anemone (*Actinia equina*):

The Cnidarian species *A. equina*, is an abundant non-calcified invertebrates that inhabit many ecologically diverse marine environments (Gadelha *et al.*, 2010). This species plays an environmentally significant role, as it is widespread in the marine environments of the North Sea (Scandinavia) and the English Channel. Its range extends to the Atlantic northwestern side of the Iberian Peninsula, and is abundant along the Portuguese coast, and also in the Mediterranean Sea and along the coast of Morocco. These sea anemones are highly adapted to the intertidal zone, where they tolerate extremes of temperature and drought. They can reach body sizes of up to 50 mm (Hook, 2008). They are able to move very slowly, and feed on whatever arrives in the currents and falls onto their tentacles and oral disc (Davenport *et al.*, 2011) (see Fig. 2.1A).

4. European Sea Squirt (*Ascidiella aspersa*):

A. aspersa are roughly block-shaped invertebrates, up to 10 cm in length (Naylor, 2005) that exist in the shallow subtidal zone at depths between 5 meters and 90 meters, and are commonly attached by their bases to marina piers (Morton and Dinesen, 2011). *A. aspersa* can also be found in the intertidal (Inglis *et al.*, 2005) and lower intertidal zone (Curtis, 2005; Mackenzie, 2011). They are widely distributed in bodies of water such as the English Channel, the North Sea, the Baltic Sea, and the Mediterranean Sea. *A. aspersa* gametes are released in spring, and it has a relatively short 18-month life cycle that lasts from mid-summer to the winter of the following year (Morton and Dinesen, 2011) (see Fig. 2.1B).

The criteria for choosing the animal species for this study included species availability and abundance on British coastal beaches, and that these species are not threatened by extinction. Another relevant criterion was the diversity of the biological characteristics of the selected species. We selected two main groups: the first group includes two species of calcified marine invertebrates and the second group includes two species of non-calcified marine invertebrates. Some of the study species are semi-mobile (*A. equina and L. littorea*) and others are sessile (*A. aspersa*) and sedentary

(*M. edulis*). There is also diversity in the manner in which each organism feeds: some are carnivorous (*A. equina and M. edulis*), and some feed on algae and plankton (*L. littorea, A. aspersa, and M. edulis*). Some of these animals actively seek and collect their food (*A. equina, L. littorea and M. edulis*), while others feed by filtration (*A. aspersa and M. edulis*).

A B

Figure 2.1 A: Photograph above show collected marine invertebrates; two of the species, *M. edulis* and *L. littorea*, are calcified, and the other one, *A. equina*, is non-calcified (photo by researcher). B: Photograph of non-calcified species: *A. aspersa* (photo by researcher).

2.1.2 Collection Methodology:

All of these species of coastal marine invertebrates were collected during low tide between the high and low water marks. Animals were collected during the winter 2011/2012 and the summer 2012, near Newbiggin-by-the-Sea, Northumberland County (55°10’38”N, 001°31’59”W) and Hartlepool Marina, Hartlepool, North England, United Kingdom (54°41’10”N, 001°12’45”W) (Fig. 2.2 and Fig 2.3). Animals were collected on a total of six occasions (Table 2.1).

Table 2.1 Dates of collection of animal species under study and when animals from each collection date were used. The first collection was performed to ensure that animals could survive in aquaria under average summer temperature conditions. Results from the second collection were not used because the experiment missed samples of *A. aspersa.*

Dates collected	**Species**	**Experimental group**
01 February 2012	All species	Winter conditions
24 May 2012	All species	Summer conditions
19 July 2012	All species	Summer conditions
24 September 2012	All species	Winter conditions

Animals were collected by hand and placed in plastic containers containing local seawater. They were then transported to the laboratory at Newcastle University and held in seawater at temperatures near those of the environment from which they were collected. Debris such as small bits of rock and shell (such as shells of barnacles) were cleaned from the study animals, parasites such as worms and *Gammarus spp.* that were visible on *A. aspersa* were also removed. Table 2.2 Shows the number of animals that were used in each experiment in summer and winter, and in each microcosm.

Table 2.2 Number of animals surviving after acclimatization period. Mortality percentage over a two-week period of acclimatization was not calculated.

Seasons	Treatments											
	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
Winter1	41	28	36	30	43	32	32	22	25	24	34	23
Winter2	43	38	17	72	38	37	14	75	33	39	15	53
Summer1	49	55	25	18	45	53	25	18	54	52	25	19
Summer2	22	47	28	15	26	55	25	19	24	54	21	21

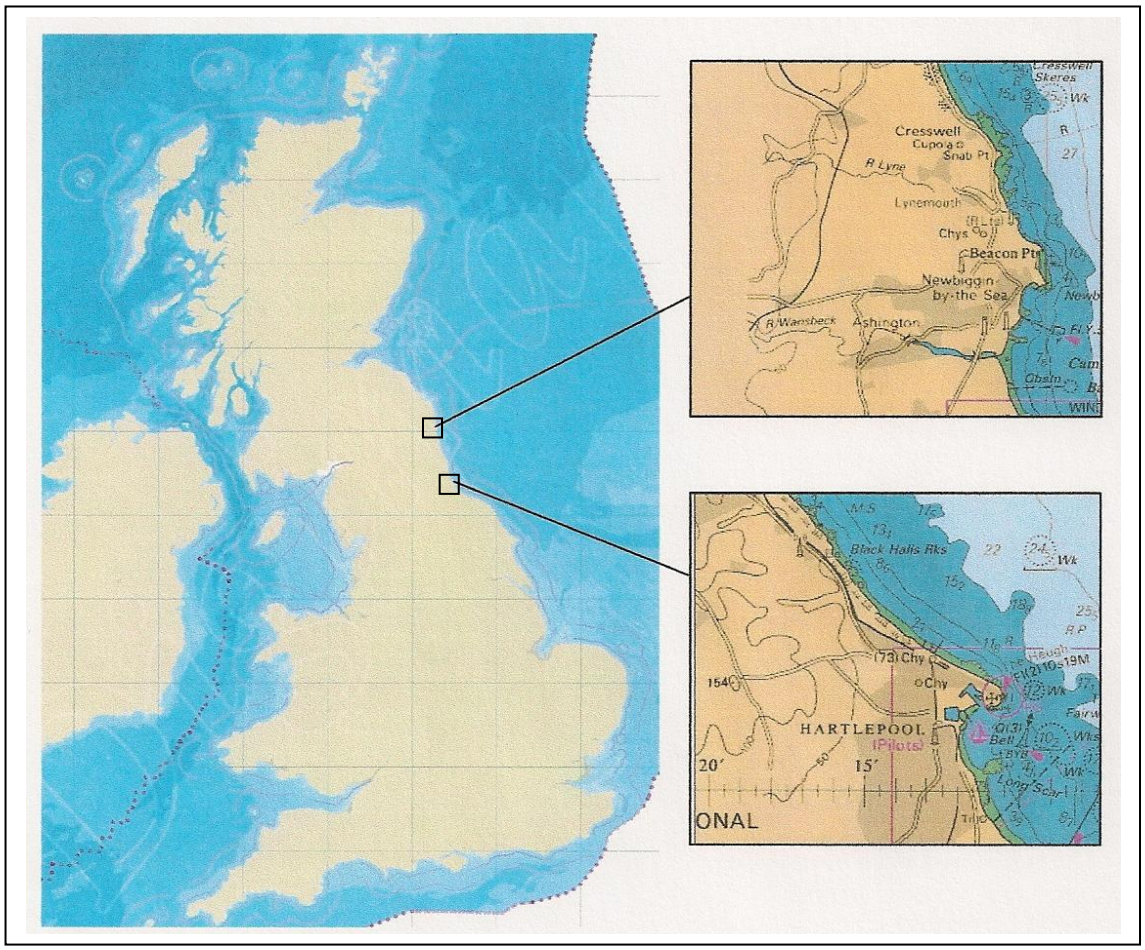

Figure 2.2 Maps showing the locations of sample collection. (Box top right): Newbiggin-by-the-Sea; species collected included *Mytilus edulis*, *Littorina littorea*, and *Actinia equina*. (Box bottom right): Hartlepool Marina; species collected included *Mytilus edulis* and *Ascidiella aspersa*. The maps from: (Marine Digimap Service).

2.2 Experimental Design:

The experimental designs used here followed that reported by Widdicombe and Needham (2007), Gazeau *et al.* (2007), Findlay *et al.* (2008), Dashfield *et al.* (2008), Munday *et al.* (2009) and Suwa *et al.* (2010) with some modifications. Experiments took place in a constant temperature room (C.T.R.) on the 5th floor, School of Biology, Newcastle University. Sterile filtered (5 µm filter) seawater was used throughout. Seawater was placed in three header tanks (mixer tanks) representing the conditions of pH and temperature predicted for three current and future time periods: current 2011, 2050, and 2100. Each header tank (mixer tank)

consisted of a plastic container (depth = 40 cm; width = 60 cm; height = 42 cm; volume = 80 l) filled with ~40 litres of seawater. These tanks were placed beneath the microcosm tanks (see Fig. 2.4) that were provided with ceramic heaters (Aquael Plastic Neo Heater), one or more as needed, to raise the microcosm temperature to the required levels. The water was aerated using an air pump (Clear Seal LP-60) connected to an airstone diffuser (from Aqua Medic) placed at the bottom of the tank.

Figure 2.3 Photographs of the sites of study animal collections. A: Hartlepool Marina; species collected from the marine pier included *Mytilus edulis* and *Ascidiella aspersa*. B: Newbiggin-by-the-sea; species collected from the rocky shore included *Mytilus edulis*, *Littorina littorea*, and *Actinia equina*. (photo A and B taken by researcher).

A pH computer (Aqua Medic, resolution pH 0.01) was used to maintain and modify the pH of the seawater in the header tank (mixer tank). The system consisted of a pH electrode (Aqua Medic) that constantly measured and reported the pH of the seawater to the connected pH computer that was also connected to a carbon dioxide CO_2 cylinder (Aqua Medic) via a solenoid valve (Aqua Medic) to control the passage of carbon dioxide (CO_2) that would control the seawater pH. The solenoid valve was connected via a fine 4 mm clear plastic tube (Reefphyto Company, UK) to an airstone (Aqua Medic) that was placed at the bottom of the header tank and adjusted to provide small bubbles that allowed the gas to dissolve easily into the seawater. When the pH electrode senses that the pH in the header tank has increased, the electrode signals the pH computer to open the solenoid and allow the CO_2 to be released. The gas flow rate was controlled using a gas bubble counter (Aqua Medic). When the required pH is attained, the pH computer closes the solenoid valve and stops the flow of CO_2. The pH computer was calibrated weekly using buffers of pH 7.00 and pH 4.00 (Aqua Medic). An air pump (Clear Seal LP-60) was used to pump atmospheric air into the header tank using an airstone (Aqua Medic) placed at the bottom of the tank.

Approximately 20 l of seawater was pumped from each header tank to a microcosm (D: 40 cm; W: 80 cm; H: 25 cm; Vol: 62 l) that was placed above the header tanks. The water pump (Rio 3100, Powerhead) constantly pumped the seawater from the header tanks to the microcosms at a rate of approximately 60 $l.h^{-1}$. Seawater was recycled back to the header tank via several overflows (see Fig. 2.4).

Acidity and temperature levels in the microcosms were monitored constantly using a pH electrode (Pico Technology; resolution pH 0.02) and a thermistor (Pico Technology; 0.01 °C at 25 °C) connected to a laptop computer via an analogue–to-digital converter (Pico Technology; DrDAQ Data Logger) (see Fig. 2.5). The laptop ran proprietary software (PicoLog; Pico Technology) and was used for data collection.

Five litres of seawater of the same composition and pH as the seawater in each header tank was added to each header tank daily (equivalent to 25% of the total seawater in the header tank) up to five days a week. As the extra seawater was mixed with the existing seawater, a further overflow siphoned the extra seawater into a waste container. The new seawater was added slowly to the header tank at a speed of

approximately (2 $l.h^{-1}$) to avoid any sudden changes in the acidity or temperature of the seawater in the system (see Fig. 2.6).

The first system (S1) represents current levels of pH and temperature replicating the 2011 conditions and is designated treatment 2011 (TR 2011). The second and third systems (S2 & S3) represent the levels of pH and temperature expected to occur at the middle and end of this century (2050 & 2100), and are designated treatment 2050 and treatment 2100 (TR 2050 & TR 2100), respectively. Animals were divided randomly into three groups and initially placed in conditions similar to the temperature and pH conditions from which they were collected. Each treatment group was then acclimatised gradually over a period of of 10 to 15 days (Anestis *et al.,* 2008; Anthony *et al.,* 2008) to the temperature and pH levels required for each treatment (TR 2011, TR 2050 and TR 2100) (see Fig. 2.6).

In order to be able to identify individual animals and because some of the animals are able to climb out of the water, animals were studied individually. Cages were made to separate the experimental animals that can move, such as *Littorina littorea* and *Actinia equina*, and to prevent them from climbing above the level of the seawater (see Fig. 2.7). Cages were made of non-stick mesh 33 × 40 cm (Planit Products Ltd., UK). These cages isolated the animals but the mesh ensured that the animals were still exposed to the same seawater conditions as all the other animals in the same treatment. Furthermore, by using mesh cages, it was possible to visually monitor the animals.

Light was provided by two daylight fluorescent tubes and animals were exposed to 8:16 h (L:D) to simulate the winter period and 16:8 h (L:D) to simulate the summer period, where; L = light, D = dark, and h = hours.

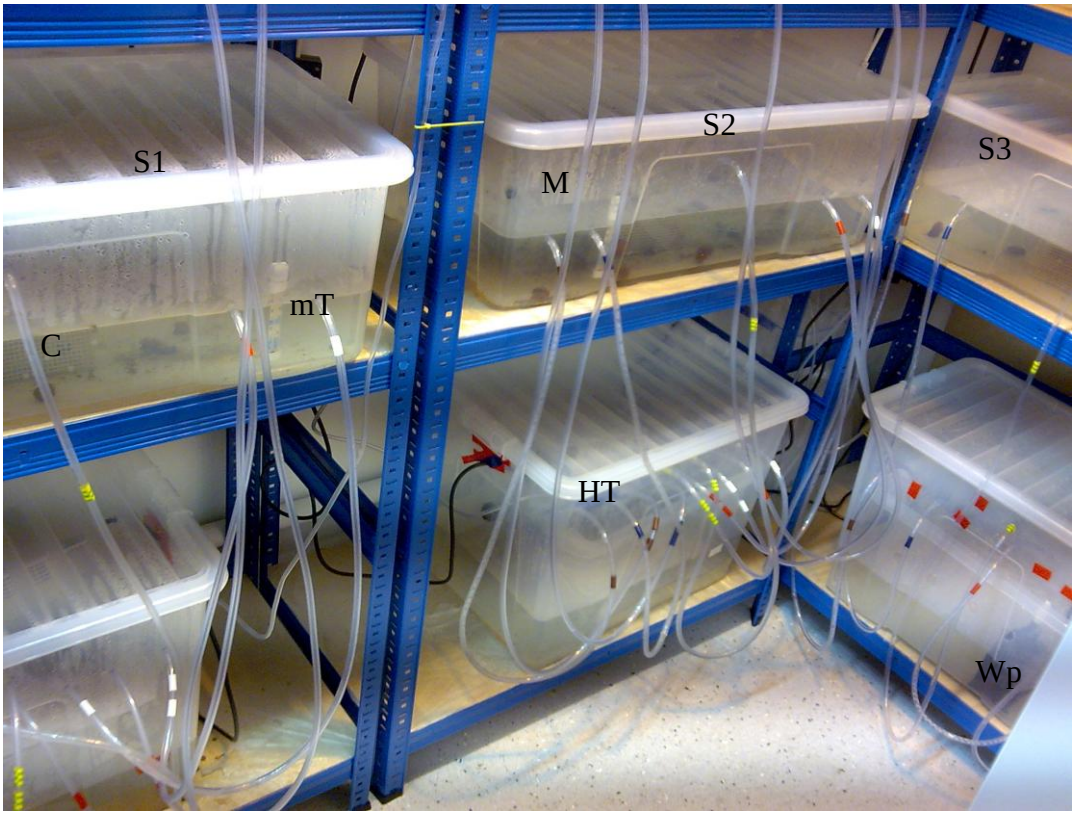

Figure 2.4 The photograph above, which was taken in the constant temperature room (CTR), shows each of the three systems (S1, S2, and S3). Each system includes a microcosm (M) to hold the animals and is linked by tubing to the header tank (mixer tank) (HT). As can be seen, condensation inside the microcosms indicates that the tanks are completely closed. Inside each enclosure, there are cages (C) to keep the animals underwater, a water pump (Wp) and a manual thermometer (mT) to monitor the temperature of the seawater even in the event that the automated monitor system were disrupted (photograph by researcher).

2.3 Feeding:

The animals under study were fed five times a week on a standard marine aquarium diet of phytoplankton (*Nannochloropsis* spp.) (purchased from Reefphyto Company). The phytoplankton were cultured in the laboratory in large conical flasks (2 and 3 l) using the same seawater that was used to fill the aquaria. At the start of phytoplankton culture, Liquid Starter Culture *Nannochloropsis* (Reefphyto Company)

was used to stimulate growth, and Phyto Nutrient – Modified F/2 Medium (Reefphyto Company) was used to provide nutrients to the algae culture during the experiments (see Fig. 2.8). Study animals were also fed brine shrimp (*Artemia* spp.). Brine shrimp eggs (Reefphyto Company) were hatched and grown in the laboratory using the same seawater source that was used to fill the aquaria. Eggs were added to the seawater in the ratio of 2.5 $ml.l^{-1}$ of seawater (see Fig. 2.7). No special diet was provided to *L. littorea*, but it fed on the algae that grew in the microcosms.

Figure 2.5 Photograph shows three DrDAQ Data Loggers (Pico Technology) with a resolution of pH 0.02 and temperature of 0.01 °C at 25 °C (enclosed in three plastic containers to maintain humidity levels around the devices), connected to a Toshiba laptop (photograph by researcher).

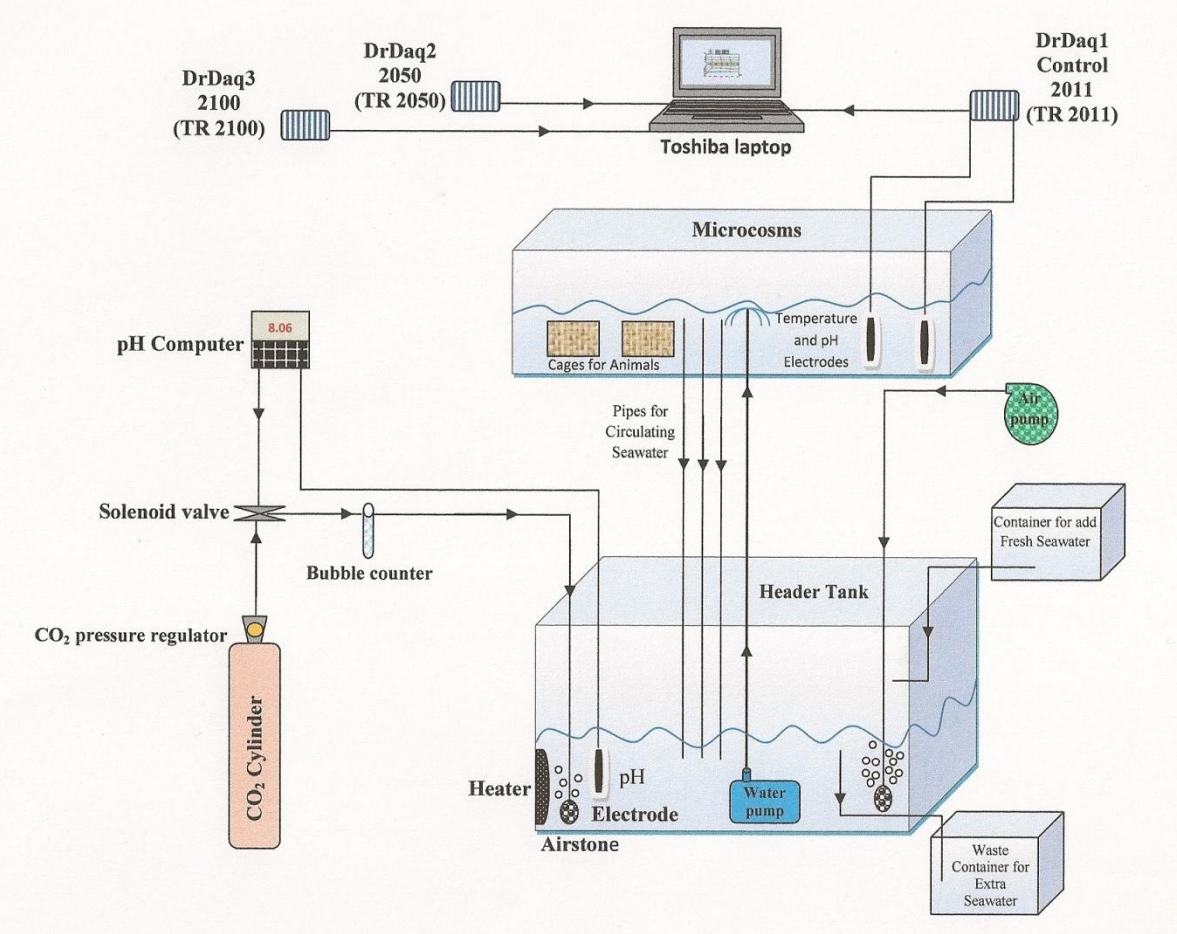

Figure 2.6 The diagram above shows the various parts of the experimental design for the control treatment (TR 2011). TR 2011 represents the first system, S1, which includes a header tank (mixer tank) linked to microcosms by tubes. Header tanks include a heater, pH electrode, a water pump, an airstone for O_2 bubbles, and an airstone for CO_2 bubbles. Microcosms include cages for animals, a pH electrode, and a temperature thermistor. Also shown is the CO_2 system, which includes a CO_2 cylinder linked to a pH computer via a solenoid valve (drawing by researcher).

2.4 Care of Aquaria:

The microcosms were cleaned weekly to remove waste and excess food by using a water siphon or vacuum made from 0.5 m tube (6 mm). Any dead animals were removed from the microcosms as soon as they were detected. All header tanks and microcosms were cleaned with 70% alcohol swabs and rinsed with water thoroughly before and after each experiment.

2.5 Measurement of Seawater Parameters:

2.5.1 Acidity (pH) and Temperature:

The pH in each header tank was measured by taking a reading every 10 minutes using a DrDAQ Data Logger and PicoLog software (Pico Technology), calibrated weekly using buffers of 4.00, 7.00 and 9.21 (Aqua Medic). Temperature was measured every 10 minutes using a thermistor connected to the computer via a converter (DrDAQ Data Logger; Pico Technology). The computer ran proprietary software (PicoLog; Pico Technology), and was calibrated weekly using a digital thermometer (Aqua Medic) with an accuracy of ± 0.5 °C. See Table 2.3.

2.5.2 Salinity:

Salinity was measured daily in the morning and before and after the addition of any fresh seawater by directly sampling from each microcosm using a handheld salinity refractometer on a scale of 0-100% (DIGI T – 100 ATC). When necessary, the salinity of the seawater was adjusted to (34-35 ppt) by adding some distilled water (Sokolova and Pörtner, 2003).

2.5.3 Dissolved Oxygen:

The amount of dissolved oxygen in the microcosm seawater was measured daily by dipping an Oakton DO 6 Dissolved Oxygen Meter (Cole-Parmer, accuracy of ± 0.3) electrode into the seawater for five minutes, then taking a reading.

2.6 Survival:

Survival was calculated after the end of a period of acclimatization, as animals lost per day; results were calculated weekly. Mortality was determined by visual observation; all dead animals were removed as soon as they were identified to avoid any contamination of the microcosms (LeBlanc *et al.*, 2010). Deaths of *M. edulis* were determined by noting any individuals with open shells in the absence of any tactile stimulation. Deaths of *L. littorea* were very difficult to determine by visual observation, so the animals checked weekly by direct examination of the aperture and

operculum. In *A. equina*, death was apparent upon the emergence of a large gap in the middle of the animal together with atrophy and early tissue decay (Fig 2.9).

Animals found in this condition were immediately removed from the microcosm to avoid spread of detrimental microbes as the process of death can take several days. Deaths of *A. aspersa* were identified upon discovery of the animals' internal organs scattered about the microcosm after an animal burst.

The following equation was used to calculate the percentage survival over the experimental period of six weeks:

$$SRV\ (\%) = (NS - NE) / NS \times 100; \qquad (2.1)$$

where SRV (%) is percentage of survival, NS is initial number of animals after the acclimatization period at the start of week 0, and NE is the final number of animals at the end of week 6.

Figure 2.7 The photograph shows sea anemones, *Actina equina*, in their separate cage, and shows a sea anemone trying to escape from the cage (photograph by researcher).

Figure 2.8 Phytoplankton and brine shrimp cultures grown in flasks (2 and 3 L) and using a deck lamp for light and heat (temperature range was 27 ºC – 30 ºC) (photography by researcher).

2.7 Growth Changes:

Individual animals were selected randomly from each species and measurements were carried out in the first week and then measured again in the sixth week (over a six-week period).

2.7.1 Body Weight:

Animals were removed from aquaria and placed on filter paper for three to five minutes to get rid of as much excess water as possible, then were weighed in ambient air using an open-top balance (Precisa 310M). The following equation was used to calculate the percentage weight change over the experimental period of six weeks:

$$PWG\ (\%) = (Wf - Wi) / Wi \times 100; \quad (2.2)$$

where Wi is the initial body weight (g) after the acclimatization period at the start of week 0, and Wf is the final body weight (g) at the end of week 6.

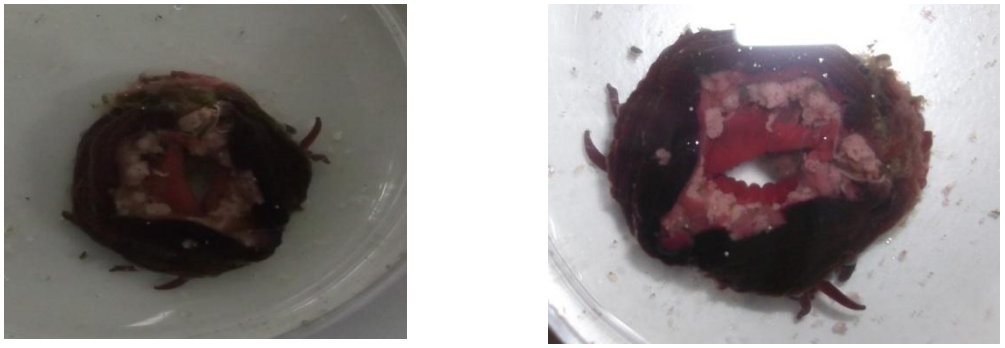

Figure 2.9 The two photos above illustrate the gap that appears in the centre of animal when individuals of *A. equina* are beginning to die.

2.7.2 Buoyant Weight:

We determined buoyant weight using the method described by Davies (1989) with some modifications. Briefly, a small cage was hung from the bottom of a balance (Precisa 310M) by a small length of string. The balance was placed on a table in which a hole had been cut to allow the string to reach a 5 l container filled with seawater (see 2.10) placed below the table, and the cage was submersed in the seawater container without touching its bottom surface. Each animal was then taken out of the microcosm, placed in the cage, and left for a period not less than four minutes to allow the balance reading to stabilise. Data were then recorded. Buoyant weight (BW) was calculated using the following equation (Jokiel *et al*., 1978; Ferrier-Pages *et al*., 2000):

$$BW\ (g) = W_{water} / [1 - (D_{water} / D_{object})] \quad (2.3)$$

where BW is buoyant weight in g, W_{water} is the weight of the sample under seawater (g), D_{water} is the seawater density in $g.ml^{-1}$, and D_{object} is the mean sample density in $g.ml^{-1}$.

The percentage of buoyant weight change (PBWR) is ultimately calculated using the following equation:

$$PBWR\ \% = [(W_f - W_i)/ W_i] \times 100 \qquad (2.4)$$

where PBWR is percentage buoyant weight %, W_f is final buoyant weight, and W_i is initial buoyant weight.

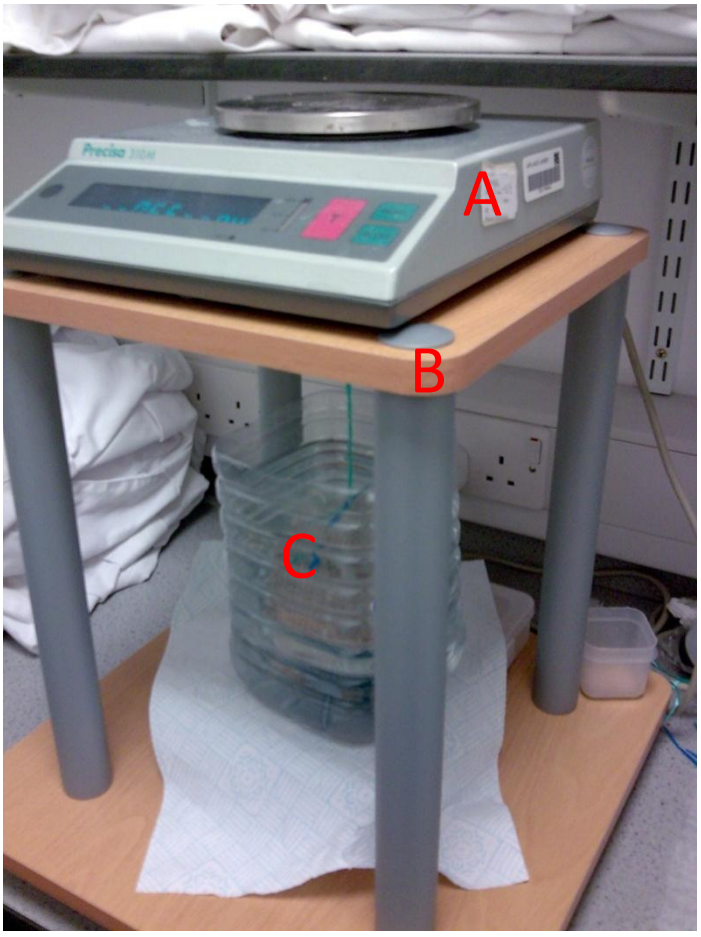

Figure 2.10 Apparatus used to measure buoyant weight. A: sensitive balance (Precisa 310M); B: table with a hole (not visible) for the string to pass through; C: a 5 l container filled with seawater. Inside the container is a small cage to put the animals in for weighing. The cage is attached to the balance by a string and the cage does not rest on the bottom of the seawater container (personal photograph).

Measurement of weight underwater was performed because buoyant weight is neutralized by the weight of water and mucus in the body (Jokiel *et al*., 1978; Rodrigues and Grottoli, 2006) and thus gives more accurate measurements of growth. This method was also used to determine calcification in calcified marine invertebrates, because most of the weight changes in calcified marine invertebrates are due to increased exoskeletal mass and are thus effective measures of calcification (Bucher and Harrison, 2000). Also, in calcified marine invertebrate changes measurement of morphological parameters (e.g. shell length and mass) one of methods to calculate the calcification (Findlay *et al*., 2009b).

2.7.3 Morphometric analyses:

Morphometric analyses of the animals were performed over a six-week period using a digital calliper. For *M. edulis*, shell lengths and widths were measured, while for *L. littorea* and *A. aspersa*, only the length measurements were taken (see Fig. 2.11). Body lengths in *A. equina* were not measured because the structure and size of these animals depends on the amount of water flowing through their bodies, which is constantly changing.

The following equation was used to calculate the percentage body length change over the experimental period of six weeks:

$$PL\ (\%) = (Lf - Li) / Li \times 100 \qquad (2.5)$$

where PL (%) is percentage body length change, Li is the initial body length (mm) after the acclimatization period at the start of week 0, and Lf is the final body length (mm) at the end of week 6. Further, the following equation was used to calculate the percentage body width change over the experimental period of six weeks:

$$PWd\ (\%) = (Wdf - Wdi) / Wdi \times 100 \qquad (2.6)$$

where PWd (%) is percentage body width change, Wdi is the initial body width (mm) after the acclimatization period at the start of week 0, and Wdf is the final body length (mm) at the end of week 6.

2.8 Metabolic Rates:

Metabolism rates were measured after the six-week experimental period using closed-system respirometry (Edmunds *et al.*, 2011). Briefly, each animal was placed in a 50 ml container with a lid and left for around 15 minutes to acclimate (Christensen *et al.*, 2011). The container was filled with seawater and left under the water to make sure that there were no air bubbles. The container was then slowly and carefully closed with Parafilm^{TM} (Edmunds *et al*., 2011) and replaced in the original microcosm to maintain the temperature. After around 2 hours (the actual duration of each experiment having been recorded), 1.5 ml of seawater was removed using a 2.5 ml syringe fitted with a needle. The needle was then removed (to minimise air bubbles) and water was injected gently into the measuring chamber and allowed to settle, then the reading was recorded. One of the sample containers contained only seawater, for which the rate of oxygen consumption was recorded after the same time period as for the experiment. Then the oxygen consumption rate (VO_2) was calculated as suggested (Zhang *et al.*, 2012) using the following equation:

$$VO_2\ (mgO_2.g^{-1}.h^{-1}) = (C_o - C_t)\ V / (WW.T) \qquad (2.6)$$

where OCR is the oxygen consumption rate, C_o is the oxygen content in the blank bottles, C_t is the oxygen content in the animal bottles ($mgO_2.L^{-1}$), WW is the wet weight of the animal in (g), T is the duration of time the animal remained in the container in (h), and V is the volume of water in the bottle (l). The oxygen meter was calibrated before and after each series of measurements. A volume of 1–1.5 ml of aerated water was placed in the measuring chamber for calibration, then 1-1.5 ml of a solution of sodium sulphite was placed in the measuring chamber until the oxygen meter reading reached zero, and thus calibration was complete. The oxygen content of the distilled aerated water and the oxygen content of the seawater in the microcosm at the start of the experiment were recorded.

2.9 Storage of Samples:

At the end of the experimentation period, the animals were weighed and then placed in 60 ml plastic containers with a screw cap (VWR, UK) and were placed in the freezer at -80 ºC for subsequent analysis.

2.10 Freeze-drying:

All samples for analysis of body composition, were freeze-dried (Kerr *et al.*, 1982; Pierson and Stack, 1988; Speakman, 2001). Animals were placed in open plastic containers that were placed in the freeze dryer (MODUL YOD Freeze Dryer, Thermo Electron Corporation). After confirming that no leakage was occurring, the samples were dried for 48 hours.

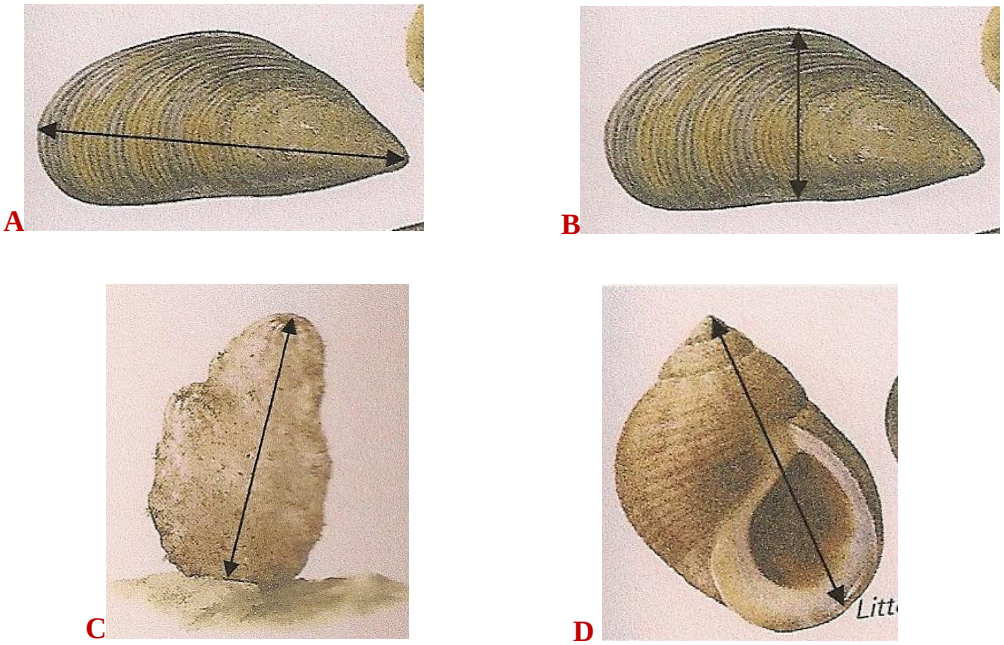

Figure 2.11 Diagrams of the morphometric parameters of the animals (A and B refer to length and width for *M. edulis*, and C and D refer to length for *A. aspersa* and *L. littorea*, respectively) that were recorded. The arrows indicate the locations of the measurements that were taken. Images were taken from (Campbell and Nicholls, 1977).

2.11 Body Composition Analysis:

2.11.1 Water Content:

The percentage total water content of the total body mass was calculated after the six-week experimental period by calculating the difference between the weight of

the sample before freeze-drying and the weight of the sample after freeze-drying, using the following equation:

$$PTW\ (\%) = (W_{air} - W_{dry}) / W_{air} \times 100 \quad (2.7)$$

where PTW is the percentage total water content (%), W_{air} is body weight before dehydration (g), and W_{dry} is body weight after dehydration (g).

2.11.2 Dry weight:

The whole body weights and total body masses after freeze-drying of animals were determined, including both soft tissues and shells of calcified organisms, or only soft tissue for non-calcified organisms. Percentage dry weight (DW) values at the end of the six-week period were calculated from the data using the following equation:

$$DW\ (\%) = W_{dry} / W_{air} \times 100 \quad (2.8)$$

where DW is percentage dry weight (%), W_{dry} is body weight after dehydration (g), and W_{air} is body weight before dehydration (g).

2.11.3 Shell weight:

This measurement was taken only for calcified animals (*M. edulis* and *L. littorea*). Shell weight was determined after the six-week experimental period by separating the soft tissue from the hard tissue (shell) then weighing hard tissue and soft tissue separately. Percentage shell weight of the total dry weight was calculated using the following equation:

$$SHW\ (\%) = DW / ShW \times 100 \quad (2.9)$$

where SHW is the percentage shell weight (%), ShW is shell weight (g), and DW is total dry weight (g).

2.11.4 Soft Tissue Weight:

All soft tissue weights including gonads, so dry weight is similar to soft tissue weight in non-calcified marine invertebrates. In calcified marine invertebrates the soft tissue weight of total body mass was determined after the six-week experimental period by separating the soft tissue from the hard tissue (shell) and then weight of

hard tissue and soft tissue separately. Then was calculated by using the following equation:

$$STW\ (\%) = STW(g) / DW(g) \times 100 \qquad (2.10)$$

where STW(%) is presented percentage soft tissue weight change; STW(g) is presented soft tissue weight (g) and DW is presented dry weight of total body mass (g).

2.11.5 Lipid Content:

The lipid content of only dried total (including gonads) soft tissue was measured after the six-week experimental period using a Soxhlet apparatus (Speakman, 2001) (see Fig. 2.12).

Analytical procedure:

Each sample was weighed (whole animal for non-calcified marine invertebrates and without shell for calcified marine invertebrates) before being put into a cellulose thimble. The samples were kept in place with a small plug of cotton wool. The thimble containing the sample was then placed into the extraction tube. Solvent (petroleum ether) was then added to the system (about 90 ml in a 100-ml flask). The solvent within the flask was heated using a hot plate until it boiled slowly. Solvent vapours condensed when they reached the condenser coils and dripped slowly into the extraction tube containing the thimble, bathing the thimble and sample in hot petroleum ether. When the solvent in the extraction tube reached a particular level, the solvent was siphoned back into the boiling flask. The solvent was then reheated and the cycle was repeated over a total period of approximately 4 h. The thimble containing the sample was then removed from the extraction tube and placed in a drying oven at a temperature of 70 ºC for 8–10 hours to completely evaporate the solvent. After drying, the sample was weighed and the fat content was determined by calculating the difference between the dry mass of the animal before extraction and the dry mass of the animal after extraction. The percentage fat content was calculated using the following equation:

$$PFC = (DM_{dry} - DM_{extr.}) / DM_{dry} \times 100 \qquad (2.11)$$

where PFC is percentage fat content (%); DM_{dry} is dry weight before extraction (g), and $DM_{extr.}$ is dry weight after extraction (g).

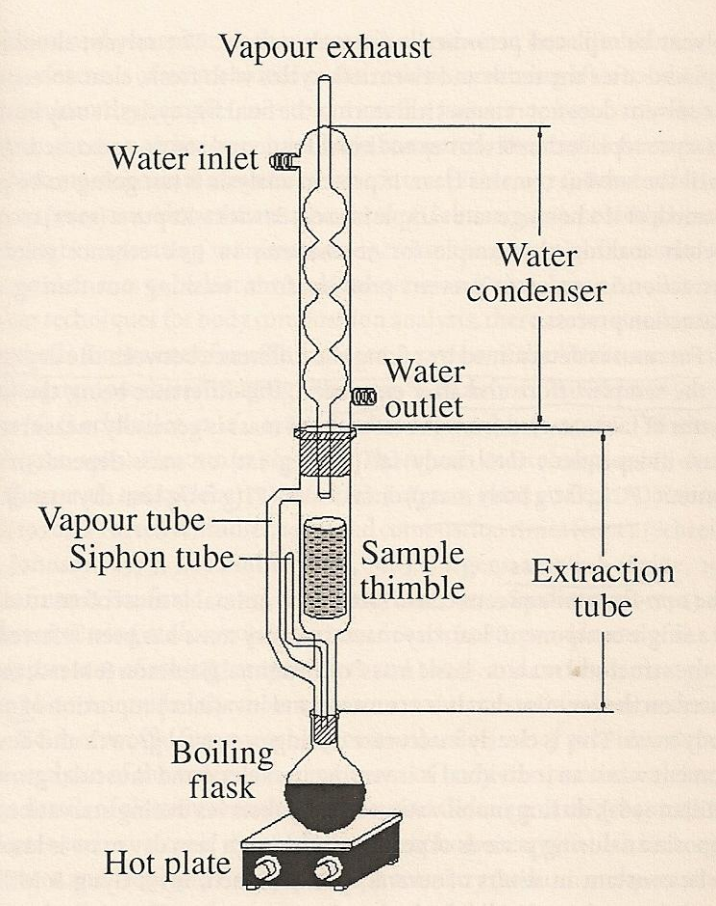

Figure 2.12 Diagram showing the various components of the Soxhlet apparatus (Speakman, 2001).

2.11.6 C:N Ratio:

C:N ratios were determined after the six-week experimental period, on only dried soft tissue, using a vario MACRO Cube CN Analyser (Elementar) linked to a computer. Briefly, these methods are based on combustion in an oxygen atmosphere and post-combustion analysis of samples in a reduction tube in the furnace of the analyser. Samples of 0.1 g are placed into the analyser, which determines C and N

content of combustion products by gas chromatography. Results are obtained in terms of carbon mass, nitrogen mass, and the C:N ratio.

2.12 Determination of Temperatures and pH Levels for Treatments:

While both air and ocean temperatures are expected to increase in the future, ocean temperatures are predicted to increase by less than half the anticipated increase in air temperatures (Brown and McLachlan, 2002). This study focuses on the physiological changes likely to result in coastal marine invertebrates that inhabit the marine tidal zone under future environmental conditions predicted to include higher temperatures and [CO_2]. Because the natural surface-water habitat of these invertebrates is in direct contact with the air, and global average air temperatures will vary only slightly from the ocean surface temperatures (Gruber, 2011), monitoring water temperatures and average air temperatures in the northeast of England, as recorded on the website of the UK Met Office (http://www.metoffice.gov.uk/) was necessary for the present study. Average water temperatures of the North Sea during the course of the year range from 18 ºC in the summer to 3 ºC in the winter (Wiltshire and Manly, 2004). Data from the Met Office website was used to determine typical temperatures during the winter and the summer for the control treatments (TR 2011) based on the average temperatures for the past 30 years (1981–2010) (see Fig. 2.13 and Fig. 2.14). We found that the temperatures ranged from 4 ºC to 5 ºC in winter and from 14 ºC to 15 ºC in the summer along the northeastern coast of the UK. Air temperatures are predicted to have increased from 4 ºC to 6 ºC by 2100 (IPCC, 2007), as the best estimate of the worst-case scenario (A1F1) in the present study. Temperatures were calculated to determine the expected temperature ranges in 2050 and 2100 (see Table 2.3).

The pH levels to be used in the experimental procedures were determined according to Turley and Findlay, (2009) (adapted from (Turley *et al.*, 2006) using data from Pearson and Palmer (2000), with a range of ± 0.5 units for each treatment). In 2011, pH of seawater in the study region was predicted to be between 8.00 and 8.10, so for the purpose of our experiment, pH was adjusted to 8.05 (see Table 2.3). These figures agree with those predicted by (Harrould-Kolieb and Savitz, 2009), which

indicated that under a 'business-as-usual' greenhouse gas emissions scenario, pH would have decreased to 8.09, decreased further to between 7.91 and 7.97 by 2050, and to 7.78 by 2100. Similarly Fabry *et al*. (2008), predicted that ocean pH would range between 8.05 and 8.06 by 2010, between 7.91 and 7.92 for 2050, and between 7.76 and 7.74 by 2100.

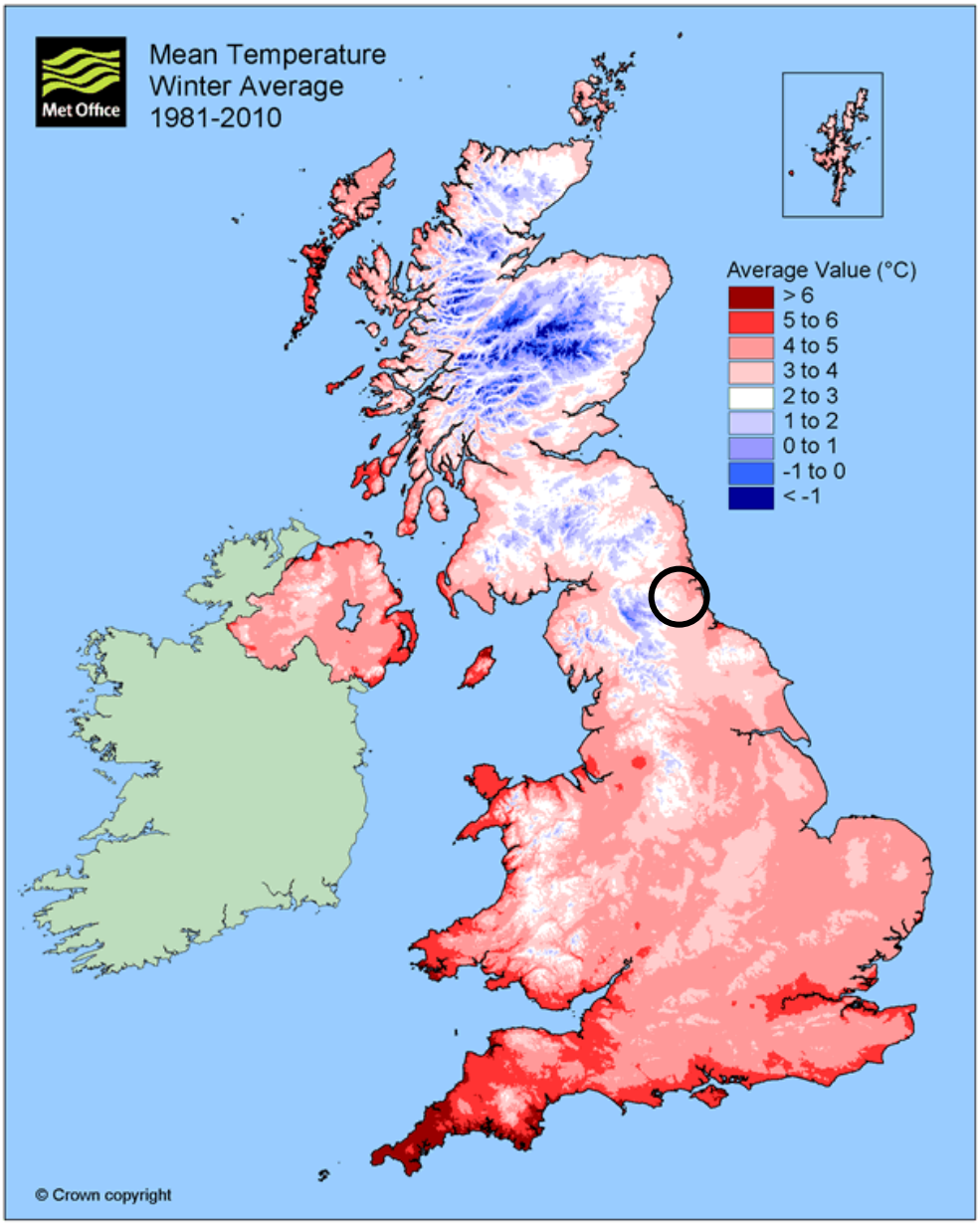

Figure 2.13 The map above shows the 30-year mean temperature winter average from 1981–2010 in degrees Celsius (ºC) (Met Office website). The circle indicates the region from which samples were collected and experiments were conducted.

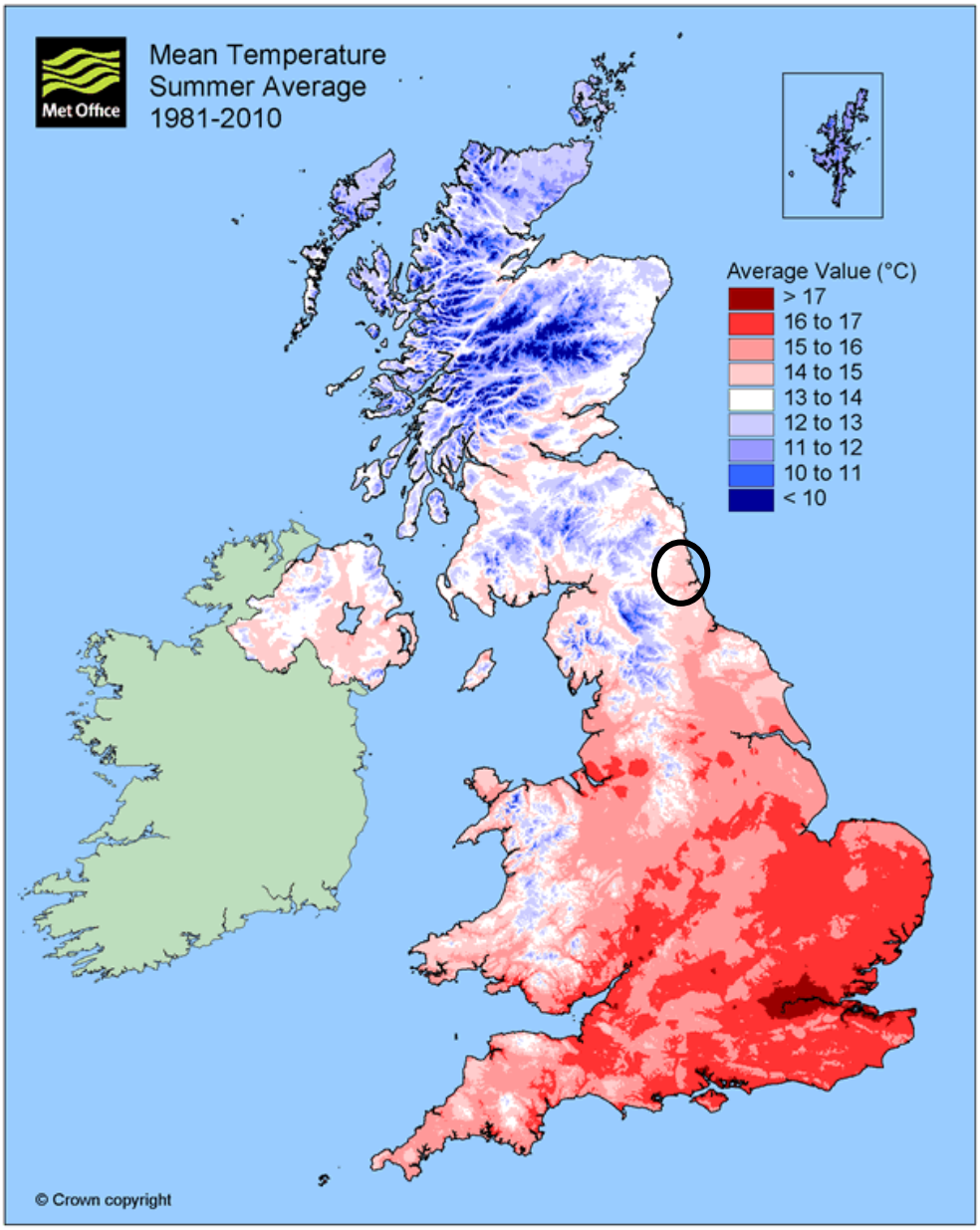

Figure 2.14 The map above shows the 30-year mean temperature summer average from 1981–2010 in degrees Celsius (ºC) (Met Office website). The circle refers to the region from which samples were collected and the experiments were conducted.

Table 2.3 Three treatments are presented: current (TR 2011) represents the control, while the conditions expected for the middle of this century (TR 2050) and the end of the century (TR 2100) represent estimates for pH and temperature based on predictions from a wide range of previous publications, a target pH range of ± 0.5 units for the present study.

	TR 2011	TR 2050	TR 2100
Predicted temperature; Experimental temperature (winter)	4 °C–5 °C; 4.7 °C	6.2 °C–6.9 °C; 6.4 °C	8.5 °C–10.5 °C; 8.7 °C
Predicted temperature; Experimental temperature (summer)	14 °C–15 °C; 14.8 °C	16.2 °C–16.9 °C; 17.0 °C	18.5 °C–20.5 °C; 19.2 °C
Predicted pH; Experimental pH (winter and summer)	8.00–8.10; 8.05	7.87–7.97; 7.84–7.90	7.73–7.83; 7.65–7.75

2.13 Statistics:

The IBM SPSS Statistics 21 predictive analytics software was used to perform the statistical analyses. All percentage data (body weight change, body length change, body width change, buoyant weight change, dry weight (and conversely, water content), soft tissue weight, shell weight, and lipid content) were converted to proportional positive data and then arc-sine square-root transformed (Dytham, 2011). C:N ratio and VO_2 (respiration rate) data were square-root or log transformed. Tests for normal distribution were performed using a Shapiro-Wilk's test, then homogeneity of variances were assessed using Levene's test. When data were normally distributed and homogeneous (equal variances), variation between treatments was tested using one-way ANOVA and specific differences were identified using the *post hoc* Scheffe's test. When normality tests indicated that data were non-normally distributed or that variances were heterogeneous, the non-parametric Kruskal-Wallis test was used to perform one-way analysis of variance, and if positive, the Mann-Whitney U test was then used to test for differences between specific treatments. An Excel program was used to create graphics from the data.

Chapter 3. Effect of Elevated Temperature and Reduced pH on Four Marine Invertebrates in Winter

Abstract:

Increased human activities and the associated increased emissions have led to higher atmospheric concentrations of CO_2, a greenhouse gas that contributes to rising ocean surface temperatures and acidity due to absorbed CO_2. The present study investigated the effects of higher ocean temperature and acidity by subjecting four marine invertebrate species (two calcified and two non-calcified) to the climatic conditions expected to occur during winter in 2050 and 2100. After being given two weeks to adapt to the experimentally altered climatic conditions, animals were exposed over six weeks to lower ph and higher temperatures predicted to occur later in this century. Survival and growth parameters based on body weight changes, morphometric changes, buoyant weight changes, and dry weights were assessed during the experimental period. The calcified invertebrates (both sedentary and mobile species) were able to survive and grow, albeit at a lower rate, under the higher temperatures and lower pH predicted for the future. Increased survival rates but slower growth rates were observed in the sea anemone *Actinia equina*, a semi-motile, non-calcified invertebrate, suggesting that future conditions may be favourable to this species. However, in the case of the sea squirt, *Ascidiella aspersa* (a non-calcified, sessile invertebrate), there was a significant increase in mortality under the higher temperatures and water acidity. These results indicate that each species is likely to respond to environmental changes in different ways. Calcified animals, such as *Mytilus edulis* and *Littorina littorea*, might be able to buffer the effects of increased acidity on their shells through increased ion regulation, facilitated by increased temperature elevating their metabolic rates (see Chapter 4). In the long term, such a strategy could only be maintained by increased food consumption to fuel an increased metabolic rate, which may not be sustainable. Surprisingly, the greatest effects of elevated temperature and decreased pH were seen in non-calcified invertebrates, in particular *A. aspersa*, which may not be able to regulate its body fluids to adapt to these environmental changes.

3.1 Introduction:

The average global ocean temperature has been increasing since at least 1976, especially during the winter (Stachowicz *et al*., 2002). This amounts to an increase of 0.13 ºC per decade because of continuing increases in atmospheric [CO_2]. Disastrous consequences are predicted if global temperatures rise by 2 ºC relative to the period before the industrial revolution if atmospheric [CO_2] in the reaches an expected 450 ppm by the middle of this century (Brierley and Kingsford, 2009). If CO_2 concentrations reach 710 ppm (pH = 7.8) in 2100 (Walther *et al*,. 2009) a temperature increase of more than 4 ºC could occur (IPCC, 2007). According to Portner and Knust (2007), at the end of this century the temperature of the North Sea could increase by 3.9 ºC. Temperatures in the North Sea range between 3 ºC and 6 ºC in the winter, and between 15 ºC and 18 ºC in the summer, with an annual average of 10.4 ºC (Sokolova and Portner, 2003). This means that if expected temperatures reach 9.9 ºC in the winter, although still below the annual average temperature, the temperature would be close to temperatures characteristic of spring.

Animal populations in the North Sea already suffer due to increased temperatures (Portner *et al.*, 2001) and will continue to do so upon exposure to low pH in the future. Usually, low winter temperatures reduce animal nutrition and biomechanical strength. Therefore, in winter they rely more on food reserves such as stored lipid to produce energy (Findlay *et al*., 2009a). Because the body temperatures of ectotherm marine invertebrates fluctuate depending on the temperature of the surrounding environment, these animals are strongly affected by changes in temperature (Brierly and Kingsford, 2009). Increased temperatures may negatively affect species' biological processes and survival (Harley *et al*., 2006), because higher temperatures lead to increased metabolic processes and consumption of larger amounts of stored lipids. With the limited availability of food during the winter, the continuation of this process for too long may lead to decreased levels of vital protein, which may promote mortality (Barnes *et al*., 1963; Findlay *et al.,* 2009a). Overwintering populations usually are the basis of breeding in the spring and summer months, so the exposure of animals to heat stress during this period (winter) could have serious implications for their survival, and even those that survive must then have enough energy reserve to continue to growth into adults and perform their vital reproductive role through the following months (Lischka *et al.*, 2011). For example,

Mytilus edulis begins to form gametes during the winter in preparation for spawning, which occurs during the spring and summer (Zagata *et al*., 2008). If the temperature rises to a sufficient degree in the spring the process of spawning is stimulated by the rise in temperature and food availability (Christian *et al*., 2010). Also, gonads of the marine snail *Littorina littorea* mature from winter to early spring, as spawning takes place from winter through the end of spring (Christian *et al.*, 2010). In addition to its impact on the timing of regeneration, the change in seawater temperatures in the winter warmer years may also affect the rate of survival (Ruiz *et al.*, 1999; Pappal, 2010). *Alitta virens* also shifts from responses expected during the winter to those responses during the summer due to increased temperatures (Godbold and Solam, 2013).

Expected increases in temperatures will be combined with expected decreases in pH due to continuing increased emissions of CO_2 into the atmosphere. The phenomenon of ocean acidification (OA) is expected to continue and will likely affect growth and mortality in addition to particular effects on calcified organisms that will find it difficult to form and maintain structures made of calcium carbonate (Raven, 2005). Many studies of the synergistic effects of high temperature with low pH have been carried out, for example in *Echinometera lacunter*, in which decreased calcification in winter compared to summer, and low buoyant weights similar to the levels expected for winter conditions in 2100 were found (Courtney *et al*., 2013; Uthicke *et al.*, 2014). Also, exposure to low pH and high temperatures led to a decrease in the rate of calcification in the coral species *Lophelia pertusa* (Maier *et al*., 2009), similar to that observed in the coral *Acropora longicyathus* (Bucher and Harrison, 2000). Combined higher temperature and lower pH also resulted in decreased shell weight in the foraminifera *Ammonia tepida* (Dissard *et al.*, 2010). Furthermore, there were synergistic and negative effects of higher temperatures and lower pH on the rate of survival in the barnacle (Findlay *et al*., 2009a) and petropod (Lischka *et al*., 2011). In *M. edulis* the growth rate and the length of the shell decreased in the winter (Melzner *et al.*, 2011). However, Landes and Zimmer (2012) showed that the marine snails *L. littorea* were better able to build their shells in response to the presence of a predator at high temperature and low pH. But Melatunan *et al.*, (2013) found decreased percentage increase in shell length in *L. littorea* under exposure to both low pH and high temperature. In coralline algae, combined high

temperature and low pH cause necrosis and death of the algae (Martin and Gattuso, 2009).

In contrast, little is known about the responses of non-calcified marine invertebrates to higher temperatures and lower pH (Suggett *et al*., 2012) due to current research bias towards calcified organisms (Connell and Russell, 2010; Suggett *et al.*, 2012). For example, a synergistic effect of increased temperature and decreased pH had a positive effect on the abundance of some seaweeds, and the biomass of algal turfs doubled (Connell and Russell, 2010).

In the following chapter, the effects of higher winter temperatures and lower pH resulting from expected future increases in [CO_2] on the mortality and growth of calcified and non-calcified marine invertebrates will be investigated.

3.2 Methods*:*

In this chapter the species under study (*M. edulis*, *L. littorea, A. equina*, *and A. aspersa*) were exposed to the increased temperatures and decreased pH levels expected in the future. Animals were collected and taken directly to the laboratory, and were cleaned. All 4 species were held together in the same microcosm, at a temperature of 4 ºC – 5 ºC at the same pH as the water in which they were found. The next day, the animals were divided randomly into three separate microcosms. Each microcosm contained a group of animals representing all four species of marine invertebrates under study. The numbers of animals of each species in the microcosm depended on the total number of animals that had been collected in the previous day. The temperature was gradually raised and the pH gradually lowered to levels expected in the future in the second microcosm (TR 2050) and the third (TR 2100) microcosm 48 h after animals were collected. The temperature in each experimental microcosm was increased at a rate of 0.5 ºC every two days, and the pH was decreased at a rate of 0.02 units per day and animals were held under these conditions for two weeks to acclimatze the animals. All measurements started after the end of this point. This point was called the (APEP) Acclimatization Period End Point. The control experiments were conducted in the winter under the conditions expected for the

winter in the period from 01 February 2012 to 28 March 2012, and from 24 September 2012 to 22 November 2012 (Table 3.1).

A total of 12 individuals of each species were used to measure wet weight, buoyant weight, metabolism, and dry weight under each treatment. In addition, body length was measured to *M. edulis, L. littorea and A. aspersa*, and body width was measured only in *M. edulis*. All animals in this set of experiments were used to determine percent survival. Some individuals of *A. equina* had to be removed from the microcosms when a large gap formed in their center before they were completely dead to avoid contaminating the microcosm as they decayed. Table 3.2 explains the numbers of samples of each species used for parameters measured in these two experiments. However, in some instances, fewer than 12 animals were studied due to reduced survival.

Table 3.1 Dates of collections and experimental periods for winter experiments.

Experiment No.	Collection date	Experimental period
1	01 February 2012	01 February – 28 March 2012
2	24 September 2012	24 September – 22 November 2012

After the completion of each experiment, some of the samples were prepared for analysis of protein contents, calorimetry, and histology (see Appendices) of the animals under study. Some samples were also preserved at -80 ºC until needed for future studies.

Table 3.2 Numbers of samples used for parameters measured in the winter condition experiments for the species under study.

Parameters	Treatments											
	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
No. of animals at start	84	66	53	102	81	69	46	97	78	63	49	89
No. of animals at end	84	66	34	98	68	67	34	63	71	62	35	16
No. of animals surviving[a]	84	66	53	102	81	69	46	97	78	63	49	89
Body weight	12	12	12	14	12	12	12	9	12	12	11	4
Body length	12	12	---	14	12	12	---	9	12	12	---	4
Body width	12	---	---	---	12	---	---	---	12	---	---	---
Buoyant weight	12	12	12	14	12	12	12	9	12	12	11	4
Dry weight	6	5	6	6	6	5	5	5	6	6	6	2

Here, [a] is the number of animals used to calculate survival, *M.e.* refers to *M. edulis*, *L.l.* refers to *L. littorea*, *A.e.* refers to *A. equina*, and *A.a.* refers to *A. aspersa.*

3.2.1 Seawater Parameters:

This study was designed to investigate the potential impacts of climate change from combined high temperature and low pH, so three sets of treatments were used. The control group was maintained at the levels of temperature (4.7 ± 0.2) and the pH (8.05 ± 0.07) surrounding the animals when they were collected. As this study is examining the effects of future conditions forecast for 2050 and 2100, the temperature was raised and the pH was lowered in each treatment. The temperatures were raised by 6.4 ±0.2 ºC and 8.7 ±0.24 ºC for 2050 and 2100 treatments, respectively, and pH was decreased to 7.9 ±0.06 and 7.75 ±0.07 units, respectively. The salinity was maintained at levels normal at Newbiggin-by-the-Sea (35 ppt) and Hartlepool (34 ppt), so salinity averages settled at 36.1 ±1.08% for the control group for year 2011 (TR 2011), at 36.0 ±1.18% for the middle of this century (TR 2050), and at 35.9 ±1.11% for the end of this century (TR 2100). Dissolved oxygen (O_2) in the water was maintained at greater than 95% for all treatments to ensure an abundant supply, as a lack of oxygen will affect the physiological processes. Seawater parameters of temperature, salinity, and dissolved oxygen (O_2) were measured daily during the experiments (Table 3.3).

Table 3.3 Seawater chemistry parameters during the winter condition experiments under control and future treatment conditions treatments (Mean ± S.E.).

Treatment	Temperature (°C)	Salinity (ppt)	pH	O_2 (%)
TR 2011	4.7 (±0.20)	36.1 (±1.08)	8.05 (±0.07)	97.4 (±1.40)
TR 2050	6.4 (±0.20)	36.0 (±1.18)	7.90 (±0.06)	97.3 (±1.60)
TR 2100	8.7 (±0.24)	35.9 (±1.11)	7.75 (±0.07)	96.8 (±1.74)

After the acclimatisation period, any dead animals were removed from the microcosms and counted daily starting from the third week until the eighth week (during the six-week experimental period) (for more about methods see Chapter 2).

3.3 Results:

3.3.1 Survival:

Survival did not differ significantly for *M. edulis*, *L. littorea*, or *A. equina* among treatments, but did differ among treatments for *A. aspersa* ($X^2 = 51.60$, df = 2, p = 0.05). A pairwise comparison was used to determine which treatments differed in *A. aspersa*, Chi-square test for binomially distributed data TR 2011 compared with TR 2050, p = 0.018; TR 2011 compared with TR 2100, p < 0.01; and TR 2050 compared with TR 2100, p < 0.01). Figures 3.1, 3.2, 3.3, and 3.4 show the changes in percent survival for all species under study.

Table 3.4 Percentage survival of four species under different treatments over a six-week period.

Species	TR 2011		TR 2050		TR 2100	
	n	Survival %	n	Survival %	n	Survival %
M. edulis	84	100	81	84	78	91
L. littorea	66	100	69	97	63	98
A. equina	53	64	46	74	49	71
A. aspersa	102	96	97	65	89	18

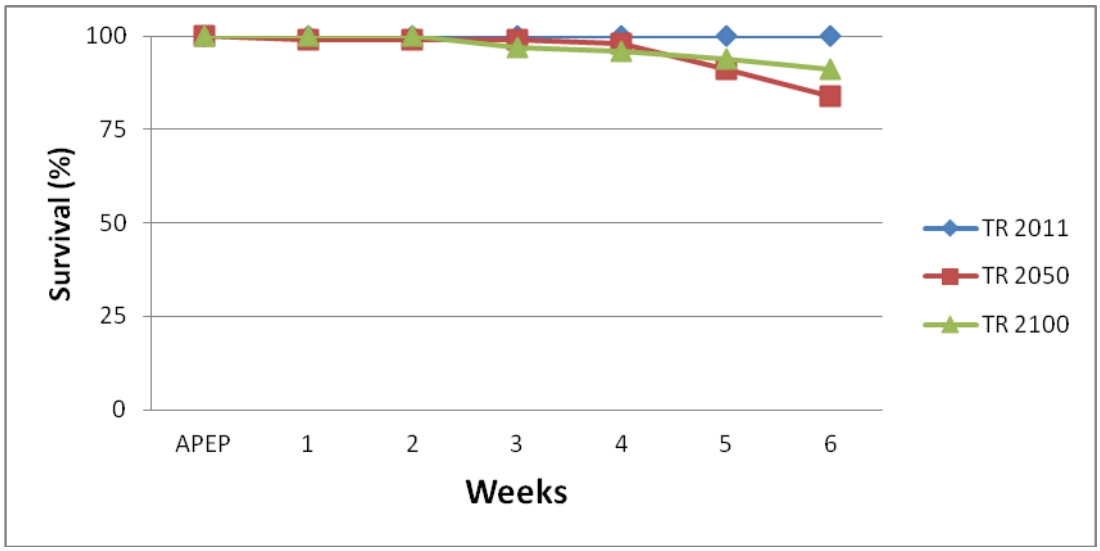

Figure 3.1 Percentage survival of *M. edulis* over a six-week experimental period for all treatments. The experimental treatment TR 2011 is represented by blue diamonds, TR 2050 is represented by red squares, and TR 2100 is represented by green triangles. APEP represents the acclimatisation period end point.

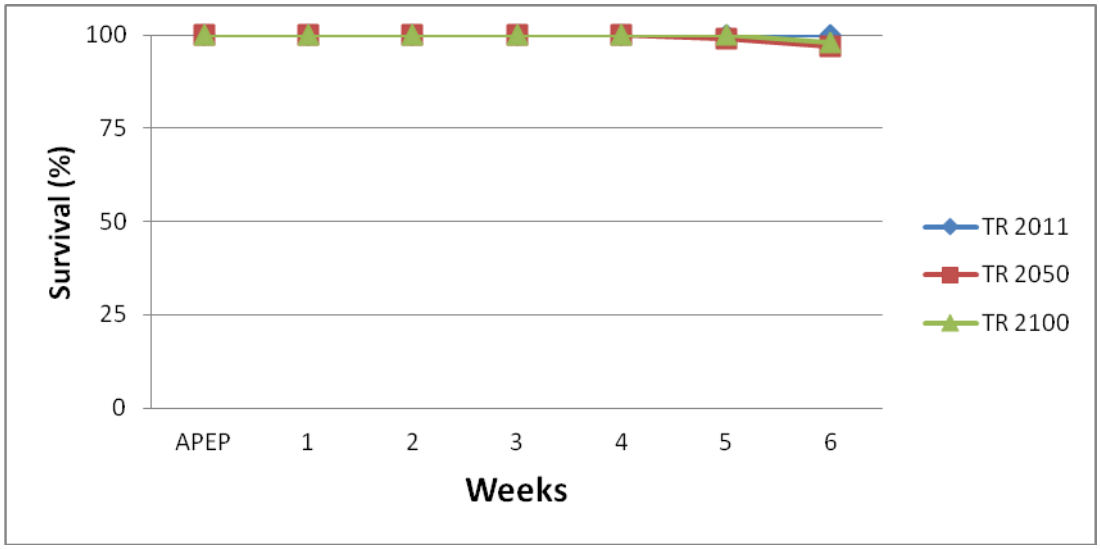

Figure 3.2 Percentage survival of *L. littorea* over a six-week experimental period for all treatments. The experimental treatment TR 2011 is represented by blue diamonds, TR 2050 is represented by red squares, and TR 2100 is represented by green triangles. APEP represents the acclimatisation period end point.

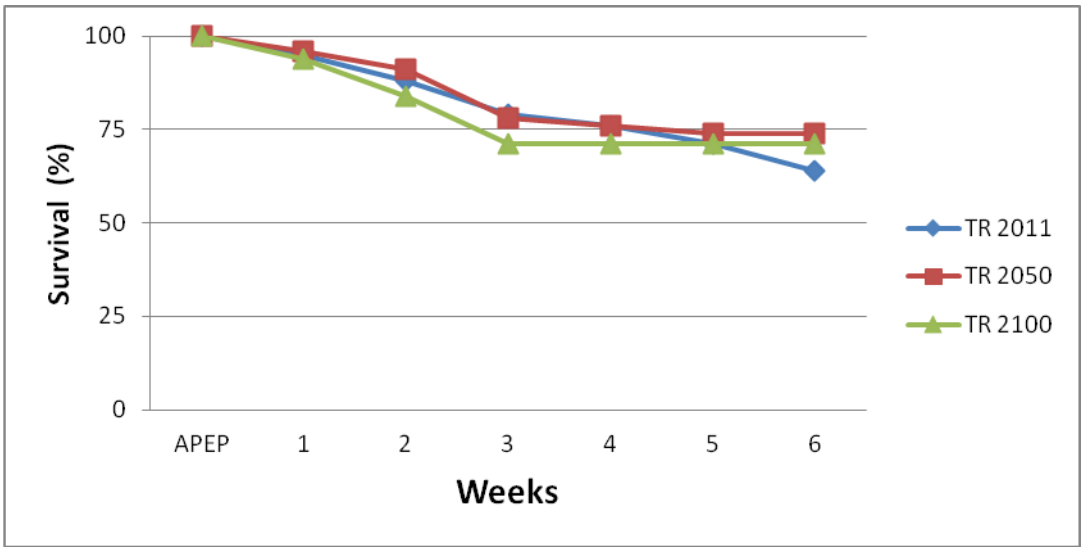

Figure 3.3 Percentage survival of *A. equina* over a six-week experimental period for all treatments. The experimental treatment TR 2011 is represented by blue diamonds, TR 2050 is represented by red squares, and TR 2100 is represented by green triangles. APEP represents the acclimatisation period end point.

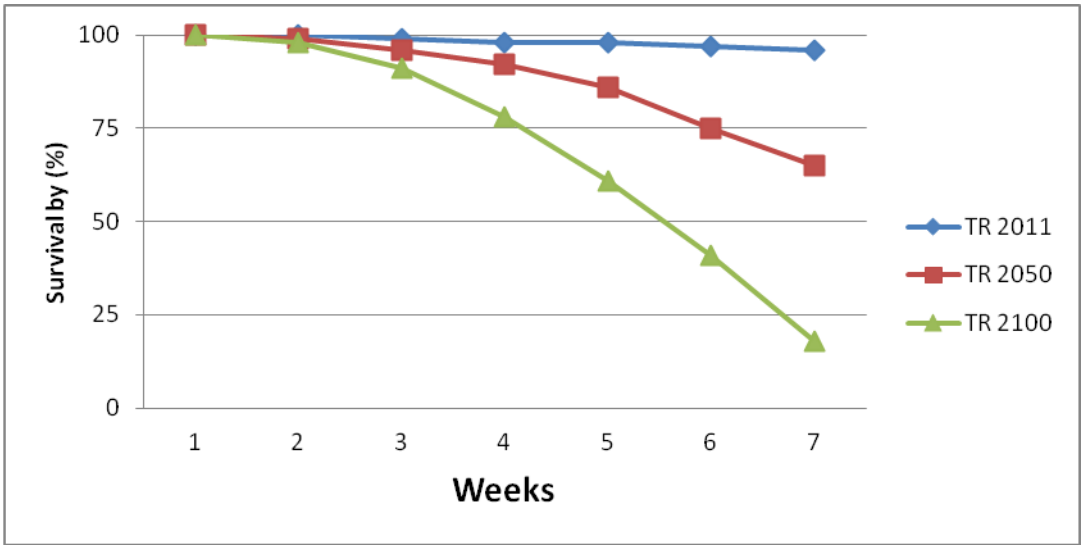

Figure 3.4 Percentage survival of *A. aspersa* over a six-week experimental period for all treatments. The experimental treatment TR 2011 is represented by blue diamonds, TR 2050 is represented by red squares, and TR 2100 is represented by green triangles. APEP represents the acclimatisation period end point.

3.3.2 Body weight change, Size change, Buoyant weight change, and Dry Weight Parameters:

M. edulis:

Data for body weight change, body length change, body width change, and buoyant weight change were not normally distributed, according to a Shapiro-Wilk's test. So, a non-parametric Kruskal-Wallis test was used to determine whether there were any significant treatment effects. A Mann-Whitney U test was then used *post hoc* to test for significant differences between treatments (Table 3.5). However, data for dry weight was normally distributed and treatments were compared by one-way ANOVA (Table 3.6).

Table 3.5 Statistical analysis of body weight change, body length change, body width change, and buoyant weight change in *M. edulis*.

	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-square	df	*p* value
Body weight	0.815	36	0.001	0.662	2	0.718
Body length	0.836	36	0.001	4.236	2	0.120
Body width	0.919	36	0.012	5.497	2	0.064
Buoyant weight	0.920	36	0.013	7.222	2	0.027

Table 3.6 Statistical analysis of dry weight in *M. edulis.*

		ANOVA test		Normality test			Homogeneity test	
Parameter		One-way		Shapiro-Wilk's test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Dry weight	18	0.027	0.973	0.955	18	0.505	1.867	0.189

There were no significant differences in the body weight change, body length change; body width change, or dry weight of *M. edulis* over the six-week period between three treatments (see Table 3.5 and Table 3.6). However, there was a significant difference in the buoyant weight change between TR 2011 compared with TR 2050 ($p = 0.008$) according to the Mann-Whitney U test.

Table 3.7 Mean percentage (%) ± standard error (SE) for parameters measured in *M. edulis*.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	6.33	±2.90	2.69	±1.66	4.66	±1.95
Body length change	1.51	±0.52	0.28	±0.38	0.14	±0.12
Body width change	0.92	±0.53	-0.51	±0.91	0.40	±0.23
Buoyant weight change	7.84	±1.82	1.56	±2.64	6.14	±1.95
Dry weight	49.78	±3.47	48.77	±3.05	50.02	±4.96

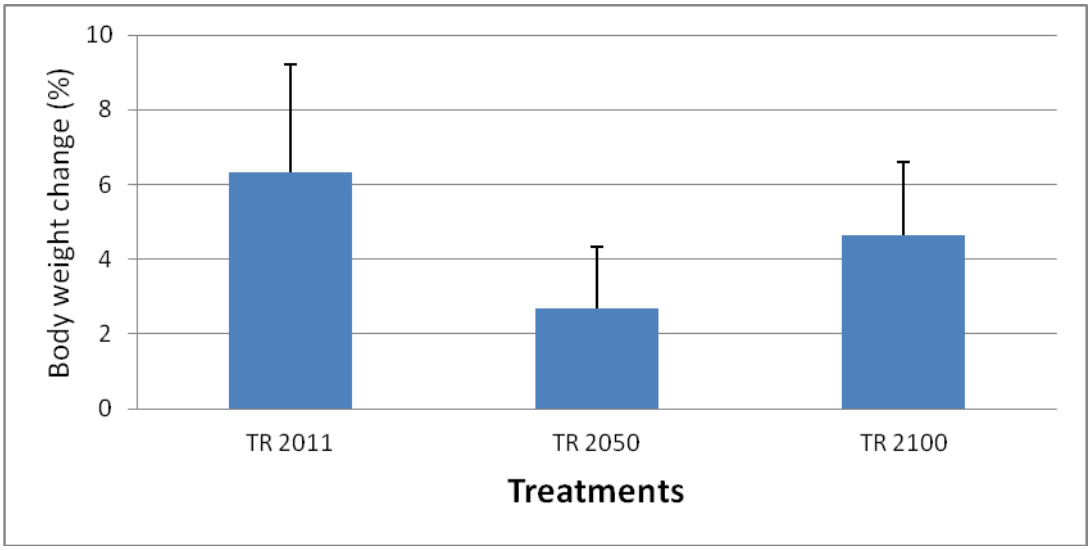

Figure 3.5 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body weight change in *M. edulis* at six weeks.

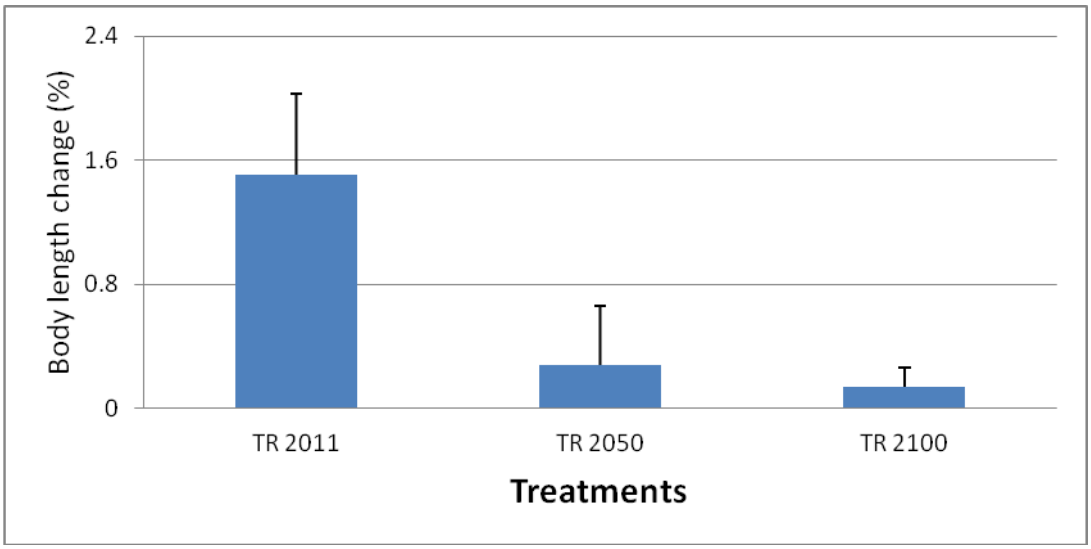

Figure 3.6 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body length change in *M. edulis* at six weeks.

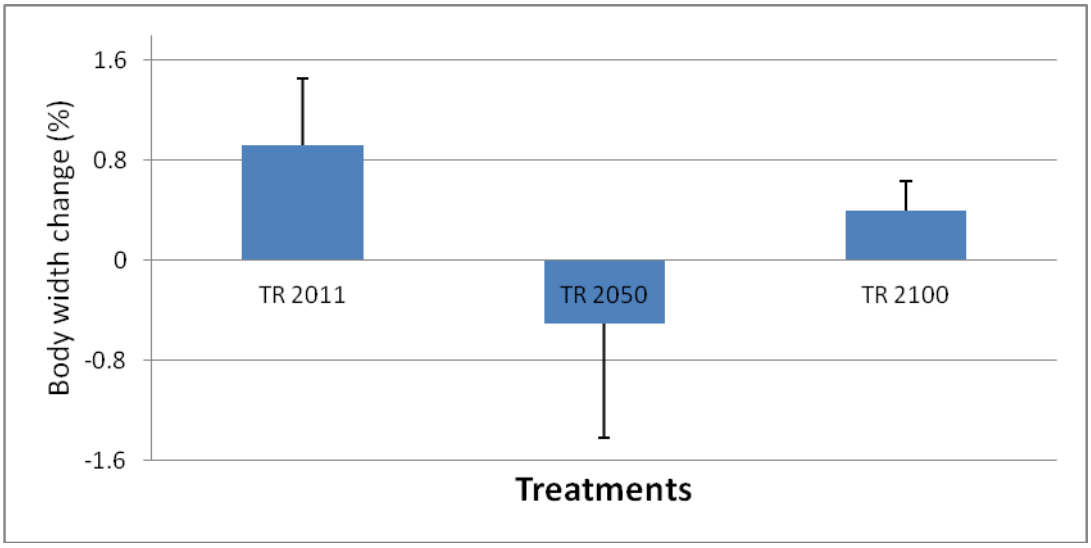

Figure 3.7 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body width change in *M. edulis* at six weeks.

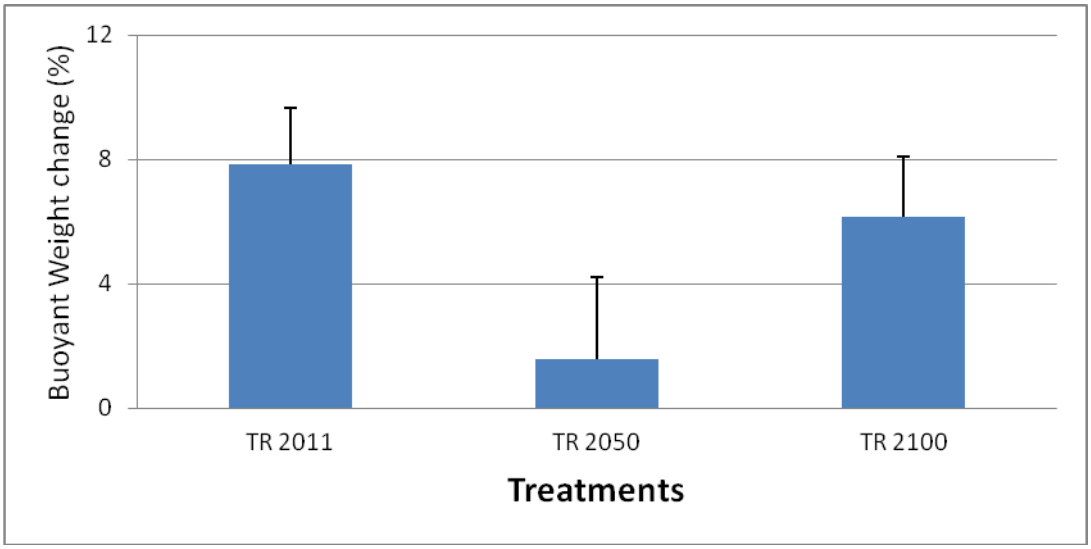

Figure 3.8 Effect of increased temperature and decreased pH on mean percentage (with SE bar) buoyant weight change in *M. edulis* at six weeks.

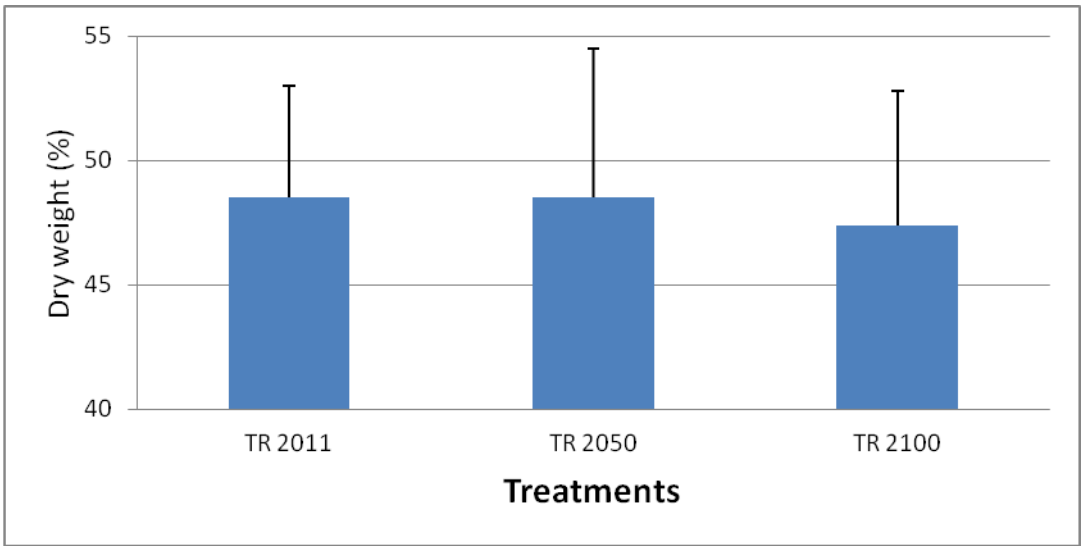

Figure 3.9 Effect of increased temperature and decreased pH on mean percentage (with SE bar) dry weight in *M. edulis* at the end of the experiment.

L. littorea:

A Shapiro-Wilk's test indicated that data for body length change was not normally distributed. So, the non-parametric test Kruskal-Wallis was used to determine whether there were any significant treatment effects (Table 3.8). However, body weight change, buoyant weight change, and dry weight were normally distributed, so treatments for those parameters were compared with one-way ANOVA (Table 3.9).

Table 3.8 Statistical analysis of body length change in *L. littorea*.

	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
Parameter	Statistics	df	p value	Chi-square	df	p value
Body length	0.930	36	0.026	3.918	2	0.141

Table 3.9 Statistical analysis of body weight change, buoyant weight change, and dry weight in *L. littorea.*

		ANOVA test		Normality test			Homogeneity test	
Parameter		One-way		Shapiro-Wilk's test			Levene's test	
	n	F	p	Statistics	df	p	Statistics	p
Body weight	36	0.128	0.880	0.978	36	0.678	1.632	0.211
Buoyant weight	36	0.303	0.740	0.951	36	0.109	3.270	0.051
Dry weight	16	0.545	0.593	0.918	16	0.158	0.113	0.894

There were no significant differences in the all parameters measured in *L. littorea* over a six-week period among the three experimental treatments (see Table 3.8 and Table 3.9).

Table 3.10 Mean percentage (%) ± standard error (SE) for parameters measured in *L. littorea*.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	2.62	±0.70	1.82	±1.05	2.18	±1.46
Body length change	0.13	±0.08	-0.14	±0.17	-0.02	±0.08
Body width change	---	---	---	---	---	---
Buoyant weight change	2.13	±0.83	1.49	±0.96	3.01	±2.03
Dry weight	80.69	±1.60	78.51	±1.71	80.14	±1.25

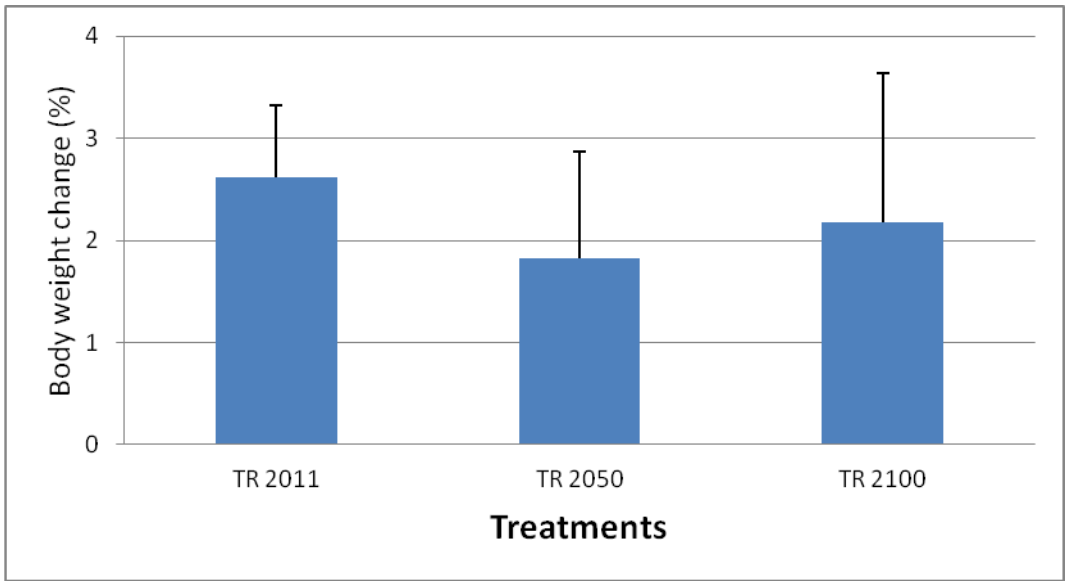

Figure 3.10 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body weight change in *L. littorea* at six weeks.

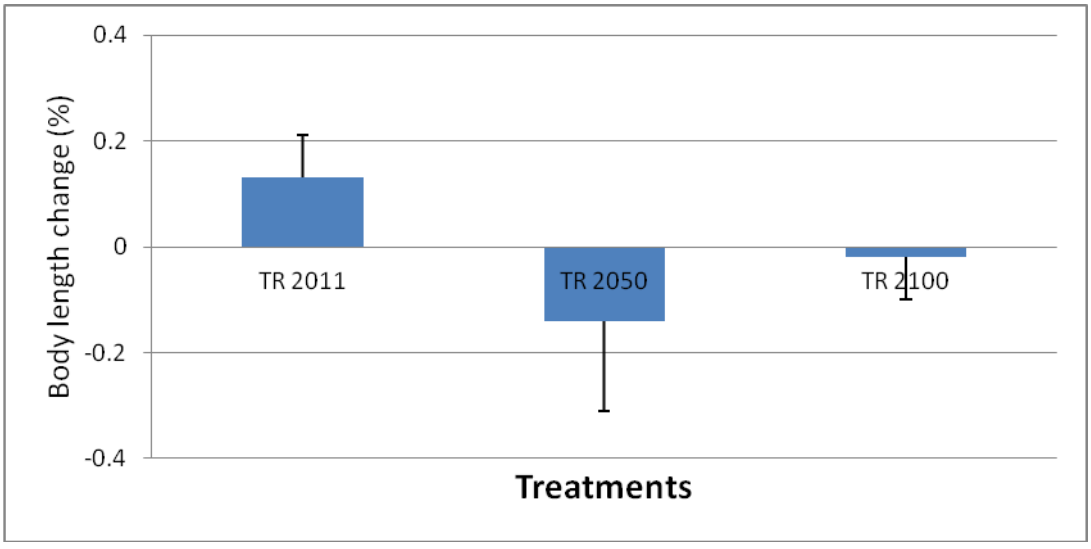

Figure 3.11 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body length change in *L. littorea* at six weeks.

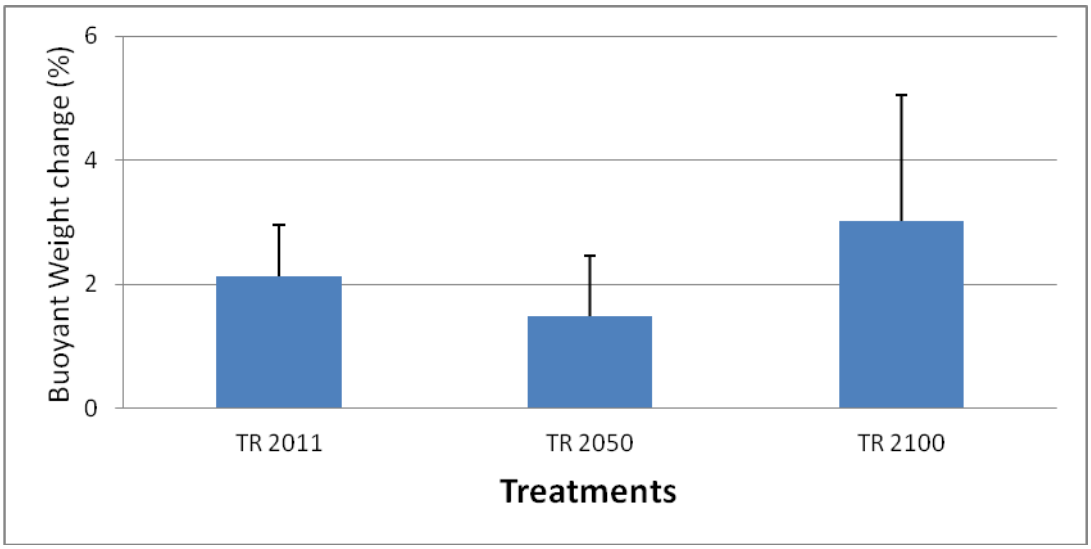

Figure 3.12 Effect of increased temperature and decreased pH on mean percentage (with SE bar) buoyant weight change in *L. littorea* at six weeks.

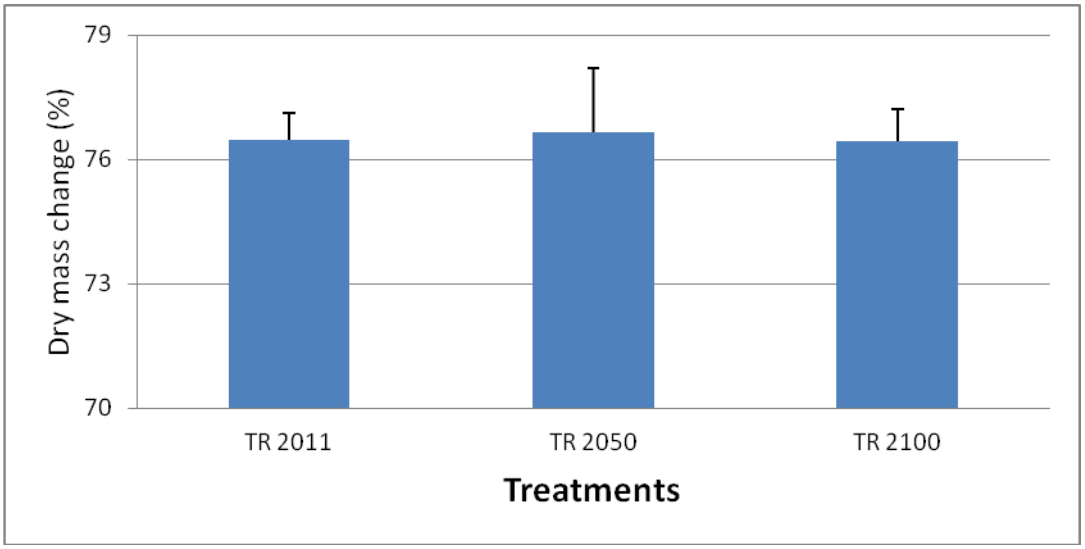

Figure 3.13 Effect of increased temperature and decreased pH on mean percentage (with SE bar) dry weight of *L. littorea* at the end of the experiment.

A. equina:

Shapiro-Wilk's tests indicated that data for body weight change, buoyant weight change, and dry weight were not normally distributed. Therefore, a non-parametric test Kruskal-Wills test was used to determine whether there were any significant treatment effects. Then the Mann-Whitney U test was used *post hoc* to test for significant differences between treatments (Table 3.11).

Table 3.11 Statistical analysis of body weight change, buoyant weight change, and dry weight in *A. equina*.

	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-square	df	*p* value
Body weight	0.815	36	0.001	10.460	2	0.005
Buoyant weight	0.920	36	0.013	0.673	2	0.714
Dry weight	0.866	17	0.019	0.295	2	0.863

There were no significant differences in buoyant weight change over six weeks or dry weight at the end of the experiment in *A. equina* between these three treatments (see Table 3.11 and Table 3.12). However, there was a significant difference in body weight change between TR 2011 and TR 2050 (p = 0.001) according to the Mann-Whitney U test.

Table 3.12 Mean percentage (%) ± standard error (SE) for parameters measured in *A. equina*.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	4.41	±6.94	-28.43	±4.74	1.12	±18.17
Body length change	---	---	---	---	---	---
Body width change	---	---	---	---	---	---
Buoyant weight change	-6.12	±8.58	-6.73	±5.26	-4.69	±4.45
Dry weight	19.73	±2.32	20.45	±0.51	20.18	±1.38

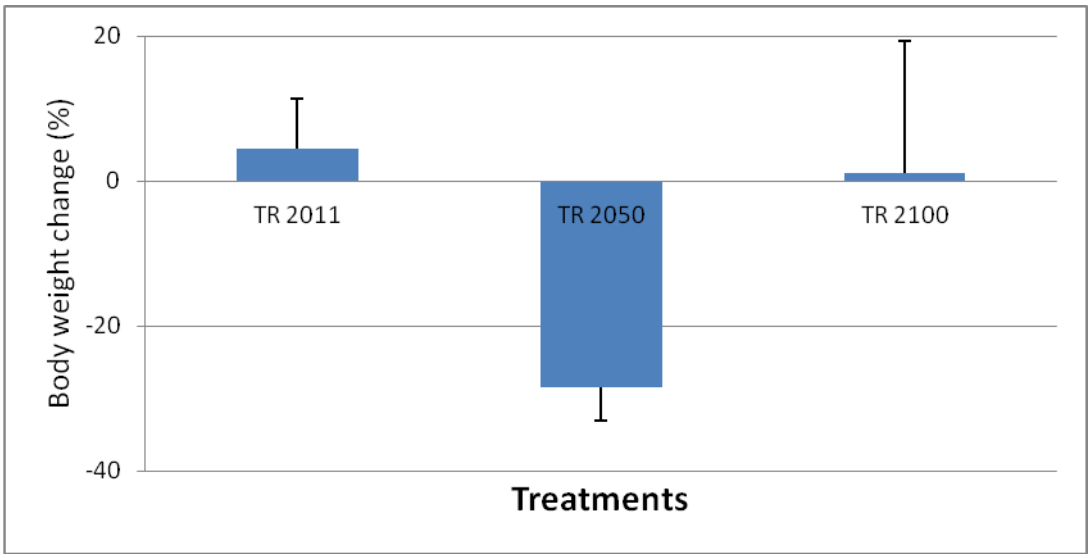

Figure 3.14 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body weight change of *A. equina* at six weeks.

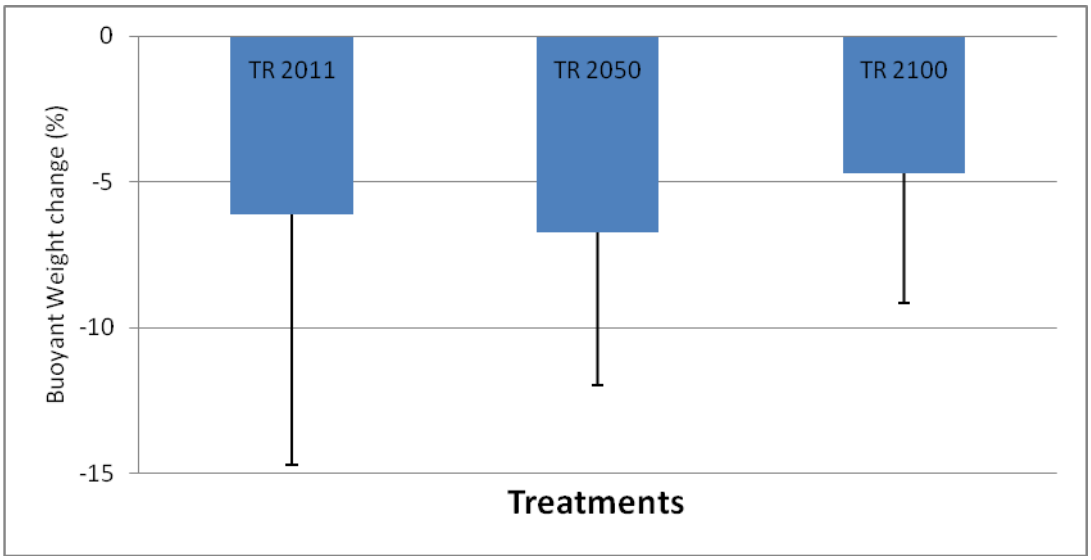

Figure 3.15 Effect of increased temperature and decreased pH on mean percentage (with SE bar) buoyant weight change in *A. equina* at six weeks.

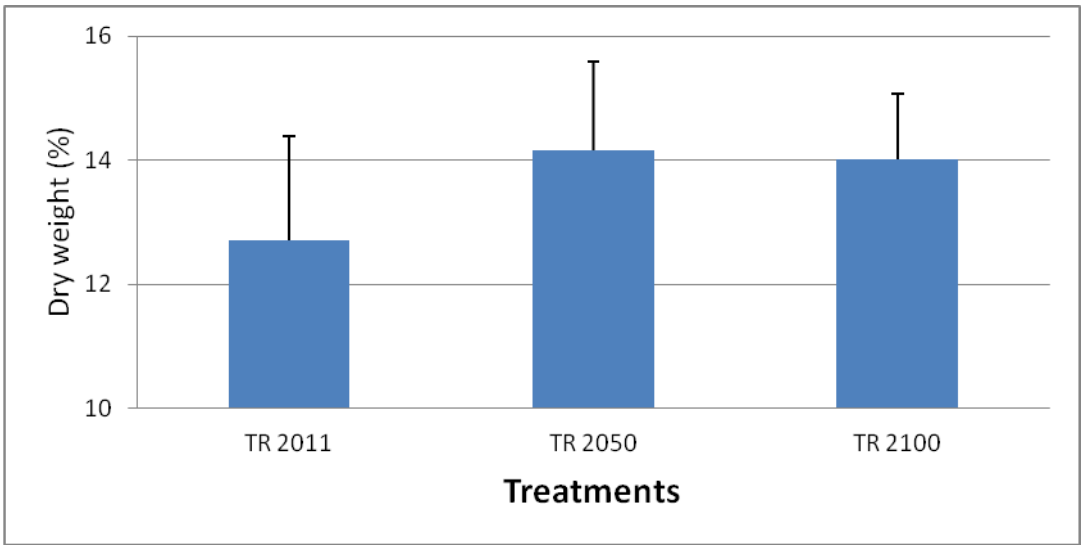

Figure 3.16 Effect of increased temperature and decreased pH on mean percentage (with SE bar) dry weight of *A. equina* at six weeks.

A. aspersa:

A Shapiro-Wilk's test indicated that data for dry weight was not normally distributed. Therefore, the non-parametric test Kruskal-Wallis test was used to determine whether there were any significant treatment effects (Table 3.13). Body weight change, body length change, and buoyant weight change were normally distributed, so treatment effects for those parameters were compared using one-way ANOVA (see Table 3.14). Scheffe's method was used *post hoc* to test for significant differences between treatments.

Table 3.13 Statistical analysis of body weight change, body length change, buoyant weight change, and dry weight in *A. aspersa.*

		ANOVA test		Normality test			Homogeneity test	
Parameter		One-way		Shapiro-Wilk's test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Body weight	27	8.895	0.001	0.977	27	0.793	1.295	0.292
Body length	27	4.078	0.030	0.954	27	0.272	0.458	0.638
Buoyant weight	27	2.446	0.108	0.972	27	0.666	0.361	0.701
Dry weight	13	0.457	0.646	0.881	13	0.074	2.906	0.101

There were no significant differences in the buoyant weight change over the six-week experimental period or in dry weight at the end of the experiment in *A. aspersa* between these three treatments (see Table 3.13). However, there were significant differences in body weight change between TR 2011 and TR 2050 ($p = 0.002$), and in body length change between TR 2011 and TR 2050 ($p = 0.040$) according to the Mann-Whitney U test.

Table 3.14 Mean percentage (%) ± standard error (SE) for parameters measured in *A. aspersa*.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	-31.79	±4.44	-56.25	±3.67	-50.48	±4.48
Body length change	-8.53	±2.09	-18.37	±3.31	-8.36	±3.42
Body width change	---	---	---	---	---	---
Buoyant weight change	-18.44	±6.29	-40.17	±8.37	-34.98	±11.25
Dry weight	6.63	±0.92	5.61	±0.65	5.56	±0.02

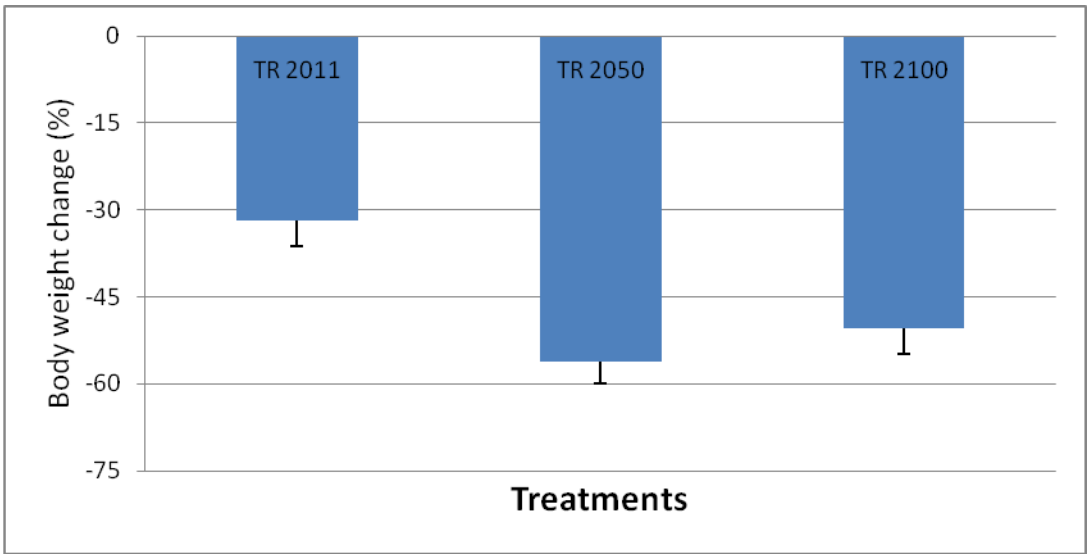

Figure 3.17 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body weight change in *A. aspersa* at six weeks.

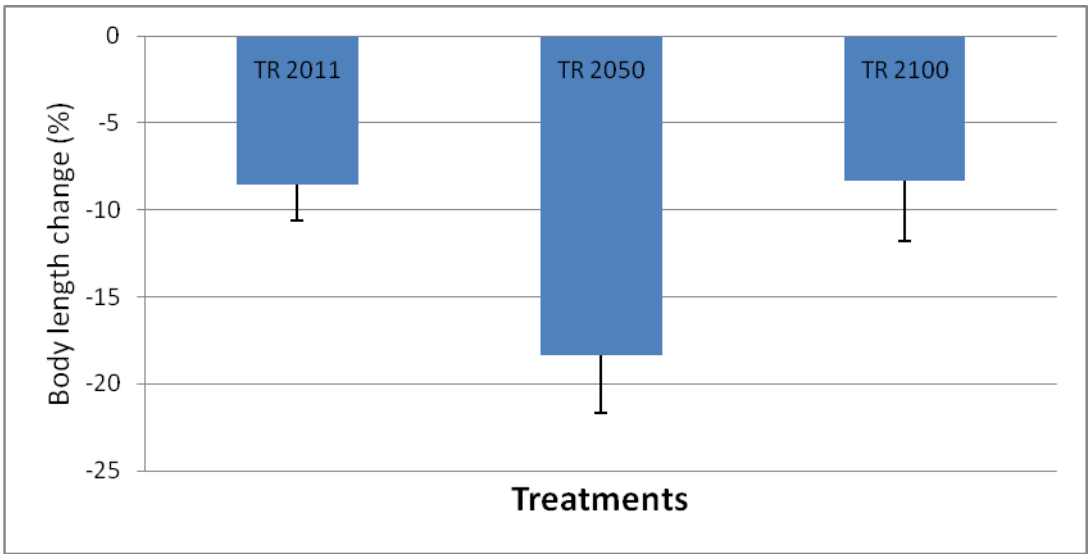

Figure 3.18 Effect of increased temperature and decreased pH on mean percentage (with SE bar) body length change in *A. aspersa* at six weeks.

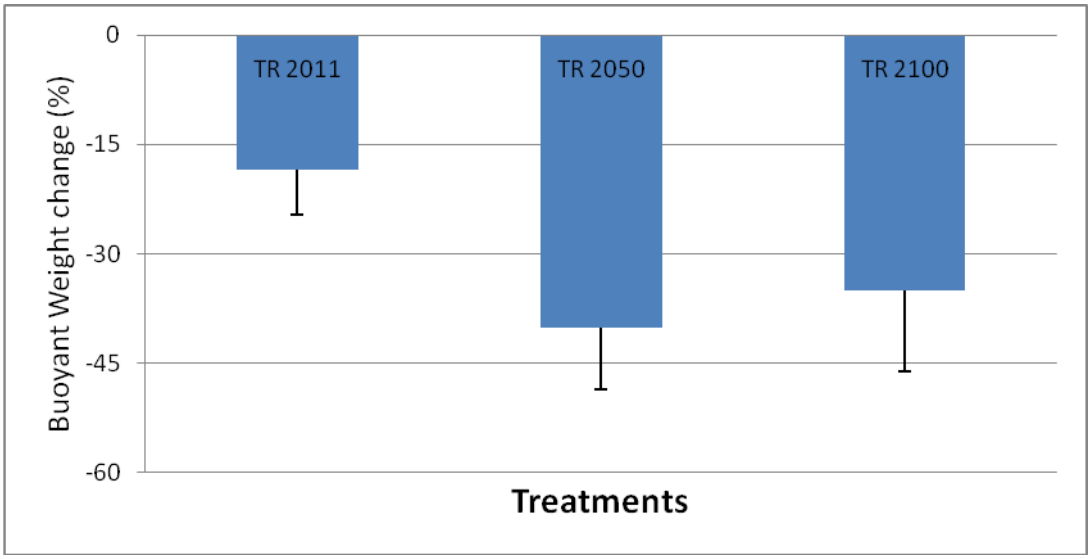

Figure 3.19 Effect of increased temperature and decreased pH on mean percentage (with SE bar) buoyant weight change in *A. aspersa* at six weeks.

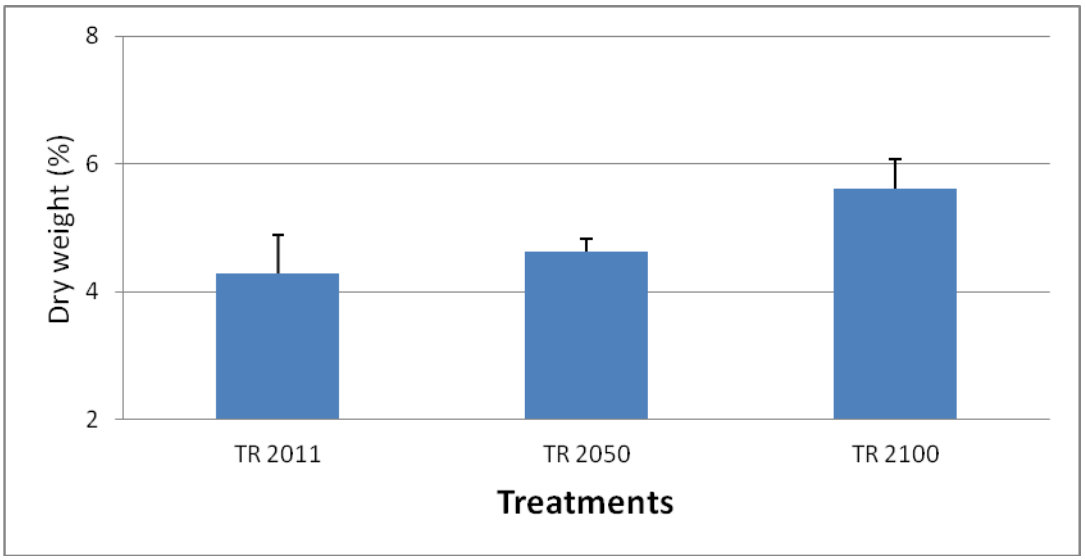

Figure 3.20 Effect of increased temperature and decreased pH on mean percentage (with SE bar) dry weight in *A. aspersa* at the end of the experiment.

3.4 Discussion:

3.4.1 Effect of Experimental Conditions on M. edulis:

These results and previous studies (Findlay *et al.*, 2009b; Ries *et al.*, 2009) show the complex differential responses of calcified coastal benthic organisms to low pH coupled with high temperatures expected to occur in the oceans at the middle and end of this century. *Mytilus edulis* mortality was very low (Table 3.4, Fig. 3.1), and under all treatments there were low levels of growth and calcification (Figs. 3.5, 3.6, 3.7 and 3.8), and no significant differences among treatments (Table 3.5), except under TR 2050, in which there was a significant decrease in buoyant weight change (p = 0.008). These results show that exposure to predicted future conditions will have a negative impact on mussels and lead to reduced calcification (1.56 ±2.64%, Table 3.7). However, these changes did not appear to result in severe damage to these organisms (Appelhans *et al.*, 2012). The results of the present study illustrate the conflicting results to date regarding the sensitivity of *Mytilus edulis* to future levels of temperature and pH (Landes and Zimmer, 2012). The slower growth observed under TR 2050 and TR 2100 (Fig. 3.5) may be due to reduced metabolic rates as the result of decreased oxygen consumption under exposure to high [CO_2] (Michaelidis *et al.*,

2005), or it could be an adaptive response to overcome and resist the stress imposed by surrounding conditions (Findlay *et al*., 2009b).

3.4.2 Effect of Experimental Conditions on L. littorea:

This study also revealed that *Littorina littorea* displays a high capacity for survival (Table 3.4, Fig 3.2), similar to some other marine snails, as mortality did not exceed 5% (Comeau *et al.*, 2009; Eklöf *et al.*, 2012). Likewise, no statistically significant differences were found for weight or length changes, or in calcification (Table 3.8 and 3.9) compared with the control treatment (TR 2011) (similar to Bibby *et al.*, 2007). Thus, these species of intertidal organisms are apparently able to adapt to and survive under predicted future changes in pH levels and temperatures. There were no significant differences in body or buoyant weights between treatments in any live snails (Fig. 3.10). However, there were conflicting results in terms of how the snail shells were affected by the increased temperature and acidification (Fig. 3.12). Although the length of the animal (shell length) decreased (Fig. 3.11), we found an increase in buoyant weight change under TR 2100 compared to TR 2011 conditions (Fig. 3.12). This could indicate concomitant building and dissolution of the shell. For example, (Melatunan *et al.*, 2013) showed that exposure to expected future levels of temperature and pH resulted in changes to the morphological characteristics of the shell. This could indicate loss of the shell on the one hand (dissolution) and rebuilding of the carbon structure on the other hand as reflected by increased buoyant weight, which supports the observations of Findlay *et al*. (2009b) who also observed an exchange between the building and the dissolution of shell. While acidification did not prevent *Littorina littorea* from undergoing increased calcification, there may be considerable dissolution of the shell. Increased calcification, especially under TR 2100 conditions (3.01 ±2.03%, Table 3.10), was greatest under the highest temperatures and levels of carbon dioxide (CO_2), which may indicate that these marine molluscs are still able to produce calcium carbonate ($CaCO_3$) under even unfavourable conditions. Such calcification under these conditions may be a reaction to the increased dissolution of the shells. In other words, it might be explained as a trade-off between calcification and dissolution (Findlay *et al*., 2009b).

3.4.3 Effect of Experimental Conditions on A. equina:

Sea anemones (*Actinia equina*) were able to survive when exposed to predicted future climate conditions (Table 3.4, Fig. 3.3) in these experiments. However, although there were no statistically significant differences in survival, these organisms may be able to adapt to the new conditions of higher temperature and lower pH. Body weight change increased over the six-week period under TR 2011 conditions (Fig. 3.10), but this increase was no longer apparent under TR 2050 conditions. Also, buoyant weight decreased in all treatments, reflecting decreased growth (Fig. 3.15). Survival was greatest under the TR 2050 conditions, but growth in terms of buoyant weight change and body weight change was lowest under that treatment. This could mean that the sea anemones are attempting to redistribute energy consumption to the maintenance and repair of body's cells, rather than to increased growth during adaptation and survival under the new environmental conditions. In any case, there were no statistically significant differences between any treatments (Table 3.11), except for body weight between TR 2011 and TR 2050 (p = 0.001). These results also support the findings of (Kroeker *et al.*, 2010) that there no statistically significant effects of ocean acidification on the growth of non-calcified non-invertebrate marine organisms (e.g. fish, multicellular algae, and seagrass). Furthermore, some Cnidarian species increase in abundance and size in more acidic environments (Suggett *et al.*, 2012). Although this Cnidarian species may tolerate higher temperatures and lower pH conditions in the oceans in the near future, exposure to low temperatures over the long term may negatively affect survival in *A. equina*.

3.4.4 Effect of Experimental Conditions on A. aspersa:

Calcified invertebrates differ in their responses to high temperatures and low pH levels. Similarly, considerable variation in response to these conditions has also been noted. Higher temperatures and increased acidity appear to have a significant negative impact on the ability of *Ascidiella aspersa* to survive (Table 3.4). Mortality rose linearly in proportion to increased temperature and [CO_2] levels Fig. 3.4), indicating a direct effect of both factors on the physiology of these invertebrates, to which they were not able to adapt and maintain viability. Under TR 2100 conditions, these sea squirts suffered from a significant increase in the number of deaths, reaching

a mortality rate of 82%. This result is contrary to the results of Dupont and Thorndyke (2009) who noted an increase in the survival rate upon lowering the pH by 0.4 units. Under TR 2050 the mortality rate reached 45%, combined with significantly decreased weight and length, which reflect lower metabolism and redirection of energy towards the maintenance and repair of cells and survival. A non-significant reduction in buoyant weight change (Fig. 3.19) was observed ($F = 2.446$, $p = 0.108$, Table 3.14).

The paucity of research on the response of filter-feeding, sessile invertebrates in the class Ascidiacea to near-future climate changes prevented any clear comparisons with similar or related species. Such sessile and filter-feeding organisms may experience strongly negative effects due to seawater conditions resulting from expected future climate changes. Responses to lower pH are direct as the animals are forced to take in water upon feeding, which could quickly lead to a pH imbalance inside the cells. Without an easy mechanism to rebalance pH, such as by shell dissolution, these organisms experience increased mortality.

3.5 Conclusion:

This study of four species of coastal marine animals showed that calcified organisms were able to cope with the rising temperatures and lower pH conditions in the sea expected in the near future slightly better if they are motile calcified organisms (*L. littorea*) rather than sedentary calcified organisms (*M. edulis*). These climate changes also appeared to be less adverse for the non-calcified sedentary invertebrates (*A. equina*) that succeeded in adapting to and surviving these conditions. In contrast, negative effects of acidification and temperature increases were observed for other non-calcified sessile invertebrates (*A. aspersa*), as reflected clearly in their substantially increased mortality.

Chapter 4. Effects of elevated temperature and decreased pH on the Body Composition of four Common British Marine Invertebrates in winter

Abstract:

It is predicted that increasing temperatures will continue in the North Sea +4°C during this century coinciding with a decrease in pH level to -0.4 units. This is on account of continuing emissions of CO_2 in the atmosphere resulting from the continued burning of fossil fuels because of anthropogenic emissions. Negative effects are also expected as regards biological activity in coastal marine invertebrates. Most research has a bias towards calcified marine invertebrates. Therefore, this study investigated the effects expected in the middle and end of this century on calcified marine invertebrates and non-calcified marine invertebrate. Four marine invertebrates (*Mytilus edulis, Littorina littorea, Actinia equina* and *Ascidiella aspersa*) were exposed to levels expected from the high temperatures and low pH in the future for a period of six weeks following two weeks of acclimatizion. At the end of the study period, the water content, somatic weight, shell weight (of calcified marine invertebrates), fat content, carbon/nitrogen ratio (C:N ratios) and the metabolic rates of organisms were measured. It was observed that the calcified and non-calcified invertebrates recorded no statistical significance in most measurements, however the study found significant positive results in the metabolic rate of *L. littorea* (mobile) at levels expected in 2100. Positive statistical significance numbers were also observed in the same species' C:N ratios, which were at levels expected in 2050. In addition, *L. littorea* recorded the best ratio of survival, while non-calcified invertebrates did not improve their ability of survival. These results indicate that each species enjoys particular personal way with a potential to adapt or not adapt to climatic conditions which are expected in the near future. This highlights the need for further study of the expected effects of these climatic changes on non-calcified invertebrates.

4.1 Introduction:

The results of the previous chapter (Chapter 3) showed variations in the growth and survival of the species under study to the different treatments. This chapter attempts to contribute to the clarification of the relationship between the effects of the interaction of the common factors of high temperature and low pH as a result of increased CO_2 in the seawater. Investigating metabolic rates may offer an explanation about the costs of maintaining or increasing calcified carbon structures. It may also be that the cost of growth or maintenance of the cells increases the chances of survival in the species under study. Adults feed on a minimum amount and do not use a lot of energy to grow in the winter (Findlay *et al*., 2009a). According to Melzner *et al.* (2011) winter is an important factor as regards reducing the rate of filtering seawater 34% less than that of the summer in *M. edulis*. The report by Sanford (2002), lent support by stating that colder temperatures lead to reduced food consumption, and substantially affect the feeding rates, however the growth change was balanced by the lower costs of metabolism. Growth may be a result of the adoption of the food reserve (fat stores) (Findlay *et al*., 2009a), thus intertidal animals under winter temperatures usually continue to feed, grow and be active (Sokolova and Portner, 2003). The temperature of the bodies of marine ectotherms fluctuate dependant on the temperature of the surrounding environment (North Sea surface water temperatures range from 3 to 6 in winter), due to critical metabolic adjustments in response to changes in temperature (Sokolova and Portner, 2003). It has been noted that, in winter, many seawater molluscs experience a reduced level of activity and feeding due to depression of metabolic rates (Sokolova and Portner, 2003). Temperature control for determining the time of spawning is important to reproduction and growth of species, which need more energy and an abundance of food to increase the ability of metabolic rate. For example: mussels begin spawning in the spring until the end of the summer due to increased temperatures and the availability of food (Nagarajan *et al.*, 2006). Gamete production usually commences in late autumn and early winter and this process will continue throughout the winter months (Gray *et al*., 1997). Increasing temperatures and the availability of food cause the spawning to begin. Temperatures may increase in winter to rates close to those in early spring, this may lead to a phenological shift and an increase in metabolic rates. It is clear that staying healthy during the winter is important for completion of the life cycle of organisms

and continuation of reproduction. Experiments carried out by Rodolfo-Metalpa *et al.* (2009) demonstrated that an increase in temperature to 3 ºC in the winter leads to stimulation of the coral metabolism. This study will also offer a contribution as regards clarifying the physiological effects on sessile organisms which may arise due to the high levels of CO_2 and temperature in the winter, as little is known about the response of this species of coastal marine invertebrates in future changes to increased temperature and decreased pH level (Findlay *et al*., 2009a).

4.2 Methods:

The methods in this chapter are similar to those previously mentioned in chapter 3, however the measurements in this chapter concerned the water content, somatic weight (including gonads), lipid content, C:N ratio and metabolic rate. Table 4.1 explains the numbers of samples used in the measurements of the two experiments together for each species (see Chapter 2 and 3 for additional information).

Table 4.1 Numbers of samples applicable for the measurements of the experiments in winter for species under study.

Parameter	Treatments											
	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
Water content	6	5	6	6	6	5	5	5	6	6	6	2
Somatic weight	6	5	6	6	6	5	5	5	6	6	6	2
Shell weight	6	5	---	---	6	5	---	---	6	6	---	---
Lipid content	6	5	6	6	6	5	5	5	6	6	6	2
C:N ratio	9	6	9	9	9	5	8	9	9	4	6	7
Metabolic rate	10	12	12	12	12	12	12	12	12	12	11	8

Where *M.e.* refers to *M. edulis*, *L.l.* refers to *L. littorea*, *A.e.* refers to *A. equina* and *A.a.* refers to *A. aspersa*.

4.3 Results:

4.3.1 Body composition analysis and metabolic rates:

M. edulis:

A Shapiro-Wilk tests showed normal distribution of C:N ratio and lipid content but the homogeneity test were not equal. The non-parametric test Kruskal-Wallis test was used to compare treatment means (Table 4.2). Content of water, soft tissue weight, shell weight, and metabolic rate were normally distributed and treatments were compared with one-way ANOVA (Table 4.3). Scheffe's method was applied *post hoc* to test the significance between the treatments.

Table 4.2 Statistical analysis of C:N ratio and lipid content of *M. edulis*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-Square	df	*p* value
Lipid content[a]	0.969	18	0.777	1.059	2	0.589
C:N ratio [b]	0.959	27	0.354	4.328	2	0.115

[a] Lipid content tested by Kruskal-Wallis test because the test for homogeneity of variances was significant ($p = 0.038$).

[b] C:N ratio tested by Kruskal-Wallis test because the test for homogeneity of variances was significant ($p = 0.004$).

Table 4.3 Statistical analysis of content of water, soft tissue weight, shell weight and metabolic rate of *M. edulis.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Content of water	18	0.027	0.973	0.955	18	0.505	1.867	0.189
Soft tissue weight	18	0.154	0.858	0.899	18	0.054	0.090	0.915
Shell weight	18	0.154	0.858	0.899	18	0.054	0.090	0.915
Metabolic rate	34	6.489	0.004	0.958	34	0.209	0.941	0.401

There were no significant differences in the content of water, soft tissue weight, shell weight, lipid content and C:N ratio of *M. edulis* at the end of the experiments (see Table 4.2 and Table 4.3). However, when the Mann-Whitney U test was used there was a significant difference in the metabolic rate between TR 2011 vs TR 2100 ($p = 0.011$) and between TR 2050 vs TR 2100 ($p = 0.024$).

Table 4.4 Mean percentage (%) ± standard error (SE) of *M. edulis* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	50.22	± 3.47	51.23	± 3.05	49.98	± 4.96
Soft tissue weight	7.00	± 1.92	6.46	± 1.70	5.61	± 1.78
Shell weight	93.00	± 1.92	93.54	± 1.70	94.39	± 1.78
Lipid content	14.03	± 3.21	12.71	± 1.61	11.45	± 1.45
C:N ratio	4.21	± 0.07	3.88	± 0.15	4.03	± 0.05
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.38	± 0.04	0.47	± 0.08	0.39	± 0.08

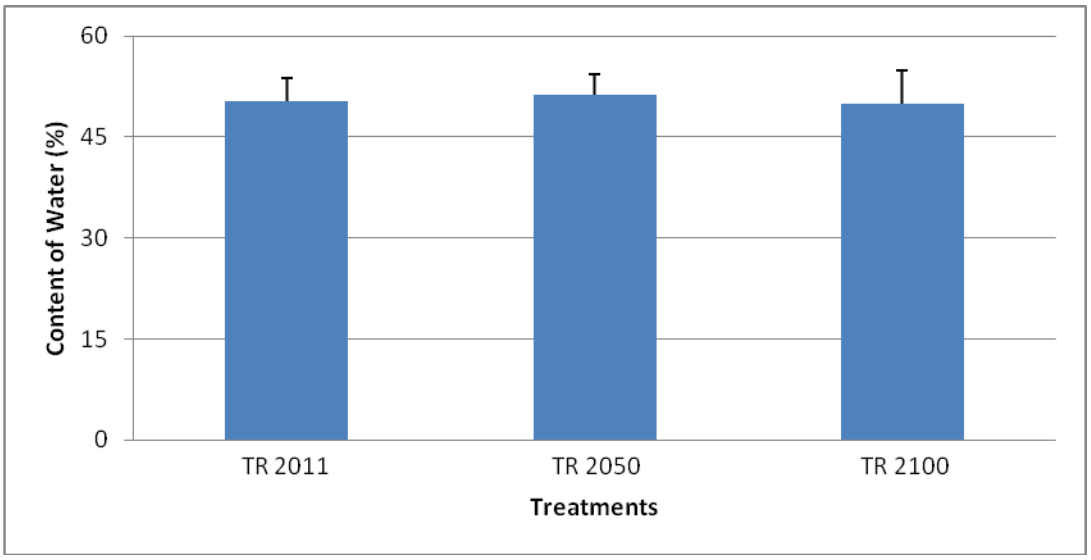

Figure 4.1 Effect of increased temperature and decreased pH level on the mean percentage water content (±SE) in *M. edulis* at the end of the experiment.

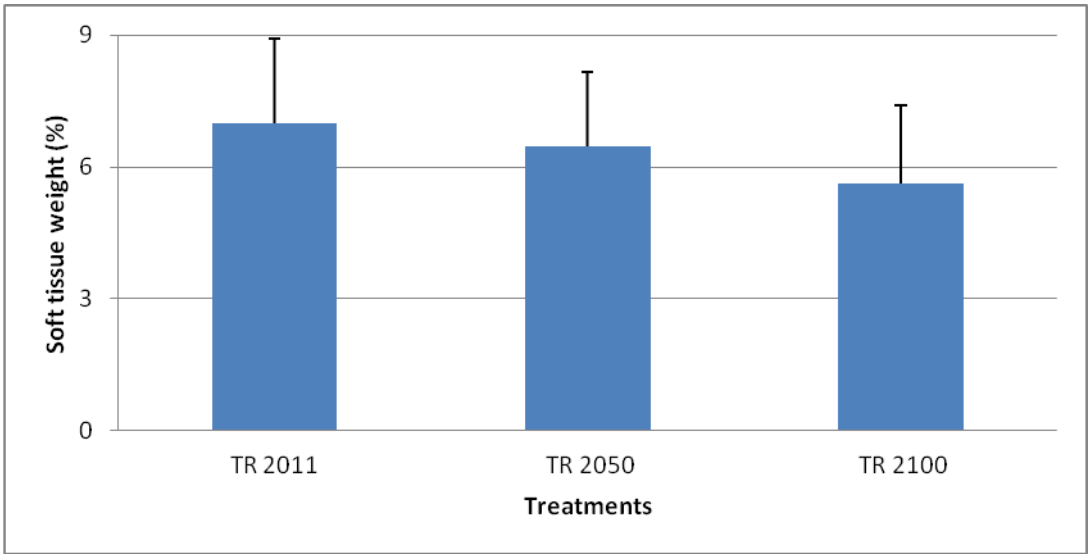

Figure 4.2 Effect of increased temperature and decreased pH level on the mean percentage soft tissue weight (±SE) in *M. edulis* at the end of the experiment.

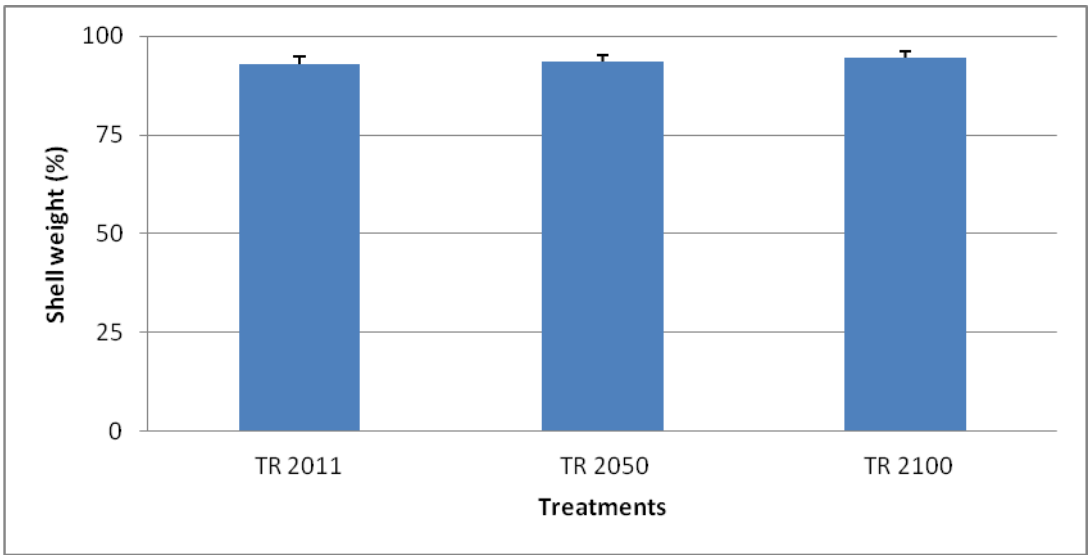

Figure 4.3 Effect of increased temperature and decreased pH level on the mean percentage shell weight (±SE) in *M. edulis* at the end of the experiment.

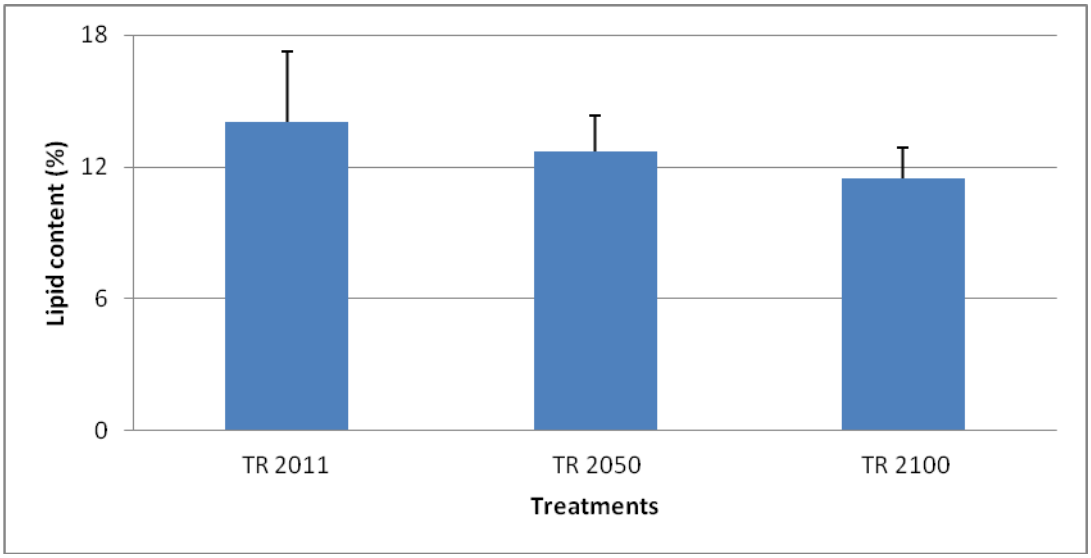

Figure 4.4 Effect of increased temperature and decreased pH level on the mean percentage lipid content (±SE) in *M. edulis* at the end of the experiment.

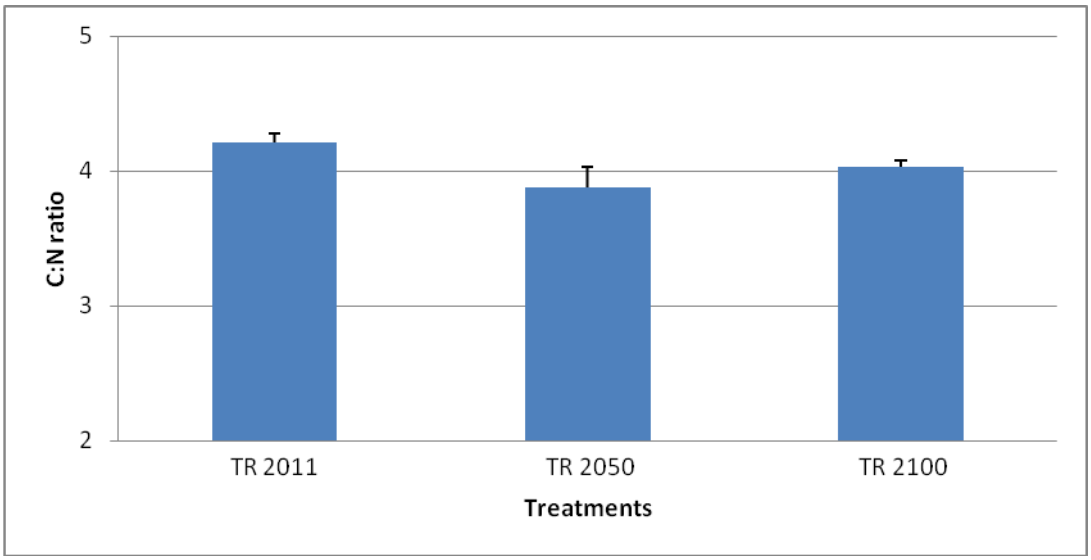

Figure 4.5 Effect of increased temperature and decreased pH level on the mean percentage C:N ratio (±SE) in *M. edulis* at the end of the experiment.

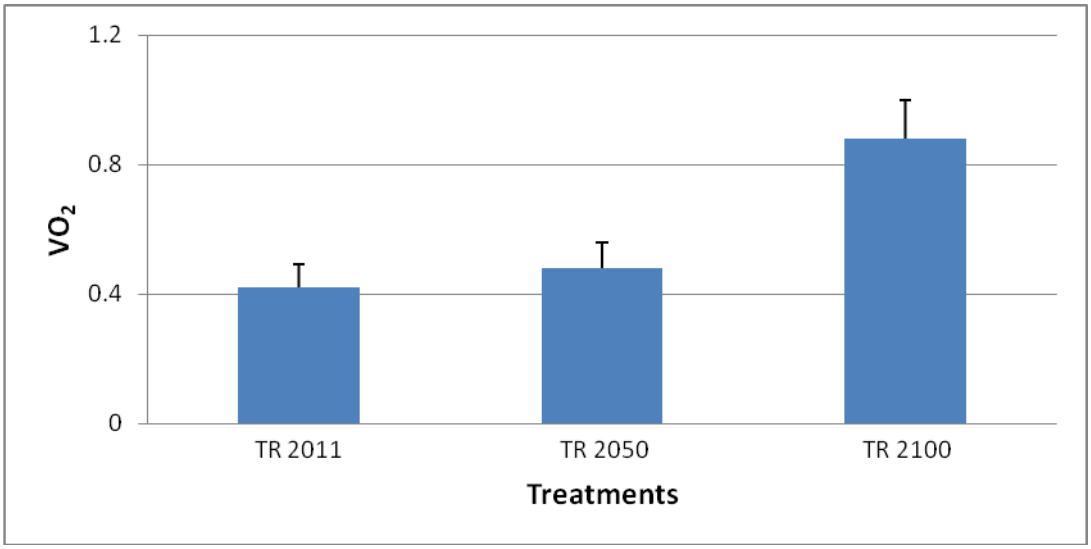

Figure 4.6 Effect of increased temperature and decreased pH level on the mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *M. edulis* at the end of the experiment.

L. littorea:

A Shapiro-Wilk test showed normal distribution of content of water, soft tissue weight, shell weight, lipid content, C:N ratio and metabolic rate, and treatments when compared with one-way ANOVA (Table 4.5). Scheffe's method was used *post hoc* to test significance between the treatments.

Table 4.5 Statistical analysis of content of water, soft tissue weight, shell weight, lipid content, C:N ratio and metabolic rate of *L. littorea.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Content of water	16	0.545	0.593	0.918	16	0.158	0.113	0.894
Soft tissue weight	16	1.102	0.361	0.991	16	1.00	2.748	0.101
Shell weight	16	1.102	0.361	0.991	16	1.00	2.748	0.101
Lipid content	16	0.773	0.482	0.905	16	0.098	0.157	0.856
C:N ratio	15	7.617	0.007	0.932	15	0.296	1.706	0.223
Metabolic rate	36	6.297	0.005	0.980	36	0.748	1.004	0.377

There were no significant differences in the content of water, soft tissue weight, shell weight and lipid content of *L. littorea* at the end of the experiments (see Table 4.5). However, there were significant differences in the C:N ratio between TR 2011 vs TR 2050 (p = 0.007) and the metabolic rate between TR 2011 vs TR 2100 (p = 0.006) when using Scheffe's method.

Table 4.6 Mean ± standard error (SE) of *L. littorea* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	19.32	± 1.60	21.49	± 1.71	19.86	± 1.25
Soft tissue weight	6.26	± 1.24	5.05	± 0.29	4.41	± 0.77
Shell weight	93.74	± 1.24	94.95	± 0.29	95.59	± 0.77
Lipid content	11.38	± 1.91	11.83	± 1.98	9.24	± 0.98
C:N ratio	4.01	± 0.07	4.67	± 0.19	4.33	± 0.08
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.43	± 0.05	0.56	± 0.04	0.63	± 0.04

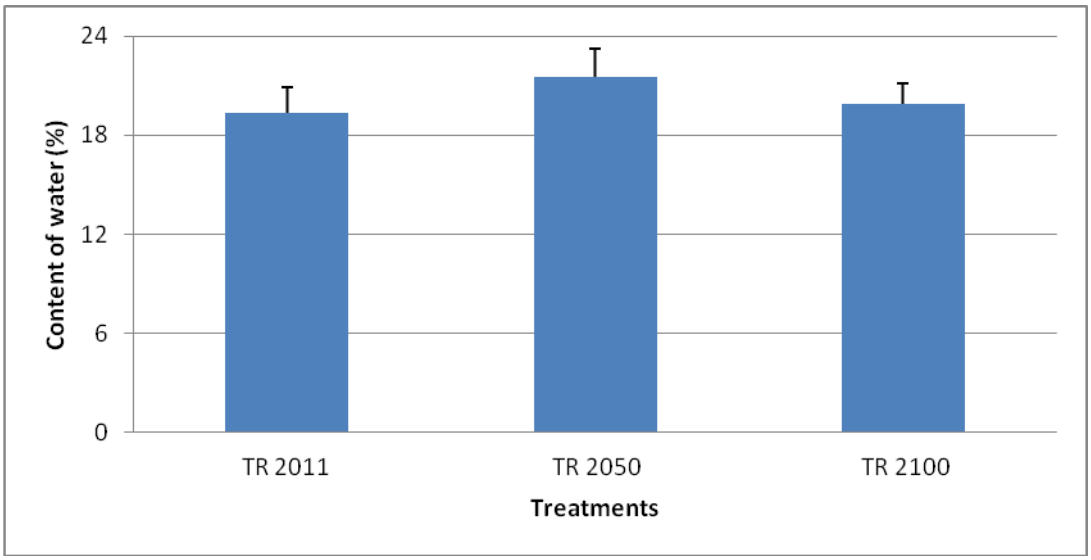

Figure 4.7 Effect of increased temperature and decreased pH level on the mean percentage content of water (±SE) in *L. littorea* at the end of the experiment.

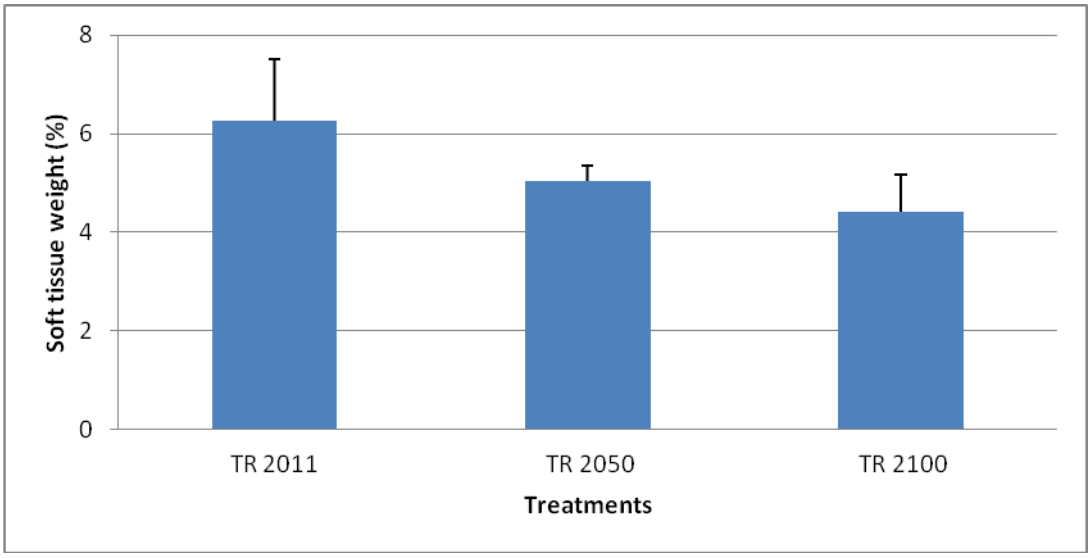

Figure 4.8 Effect of increased temperature and decreased pH level on the mean percentage soft tissue weight (±SE) in *L. littorea* at the end of the experiment.

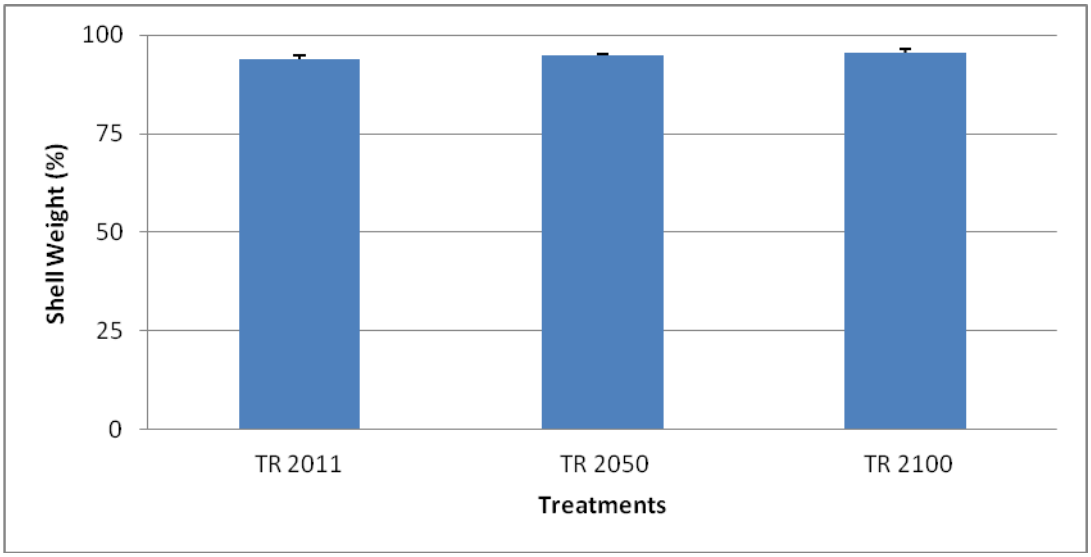

Figure 4.9 Effect of increased temperature and decreased pH level on the mean percentage shell weight (±SE) in *L. littorea* at the end of the experiment.

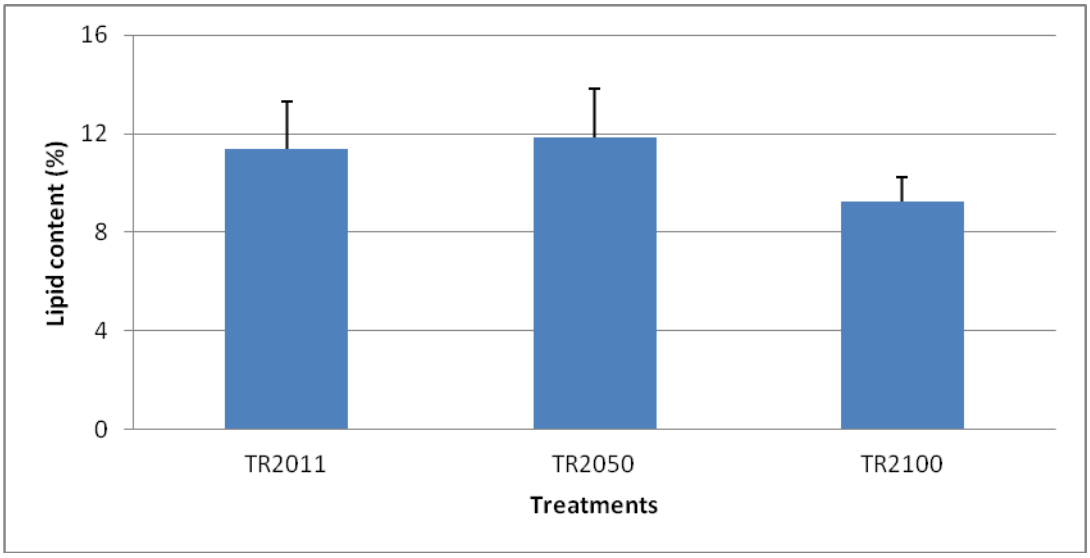

Figure 4.10 Effect of increased temperature and decreased pH level on the mean percentage lipid content (±SE) in *L. littorea* at the end of the experiment.

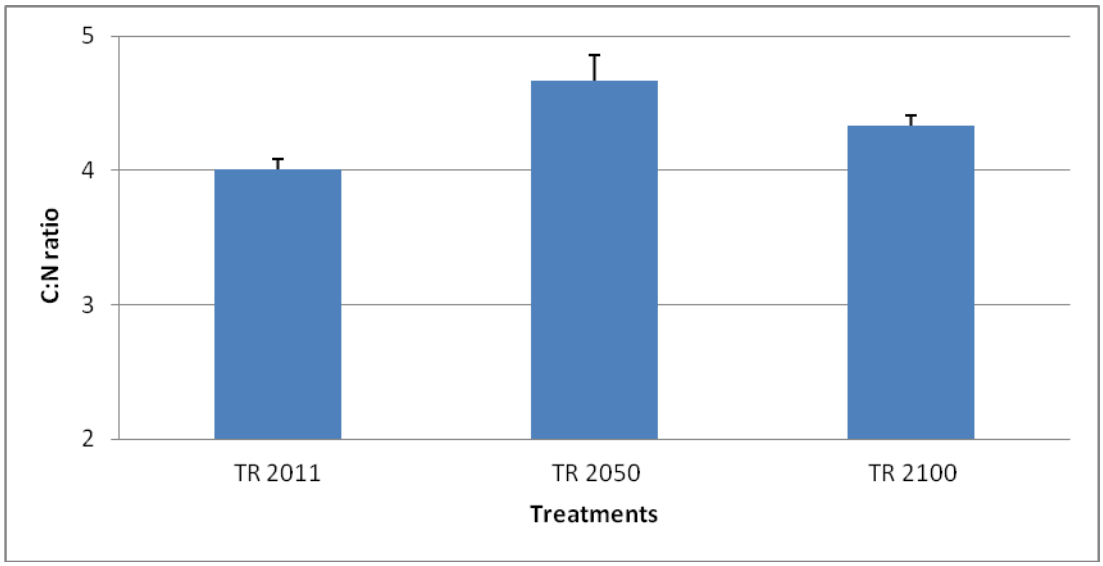

Figure 4.11 Effect of increased temperature and decreased pH level on the mean percentage C:N ratio (±SE) in *L. littorea* at the end of the experiment.

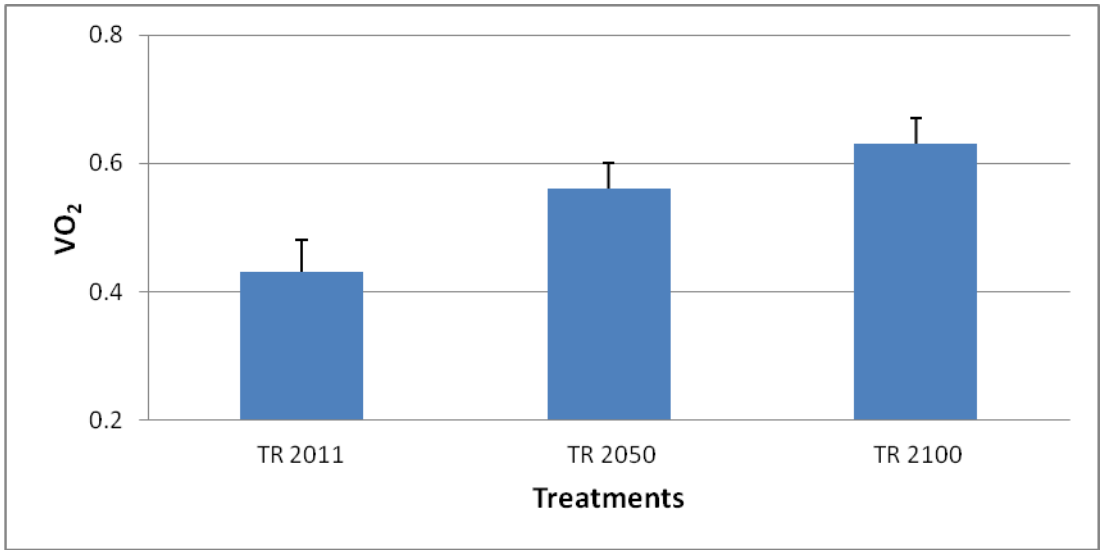

Figure 4.12 Effect of increased temperature and decreased pH level on the mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *L. littorea* at the end of the experiment.

A. equina:

A Shapiro-Wilk test showed a non-normal distribution of content of water and soft tissue weight. A non-parametric test Kruskal-Wallis test was used to compare treatment means (Table 4.7). The C:N ratio and metabolic rate were normally distributed and treatments were compared with one-way ANOVA (Table 4.8).

Table 4.7 Statistical analysis of the content of water, soft tissue weight and lipid content of *A. equina.*

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-Square	df	*p* value
Content of water	0.866	17	0.019	0.295	2	0.863
Soft tissue weight	0.866	17	0.019	0.295	2	0.863
Lipid content[a]	0.945	17	0.383	0.413	2	0.813

[a] Lipid content tested by Kruskal-Wallis test because test of homogeneity of variances was significant ($p = 0.024$).

Table 4.8 Statistical analysis of C:N ratio and metabolic rate of *A. equina.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
C:N ratio	23	0.199	0.821	0.953	23	0.339	0.050	0.952
Metabolic rate	35	2.102	0.139	0.955	35	0.161	0.313	0.733

There were no significant differences in all measurements of *A. equina* at the end of the experiments (see Table 4.7 and Table 4.8).

Table 4.9 Mean percentage (%) ± standard error (SE) of *A. equina* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	80.27	± 2.32	79.55	± 0.51	79.82	± 1.38
Soft tissue weight	19.73	± 2.32	20.45	± 0.51	20.18	± 1.38
Lipid content	12.91	± 1.26	14.44	± 1.65	11.45	± 2.19
C:N ratio	4.43	± 0.06	4.43	± 0.05	4.48	± 0.06
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.33	± 0.09	0.51	± 0.25	0.72	± 0.20

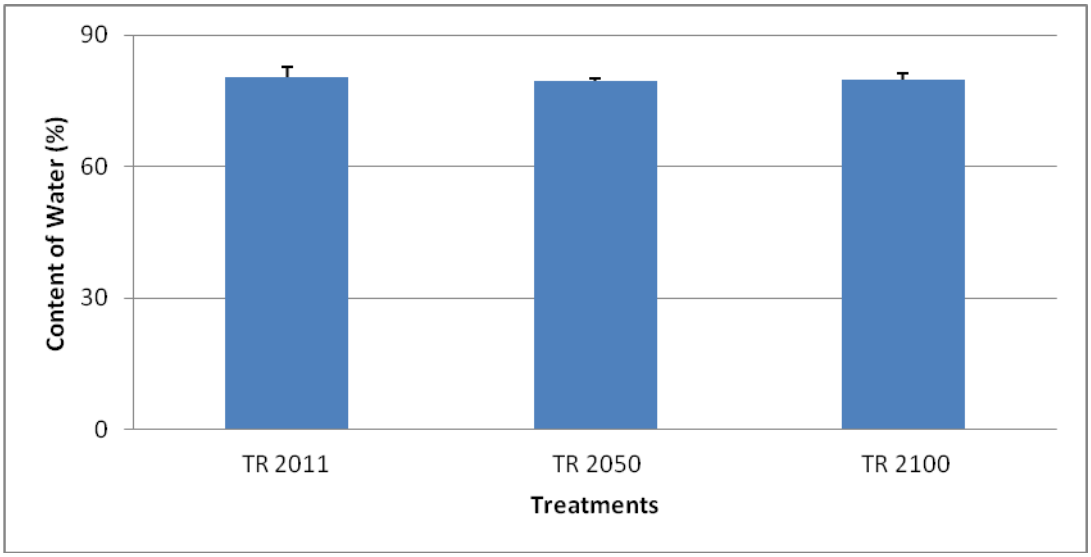

Figure 4.13 Effect of increased temperature and decreased pH level on the mean percentage content of water (±SE) in *A. equina* at the end of the experiment.

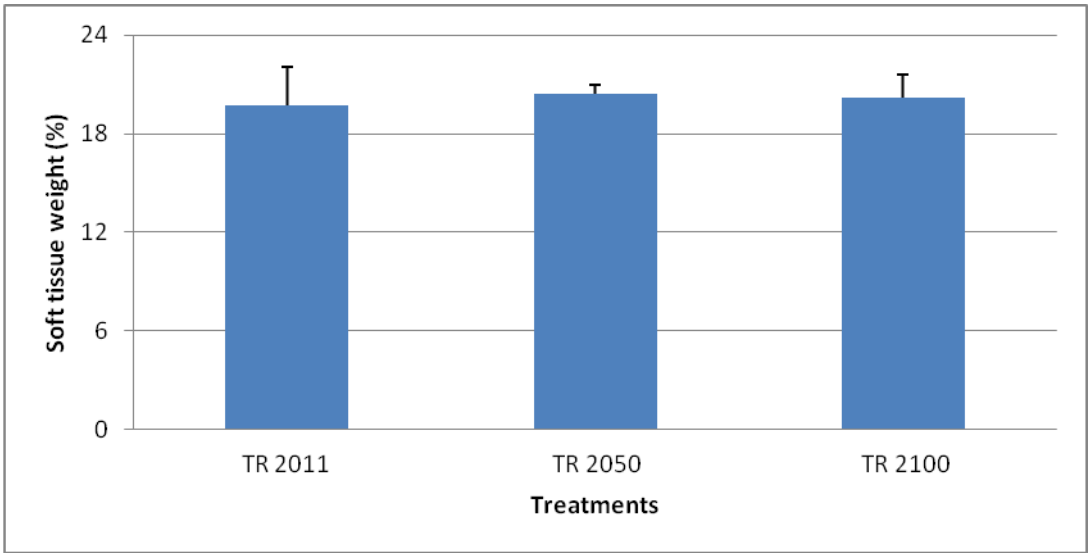

Figure 4.14 Effect of increased temperature and decreased pH level on the mean percentage soft tissue weight (±SE) in *A. equina* at the end of the experiment.

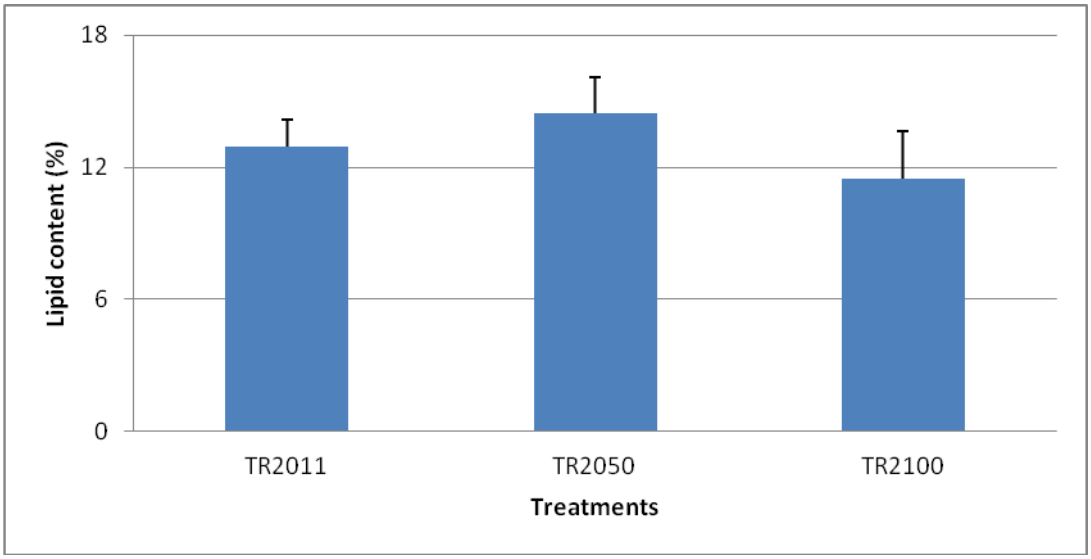

Figure 4.15 Effect of increased temperature and decreased pH level on the mean percentage lipid content (±SE) in *A. equina* at the end of the experiment.

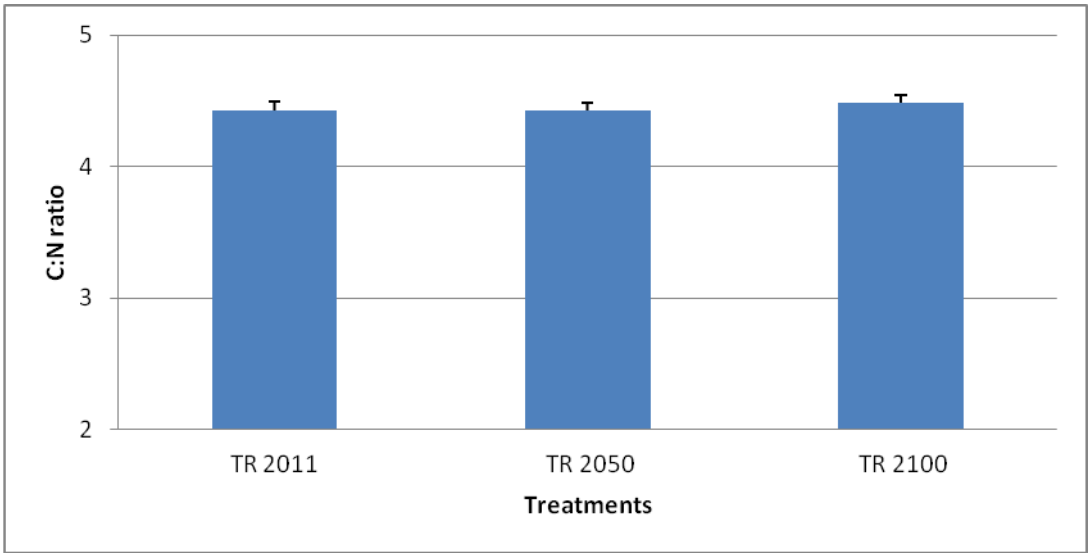

Figure 4.16 Effect of increased temperature and decreased pH level on the mean percentage C:N ratio (±SE) in *A. equina* at the end of the experiment.

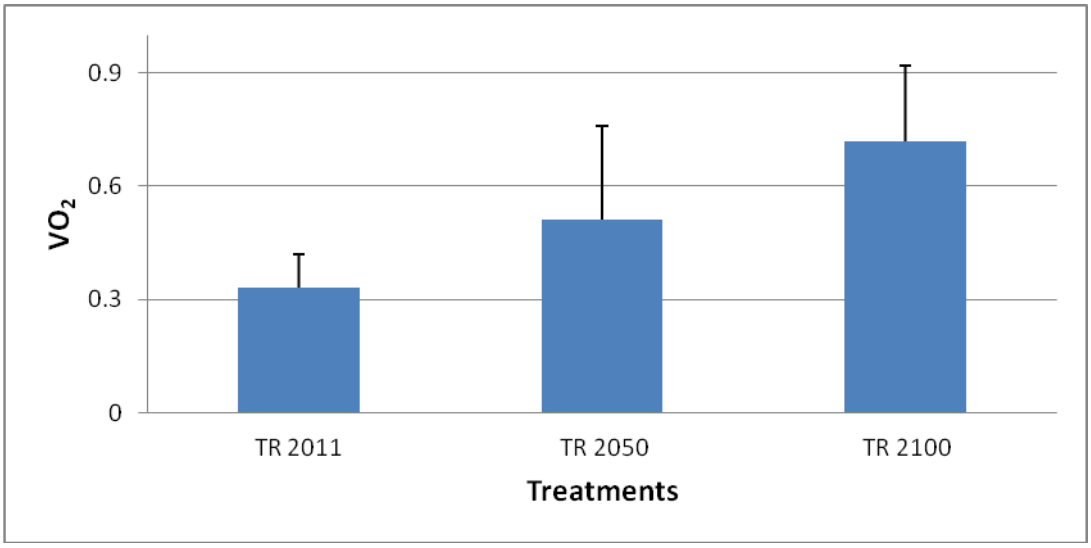

Figure 4.17 Effect of increased temperature and decreased pH level on the mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *A. equina* at the end of the experiment.

A. aspersa:

Shapiro-Wilk tests showed t normality distributed of content of water, soft tissue weight, lipid content, C:N ratio and metabolic rate. Treatments were compared with one-way ANOVA (Table 4.10).

Table 4.10 Statistical analysis of content of water, soft tissue weight, lipid content, C:N ratio and metabolic rate of *A. aspersa.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Content of water	13	0.457	0.646	0.881	13	0.074	2.906	0.101
Soft tissue weight	13	0.457	0.646	0.881	13	0.074	2.906	0.101
Lipid content	13	1.054	0.384	0.961	13	0.770	3.630	0.065
C:N ratio	25	0.174	0.841	0.948	25	0.228	2.404	0.114
Metabolic rate	32	0.909	0.414	0.974	32	0.609	1.601	0.219

There were no significant differences in all measurements of *A. aspersa* at the end of the experiments (see Table 4.10).

Table 4.11 Mean percentage (%) ± standard error (SE) of *A. aspersa* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	93.37	± 0.92	94.39	± 0.65	94.44	± 0.02
Soft tissue weight	6.63	± 0.92	5.61	± 0.65	5.56	± 0.02
Lipid content	6.47	± 0.72	6.59	± 0.50	8.86	± 2.85
C:N ratio	5.13	± 0.31	5.18	± 0.19	5.30	± 0.10
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.26	± 0.03	0.24	± 0.02	0.30	± 0.06

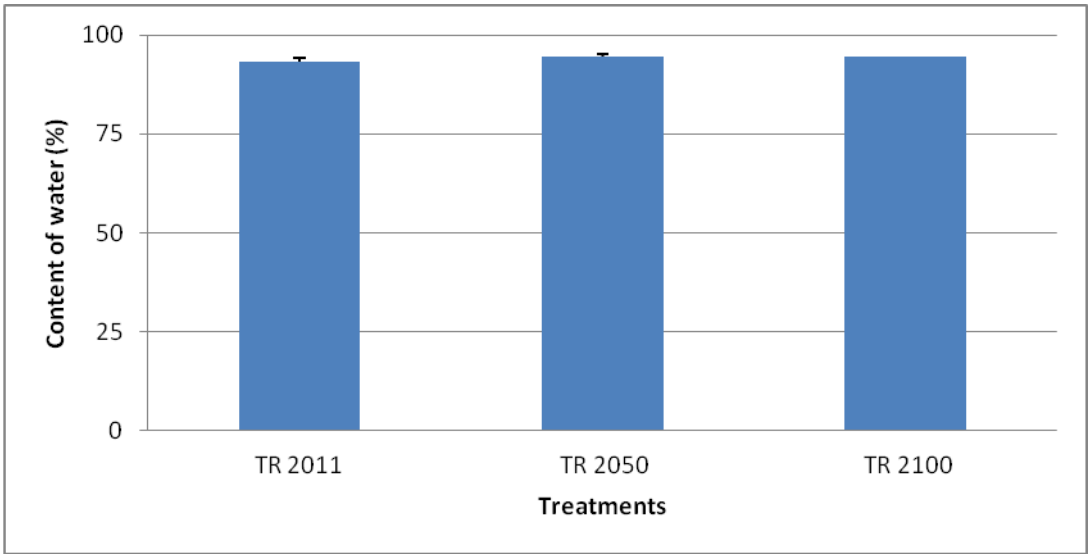

Figure 4.18 Effect of increased temperature and decreased pH level on the mean percentage content of water (±SE) in *A. aspersa* at the end of the experiment.

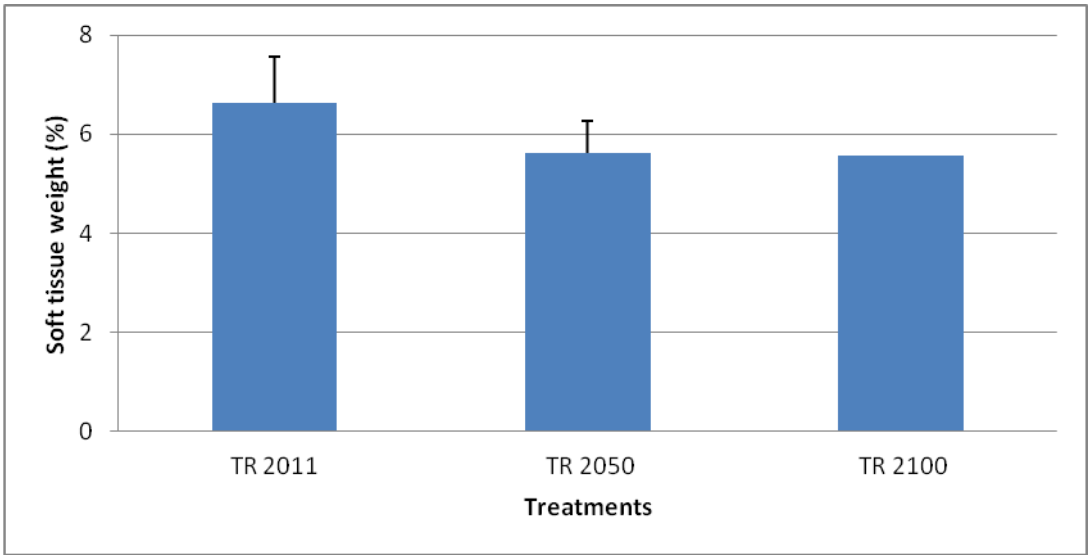

Figure 4.19 Effect of increased temperature and decreased pH level on the mean percentage soft tissue weight (±SE) in *A. aspersa* at the end of the experiment.

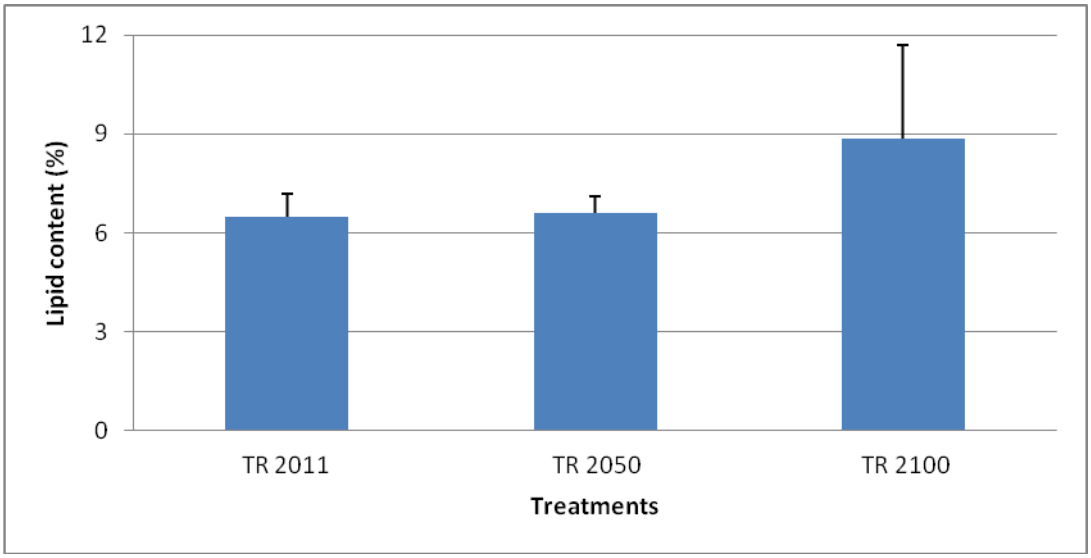

Figure 4.20 Effect of increased temperature and decreased pH level on the mean percentage lipid content (±SE) in *A. aspersa* at the end of the experiment.

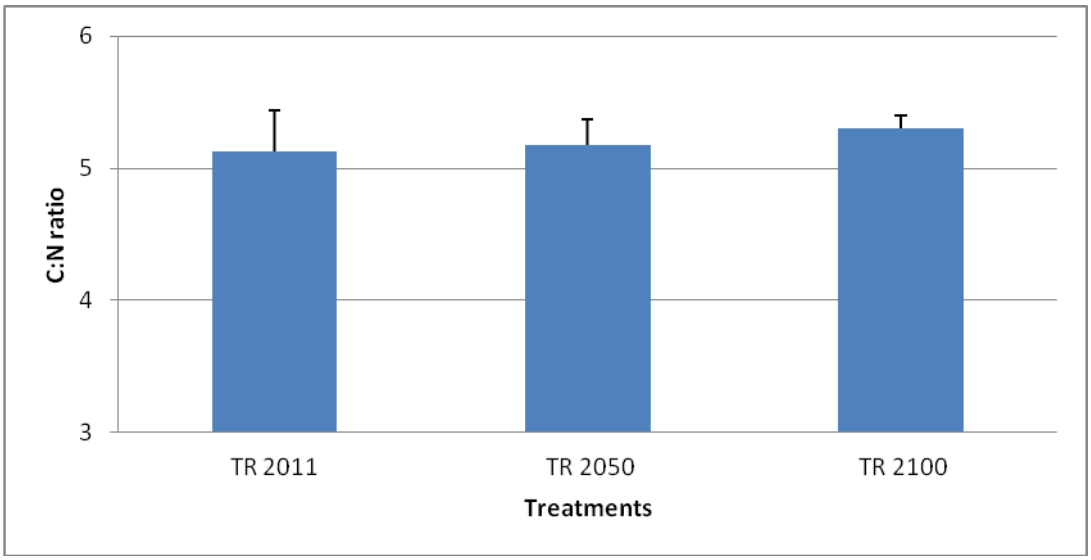

Figure 4.21 Effect of increased temperature and decreased pH level on the mean percentage C:N ratio (±SE) in *A. aspersa* at the end of the experiment.

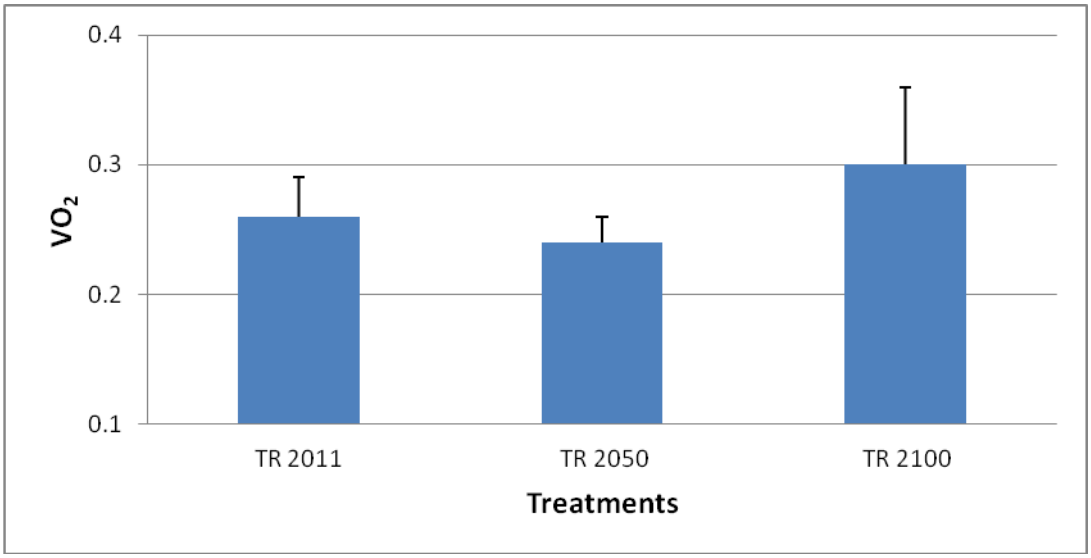

Figure 4.22 Effect of increased temperature and decreased pH level on the mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *A. aspersa* at the end of the experiment.

4.4 Discussion:

4.4.1 Effects on M. edulis:

The measurements did not show any statistically significant change between the various treatments of *M. edulis*, apart from significant differences in the metabolic rate ($F = 6.489$, $p = 0.004$, Table 4.3, Fig. 4.6). There were significant increases in the rate of oxygen consumption in the medium treatment (TR 2050) compared to the two other treatments TR 2011 ($p = 0.011$) and TR 2100 ($p = 0.024$). In Chapter 3 it was shown that the medium treatment (TR 2050) recorded the lowest body weight change and buoyant weight change, together with the highest mortality rate 16%, compared with 0% for the control treatment and 9% for TR 2100. It is clear that mussels made a major effort to use the energy resulting from metabolic processes for restoration and maintenance. They also used reduced rates of growth and calcification as a physiological reaction to mitigate the negative effects which could arise due to increased temperatures and high levels of ocean acidification. These results confirm the findings of Melzner *et al.* (2011) that there are no significant differences in soft tissue weight and shell weight. This study also concurred with Thomsen and Melzner

(2010) and Thomsen *et al.* (2010) that there is no significant difference in soft tissue weight of *M. edulis*.

4.4.2 Effects on L. littorea:

The results for *L. littorea* showed an increase in the metabolic rate which was directly proportional to increases in temperature and decreased pH levels (Fig. 4.12). This increase was a significant rise in TR 2100 ($p = 0.006$) compared with that of the control. When these results were compared, as noted in Chapter 3, it is clear that this rise in metabolism also led to an increase in calcification in TR 2100. This coincides with a reduction in soft tissue weight and lipid content in TR 2100 (Fig. 4.8 and 4.10). This decrease may be explained as a successful attempt by *L. littorea* to resist the changing environmental conditions from the increase in temperature and levels of acidification of seawater. A slight reduction of growth was accompanied by an increase in calcification but with a high cost to compensate for the decay of the shell (Findlay *et al*., 2009b). This adaptation resulted in the maintenance of low mortality rates that did not exceed 2%; the first death was recorded in the fifth week of the experiment. These results show that *L. littorea* has a high potential to adapt to new circumstances and to make changes in the mechanism of energy expenditure appropriate to the physiological changes required to maintain the vitality of the cells. On the other hand, a decrease in soft tissue weight and lipid content were observed (Table 4.6), although they were not significant (Table 4.5). Nevertheless, the increased use of lipid content for vital maintenance could lead to a decrease in the vital protein that can promote death (Barnes *et al.*, 1963; Findlay *et al.*, 2009a) in the long term.

The findings were similar to those of Eklof *et al.* (2012) that there was no significant difference in the percentage of water content for every treatment. The results of this study contradict the results obtained by (Melatunan *et al.*, 2011), possibly because they conducted their experiments at temperatures higher than those used in this study. The study's findings contrasted with the conclusions made by Melatunan *et al.* (2013) that exposure to the future dual factors of heat and acidity may disrupt metabolism, which could be on account of exposing the animals to temperatures up to 20° C. This may confirm that reactions to acidification and rising temperatures are different and complex, not only because of the different species of

organisms and rates and durations of exposure, but also due to reactions which vary by seasons of the year or even by months. It can be concluded, that the marine snail *L. Littorea* managed to cope with the surrounding environmental conditions in various ways, either by increasing calcification or by reducing growth and directing more energy to vital maintenance operations. This offers evidence of its ability to adapt to the temperature and acidification levels expected during this century, without which there would be a serious threat of extinction.

4.4.3 Effects on A. equina:

*A. equina'*s results showed no significant differences between the measurements of the different treatments (Tables 4.7 and 4.8). These results support the Chapter 3 discussion, in which it was stated that there were no significant differences among treatments. Metabolic levels did not show significant differences but rose proportionally in accordance with the increases in temperature and decreased pH levels (Fig. 4.17). Sea anemones did not reduce their metabolism as an adaptation strategy but in future treatments their metabolic rates increased, which led to a very slight increase in soft tissue weight compared with the control treatment (Fig. 4.14). In view of the results obtained in the previous chapter, it was found that growth was significantly lower in the medium treatment (TR 2050). This indicates that the sea anemone reduced growth changes whilst increasing metabolic rates as a reaction to the increased levels of acidification and temperature. This resulted in a reduction in mortality rates for future treatments compared with the control treatment. The study found that TR 2100 produced an increased metabolism and lower growth changes, resulting in a survival rate of 71% and adaptation to the surrounding circumstances. No death cases were recorded after the fourth week. The same process and results occurred in TR 2050, however, in TR 2050, the metabolic rate was slightly lower, and the growth change was clearly reduced compared with TR 2100. In spite of these changes, TR 2050 had the highest survival rate, at 74%, and did not record any cases of mortality in the sixth week. An increase in somatic weight due to rising temperature and CO_2 levels was recorded by Widdicombe and Needham (2007). All of these results may be interpreted as responses which may be slightly positive to the extent that it eventually led to reduced mortality rates in

future treatments compared with the control, with taking in account that the minor differences were not statistically significant.

4.4.4 Effects on A. aspersa:

In *A. aspersa* the soft tissue weight decreased in TR 2050 and TR 2100 compared with TR 2011 (Table 4.11, Fig. 4.19). This decline was accompanied by an increase in the content of lipid (Fig. 4.20), especially in treatment expected at the end of this century (8.86 ± 2.85%, Table 4.11). The same changes occurred with the rate of oxygen consumption but were not statistically significant ($F = 0.909$, $p = 0.414$, Table 4.10). This rise in metabolism could not build and repair cells and soft tissues, so it had a negative impact on the significant increase in the proportion of survival which did not exceed 18% (Chapter 3). On the other hand, there was a slight decrease in metabolism in TR 2050 (0.24 ± 0.02, Table 4.11) which coincided with a small decline in soft tissue weight (5.61 ± 0.65%). In the third chapter, the presence of significant fall in body weight change and a clear reduction in buoyant weight change compared to the control was noted. In spite of these changes, the survival rate was 65% (Chapter 3). This result indicates that TR 2050 used the strategy of reducing metabolism and growth and directing more energy to be spent on the restoration and maintenance of cells in order to lower the mortality rate as much as possible. This strategy may have succeeded when compared to the mortality rate in TR 2100. This strategy is known to be adaptive and may be useful in the short term, but long-term use could have negative effects on living organisms (Widdicombe and Needham, 2007). This major damage found in the future treatments conflicted with the expectations of some studies (e.g., Connell and Russell, 2010) that rising temperature and acidity levels expected in the future would have a positive effect on non-calcified marine invertebrates, and on *A. aspersa* in particular (Dupont and Thorndyke, 2009).

4.5 Conclusion:

A study of four species of coastal marine animals showed that body composition analyses which measured calcified invertebrates reactions to different climate conditions did not significantly disrupt metabolism rates or reduce soft tissue

weight. However, the results discussed in the previous chapter make clear the difficulty and complexity of interpreting the various reactions of different organisms to climate change. The previous results also show that, responses to different biological measurements varied within a single species of marine invertebrates. Calcified organisms (*L. littorea* and *M. edulis*) could cope better with the expected near-future climatic conditions of rising temperatures and lower pH levels in the ocean. It was also noted that these climate changes did not prevent (*A. equina*) from successfully adapting and biological maintaining its survival. By contrast, very bad effects arose on non-calcified (*A. aspersa*) from acidification and the expected rise in temperature in the mid and late 21st century (2050 and 2100).

Chapter 5. The Effects of Decreased pH level and increased Temperature on Four Common British Marine Invertebrates in summer

Abstract:

Increases in global temperatures and ocean acidification are predicted to have a significant impact on coastal marine invertebrate organisms. In order to understand the effects that these environmental changes will have on marine organisms, four species of marine invertebrates (two calcified and two non-calcified) were exposed to the climatic conditions expected to prevail in 2050 and 2100. Animals under study were gradually adapted over two weeks to the levels of acidity and temperatures predicted for 2050 and 2100, and were then held under those conditions for a further six weeks. During the study period, the mortality rate was monitored as well as the body weight, body morphometrics, buoyant weight, and dry weight. The calcified invertebrates were observed to be able to survive and grow, albeit at a lower rate than under control conditions. Increased survival but slower growth was observed in the sea anemone *Actinia equina* (non-calcified invertebrate), suggesting that future warmer, more acidic conditions may be favourable to this species. Also, there was no significant increase in mortality at the higher temperatures and lower pH in the case of the sea squirt *Ascidiella aspersa* (a non-calcified, sessile invertebrate). These results indicate that each species is likely to respond to environmental changes in different ways. Calcified animals, such as *Mytilus edulis* and *Littorina littorea*, may be able to mitigate the effects that decreased pH may have on their shells through an increase in ion regulation, which might be facilitated by increased temperature through elevation of the metabolic rate (see Chapter 6). Such an enhanced metabolic rate could probably only be maintained through an increase in food consumption, this may not be sustainable in the long term.

5.1 Introduction:

Under warmer temperatures in the spring months that then reach a maximum in the summer when food is plentiful, the adult organisms begin to regain full physiological activity and increase growth rates. The combined effects on four species of coastal marine invertebrates of increased temperature and decreased pH at the levels expected during the summer at mid-century in 2050 (+0.2 pH, +2 °C) and at the end of this century in 2100 (+0.4 pH, +4 °C) will be investigated in this study. Several previous studies (e.g. Berge *et al.*, 2006; Gazeau *et al.*, 2007) have shown negative effects of ocean acidification on the growth of some calcified organisms such as *M. edulis*. In contrast, some other research has shown enhanced growth in *M, edulis* under higher pCO_2 (Thomsen *et al*., 2010). On the other hand, there was no response to lower pH in growth of *M. edulis* in the study conducted by Ries *et al.* (2009), although the same study reported a decrease in the degree of calcification in *L. littorea*. Such expected decreases in pH due to elevated [CO_2] coincide with the expected increase in average temperatures in the troposphere in the future. Many researchers have studied the combined effects of elevated temperature and decreased pH on many marine invertebrates and observed some negative effects. For example, Martin and Gattuso (2009) noticed decreased calcification and increased necrosis and mortality of coralline algae under higher temperature and lower pH. According to Maier *et al.*, (2009) both increased temperature and decreased pH cause reductions in coral calcification. Lischka *et al.*, (2011) recorded an increase in the mortality rate of a pteropod species *Limacina helicina* after exposure to high temperature and low pH. Climate has changed and likely will continue to change; the 21st century has been characterized by the creation of significant new and conditions that are harmful to marine organisms (Brierley and Kingsford, 2009).

In general, different species of organisms differ in their ability to adapt to temperature variation (Peck *et al*., 2004). Continuation of the rise in temperatures due to climate change will be the most difficult challenge for ectothermic organisms. This temperature increase may reduce the scope of growth, and under certain temperatures animal activity would stop and they would be unable to perform biological functions (Levinton, 2009). Temperatures affect the rates of physiological processes of aquatic ectothermal organisms (Hochachka and Somero, 2002; Bolton and Havenhand, 2005) and will likely have a negative impact on survival in marine organisms (Harley *et al*.,

2006). These expected changes in the physical and chemical properties of seawater will determine which among extant calcified and non-calcified marine invertebrates will survive in the future. Competition within the same habitat, such as between *A. aspersa* and *M. edulis*, which both feed by filtering seawater, will likely become more intense. Species with a greater capacity to acclimate to the new environmental conditions in the future will be able to dominate the habitat and displace other species.

5.2 Methods:

The methods described in this chapter follow the methods reported in Chapter 2 and Chapter 3, with some adjustments. For example, species and parameters measured were the same, but the date of collections and temperatures differed. The two experiments in this chapter were conducted under artificial summer conditions from 01 February 2012 to 28 March 2012 and from 24 September 2012 to 22 November 2012 (Table 5.1). Animals were collected and taken directly to the laboratory, and were cleaned. All four species (*Mytilus edulis*, *Littorina littorea*, *Actinia equina*, and *Ascidiella aspersa*) were held together in the same microcosm, at a temperature of 14-15 °C and current pH level (see Chapter 2). Table 5.2 explains the numbers of samples used to measure parameters in both experiments for each species. However, sometimes the final number of replicates was less than 12 due to reduced survival.

Table 5.1 Dates of collections and experimental periods for summer condition experiments.

Experiment No.	Collection date	Experimental period
1	24 May 2012	24 May – 17 July 2012
2	19 July 2012	19 July – 13 September 2012

After the completion of each experiment, some of the samples were prepared for the detection of protein contents, histology (see Appendices) and test energy in the animals under study. However, some samples were preserved at -80 °C until needed for future studies. These contingencies explain why not all the available samples were used for the current set of experiments.

Table 5.2 Numbers of samples used for summer condition measurements of parameters for the species in this study.

Parameters	Treatments											
	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
Start No.	71	102	53	33	71	108	50	37	78	106	46	40
End No.	64	98	53	5	56	100	50	6	61	95	46	9
No. of Individuals Surviving[a]	71	102	53	33	71	108	50	37	78	106	46	40
Body weight	12	12	12	4	11	11	12	3	12	11	12	2
Body length	12	12	---	4	11	11	---	3	12	11	---	2
Body width	12	---	---	---	11	---	---	---	12	---	---	---
Buoyant weight	12	12	12	4	11	11	12	3	12	11	12	2
Dry weight	5	5	5	3	5	5	5	3	5	5	5	3

Above, [a] is the number of animals used to calculate survival, *M.e.* refers to *M. edulis*, *L.l.* refers to *L. littorea*, *A.e.* refers to *A. equina*, and *A.a.* refers to *A. aspersa*.

5.2.1 Seawater Measurements:

This study was designed to investigate the potential combined impacts of high temperatures and low pH levels together, as could result from climate change, so three sets of treatments were used. The control treatment comprised an ambient temperature of 14.8 ±0.13 °C and pH of 8.05 ±0.14. As this study is examining the potential future impacts of conditions forecast for 2050 and 2100, the temperature was raised and the pH level was lowered in the treatment tank. The experimentally applied temperatures were 17.0 ±0.20 °C or 19.2 ±0.20 °C, and the experimental pH was 7.84 ±0.11 or 7.65 ±0.08 for, respectively. The salinity was kept at the level normal for Newbiggin-by-the-Sea (35 ppt) and for Hartlepool (34 ppt), so salinity averages were 36.3 ±1.23 for the control treatment in year 2012 (TR 2011), 36.6 ±1.20 in the middle of the century (TR 2050), and 36.6 ±1.22 at the end of this century (TR 2100). The levels of dissolved oxygen (O_2) in the seawater were maintained at greater than 95% for all treatments in these experiments to ensure an abundant supply of oxygen, the lack of which may adversely affect physiological processes. Therefore, seawater parameters including temperature, salinity, and dissolved oxygen (O_2) were measured daily during these experiments (Table 5.1).

Table 5.3 Seawater chemistry parameters during the winter condition experiments relative to the control treatment and model future condition treatments (Mean ±S.E.).

Treatment	Temperature (°C)	Salinity (ppt)	pH	O_2 (%)
TR 2011	14.8 (±0.13)	36.3 (±1.23)	8.05 (±0.14)	96.6 (±1.45)
TR 2050	17.0 (±0.20)	36.6 (±1.20)	7.84 (±0.11)	97.0 (±1.45)
TR 2100	19.2 (±0.20)	36.6 (±1.22)	7.65 (±0.08)	96.8 (±1.46)

After the acclimatisation period, any dead animals were collected and counted daily during 6 weeks period starting from the third week until the eighth week (for more detail about methods, see Chapter 2).

5.3 Results:

5.3.1 Survival:

After the end of the acclimatisation period, dead animals were collected and counted weekly starting from the third week until the eighth week (Table 5.2). No significant differences in survival rate among all species were found using the Chi-square test. However, a linear decrease in the survival of animals under the treatments TR 2050 and TR 2100 compared with the Control TR 2011 was observed for *M. edulis*, which was accompanied by a very slight decline in the survival rate of *L. littorea*, while no mortality was recorded for *A. equina*. On the other hand, there was a linear decrease in mortality for *A. aspersa*, which showed improvement in survival rates under TR 2100, despite the huge increase in mortality for this species observed under all treatments.

Table 5.4 Percentage survival of four species under different treatments over six weeks.

Species	TR 2011		TR 2050		TR 2100	
	n	Survival %	n	Survival %	n	Survival %
M. edulis	71	90	71	79	78	78
L. littorea	102	96	108	93	106	90
A. equina	53	100	50	100	46	100
A. aspersa	33	15	37	16	40	23

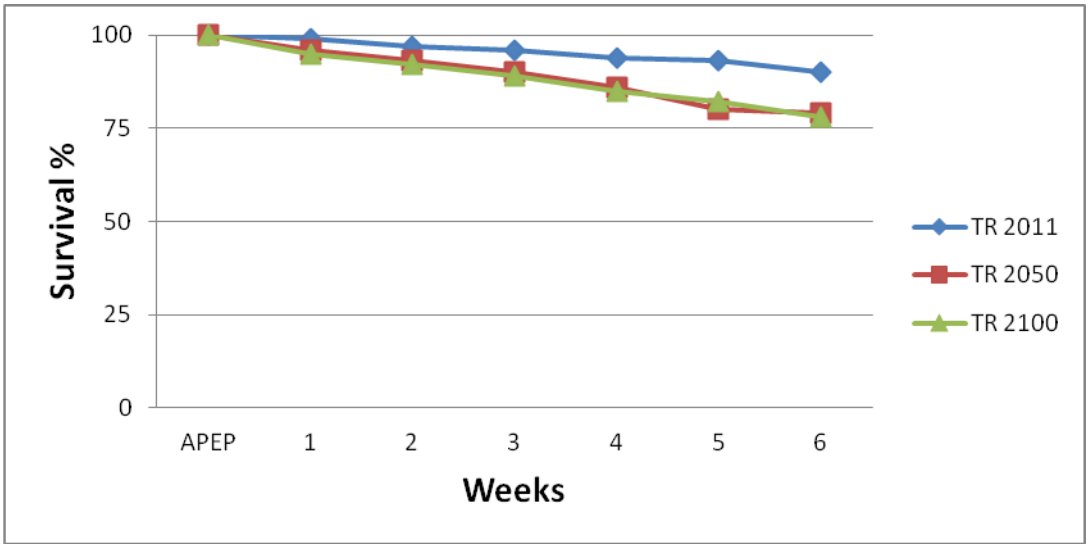

Figure 5.1 Percentage survival of *M. edulis* over a six-week experimental period under all treatments. Blue diamonds represent the experimental treatment TR 2011, red squares represent treatment TR 2050, green triangles represent treatment TR 2100, and APEP represents the acclimatisation period end point.

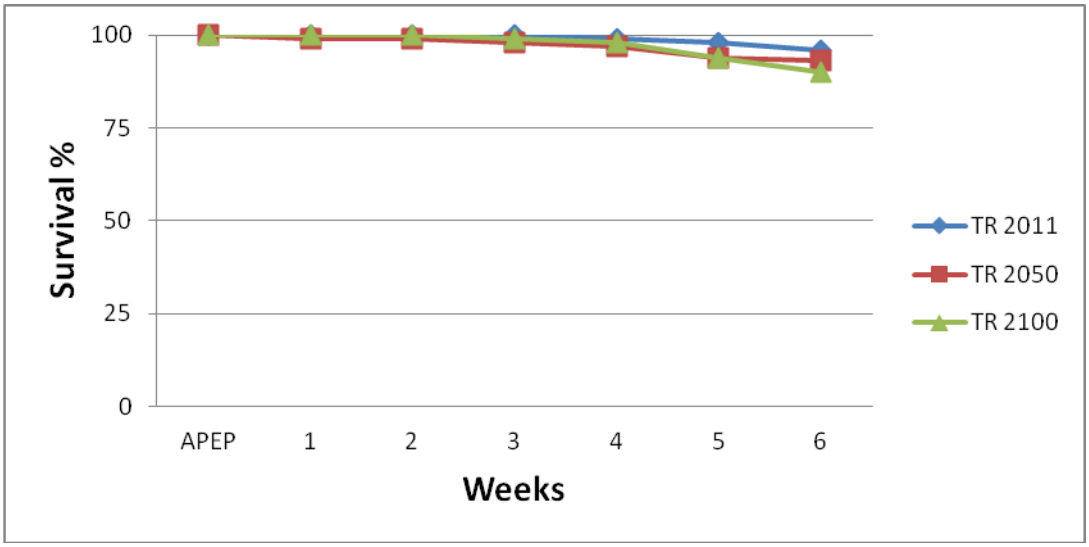

Figure 5.2 Percentage survival of *L. littorea* over a six-week experimental period under all treatments. Blue diamonds represent the experimental treatment TR 2011, red squares represent treatment TR 2050, green triangles represent treatment TR 2100, and APEP represents the acclimatisation period end point.

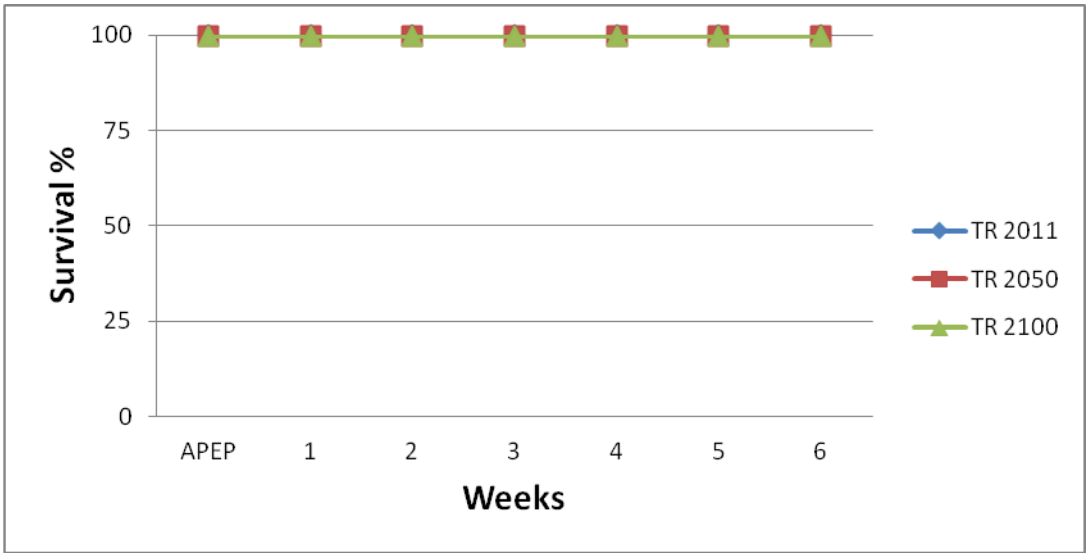

Figure 5.3 Percentage survival of *A. equina* over a six-week experimental period under all treatments. Blue diamonds represent the experimental treatment TR 2011, red squares represent treatment TR 2050, green triangles represent treatment TR 2100, and APEP represents the acclimatisation period end point.

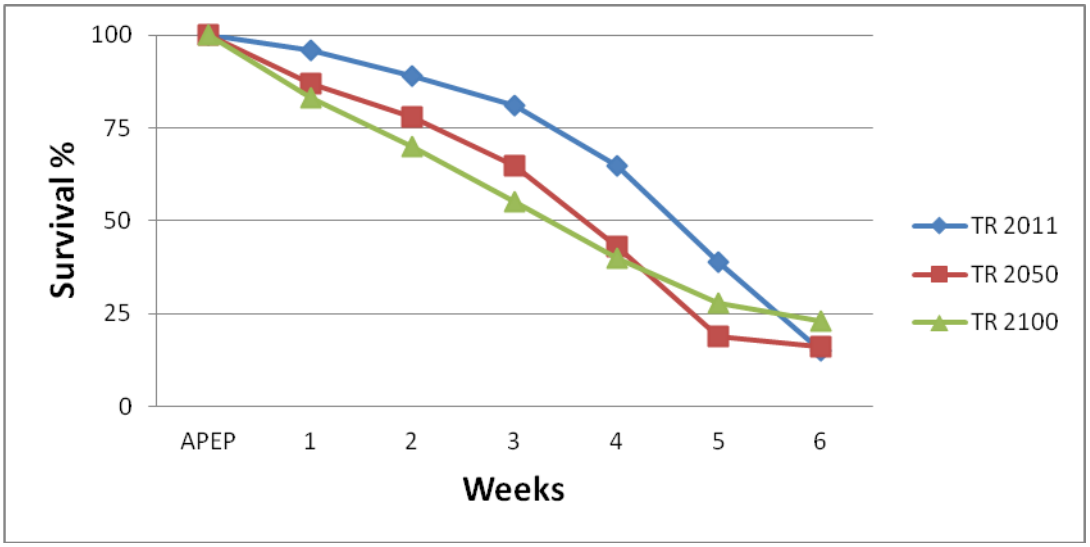

Figure 5.4 Percentage survival of *M. edulis* over a six-week experimental period under all treatments. Blue diamonds represent the experimental treatment TR 2011, red squares represent treatment TR 2050, green triangles represent treatment TR 2100, and APEP represents the acclimatisation period end point.

5.3.2 Body weight change, size change, buoyant weight change, and dry weight parameter measurements:

M. edulis:

Data for body weight change, body width change, buoyant weight change, and dry weight were not normally distributed according to the Shapiro-Wilk test. Therefore, a non-parametric Kruskal-Wallis test was used to test for significant differences in these parameters under each condition (Table 5.5). However, data for body length were found to be normally distributed, so the effects of treatments were compared using one-way ANOVA (Table 5.6).

Table 5.5 Statistical analysis of body weight change, body width change, buoyant weight change, and dry weight of *M. edulis*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	p value	Chi-square	df	p value
Body weight	0.918	35	0.013	2.017	2	0.365
Body width	0.879	35	0.001	0.510	2	0.775
Buoyant weight	0.879	35	<0.001	3.154	2	0.207
Dry weight	0.843	15	0.014	0.260	2	0.878

Table 5.6 Statistical analysis of body length change of *M. edulis.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Body length	35	2.936	0.068	0.947	35	0.090	2.431	0.104

Table 5.7 Mean percentage (%) ± standard error (SE) of *M. edulis* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	2.19	±0.69	1.44	±0.54	1.02	±0.39
Body length change	0.05	±0.02	0.02	±0.02	0.08	±0.03
Body width change	0.009	±0.02	0.03	±0.04	0.04	±0.04
Buoyant weight change	7.25	±2.80	2.60	±2.10	2.26	±1.06
Dry weight	48.5	±4.49	48.5	±6.00	47.4	±5.38

There were no significant differences among the three treatments for parameters measured in *M. edulis* over six weeks (see Table 5.5 and 5.6).

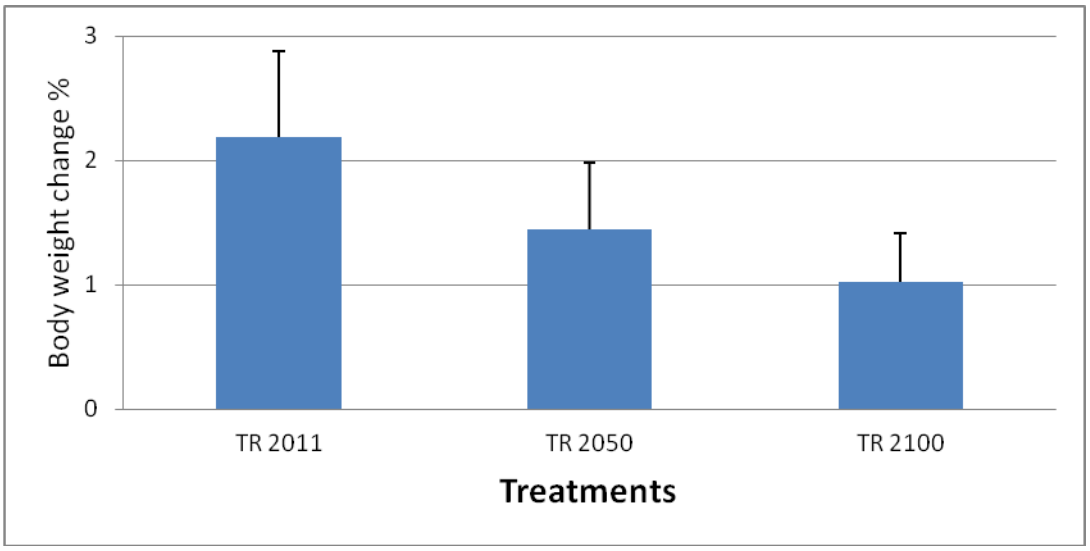

Figure 5.5 Effect of increased temperature and decreased pH on mean percentage body weight change (±SE) in *M. edulis* at six weeks.

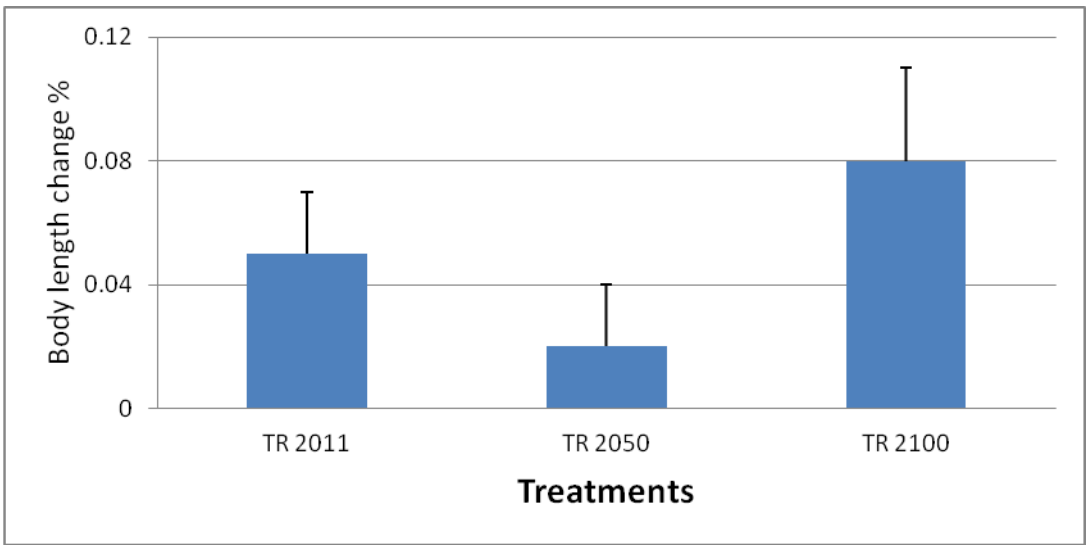

Figure 5.6 Effect of increased temperature and decreased pH on mean percentage body length change (±SE) in *M. edulis* at six weeks.

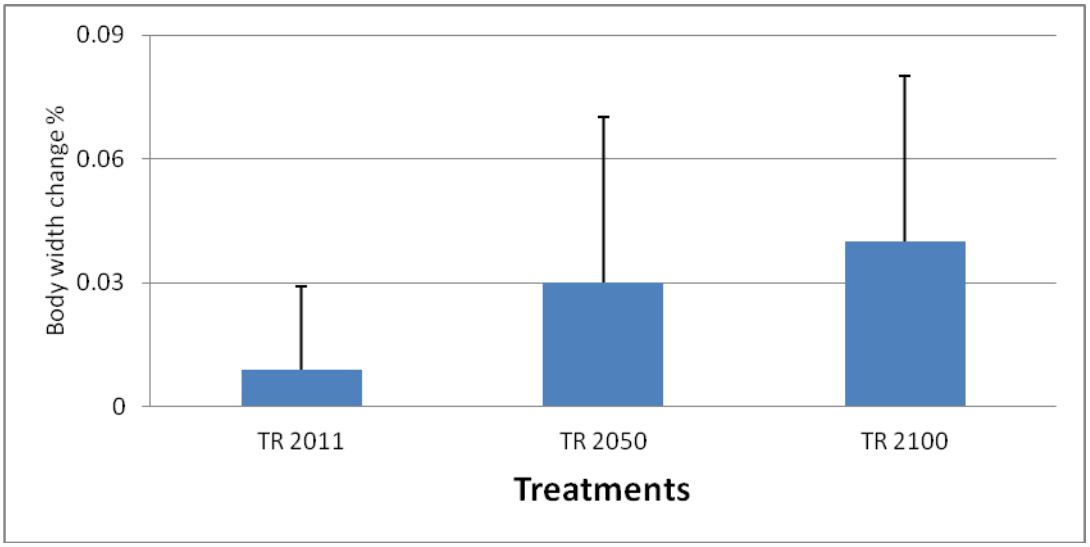

Figure 5.7 Effect of increased temperature and decreased pH on mean percentage body width change (±SE) in *M. edulis* at six weeks.

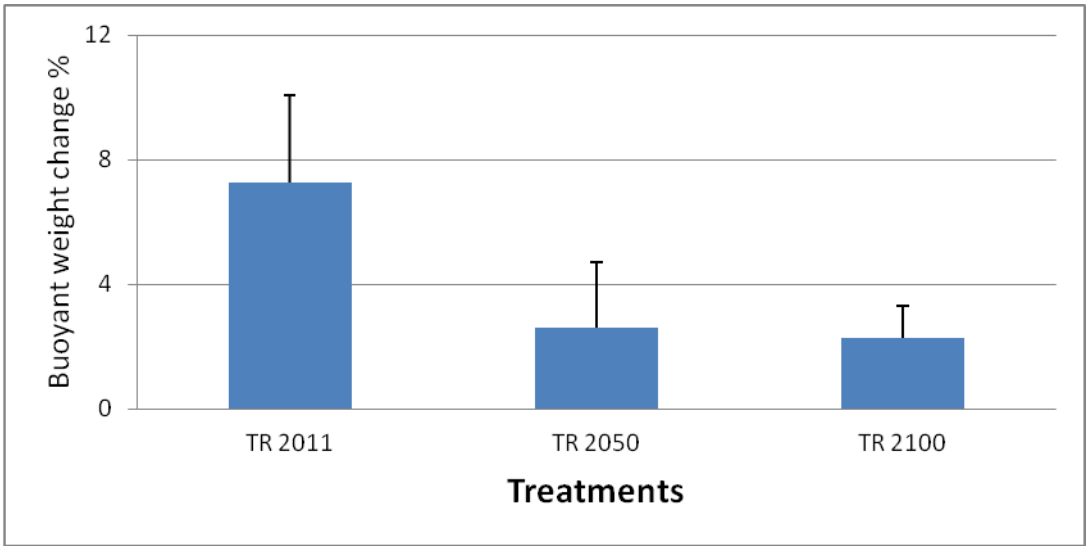

Figure 5.8 Effect of increased temperature and decreased pH on mean percentage buoyant weight change (±SE) in *M. edulis* at six weeks.

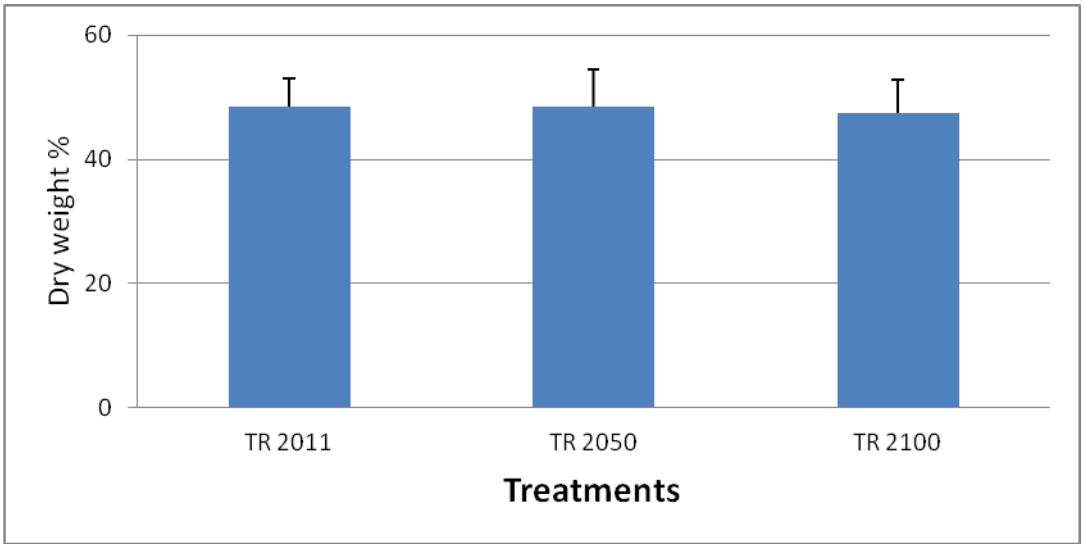

Figure 5.9 Effect of increased temperature and decreased pH on mean percentage dry weight (±SE) in *M. edulis* at the end of the experiment.

L. littorea:

A Shapiro-Wilk test indicated that data for body length change were not normally distributed. Therefore, the non-parametric Kruskal-Wallis test was used to compare treatment means (Table 5.8). Body weight change, buoyant weight change, and dry weight were found to be normally distributed, so treatment effects on the latter parameters were compared using one-way ANOVA (Table 5.9). Scheffe's method was used as the *post-hoc* test for significant differences among treatments.

Table 5.8 Statistical analysis of body length change in *L. littorea*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	p value	Chi-square	df	p value
Body length	0.871	34	0.001	1.959	2	0.376

Table 5.9 Statistical analysis of body weight change, buoyant weight change, and dry weight of *L. littorea.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Body weight	34	8.062	0.002	0.962	34	0.287	3.193	0.077
Buoyant weight	34	7.426	0.002	0.969	34	0.422	2.790	0.077
Dry weight	15	0.020	0.980	0.956	15	0.627	3.134	0.080

There were no significant differences in body length change over a six-week period or dry weight of *L. littorea* at the end of experiment among these three treatments (see Table 5.8 and Table 5.9). However, significant differences were found in body weight change for TR 2011 compared with TR 2050 (p = 0.003) and for TR 2011 compared with TR 2100 (p = 0.015). However, a significant difference in buoyant weight change for TR 2011 compared with TR 2050 (p = 0.003) was found using Scheffe's method.

Table 5.10 Mean percentage (%) ± standard error (SE) of *L. littorea* for parameters measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	1.42	±0.23	-0.03	±0.25	0.21	±0.30
Body length change	0.001	±0.004	-0.02	±0.013	0.01	±0.01
Body width change	---	---	---	---	---	---
Buoyant weight change	4.62	±1.38	-1.3	±1.26	1.74	±0.75
Dry weight	76.46	±0.65	76.65	±1.55	76.44	±0.77

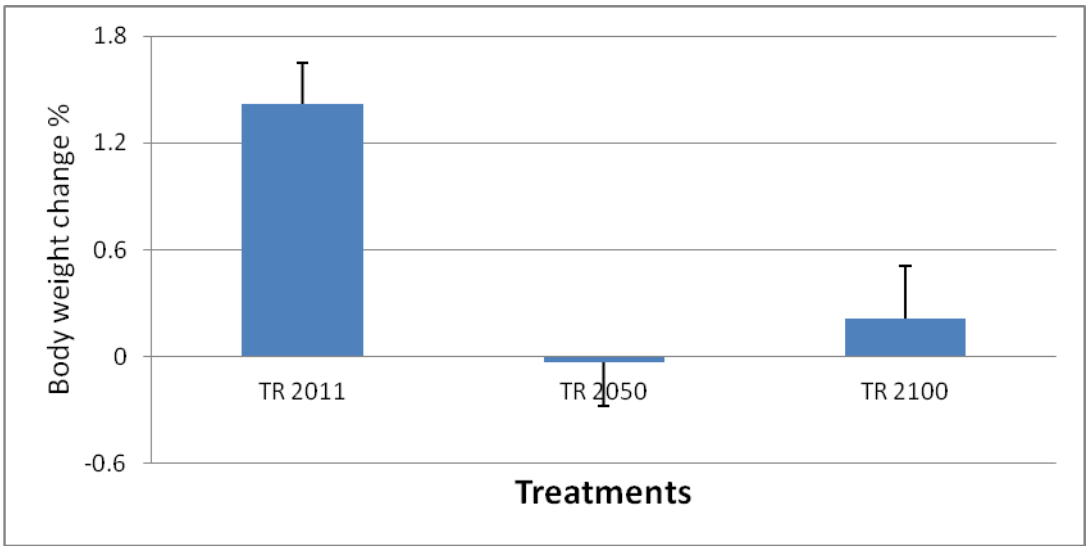

Figure 5.10 Effect of increased temperature and decreased pH on mean percentage body weight change (±SE) in *L. littorea* at six weeks.

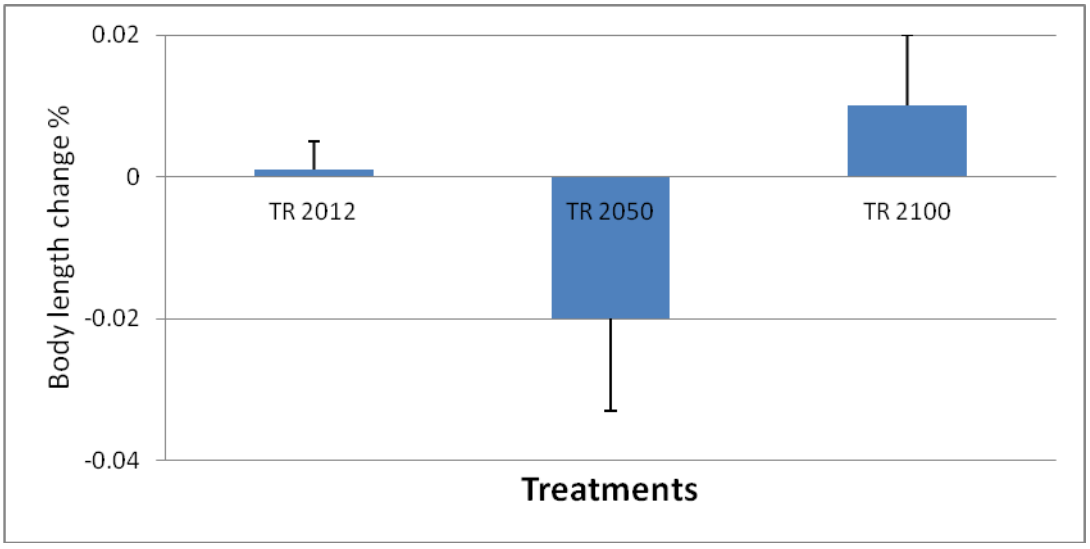

Figure 5.11 Effect of increased temperature and decreased pH on mean percentage body length change (±SE) in *L. littorea* at six weeks.

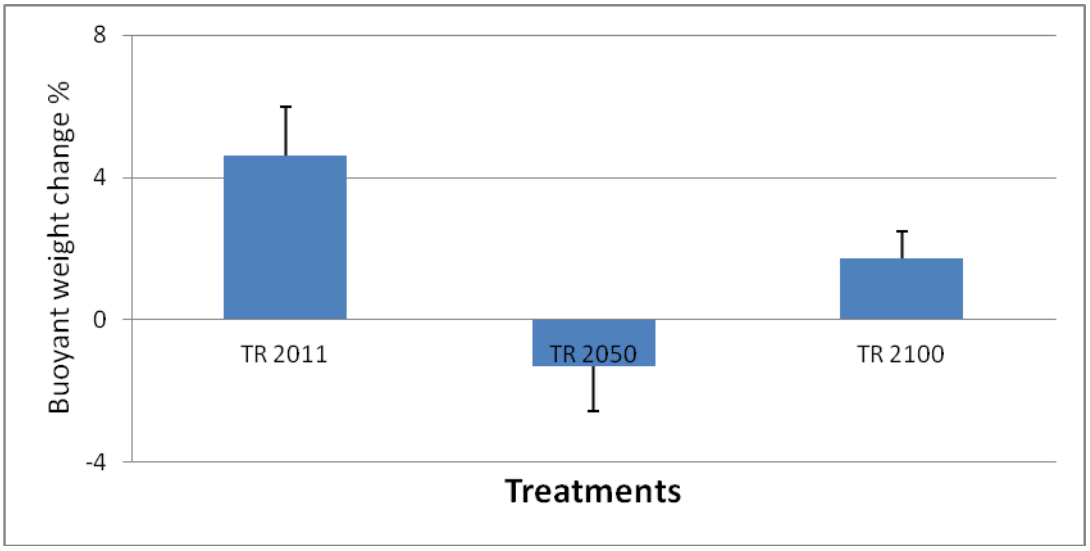

Figure 5.12 Effect of increased temperature and decreased pH on mean percentage buoyant weight change (±SE) in *L. littorea* at six weeks.

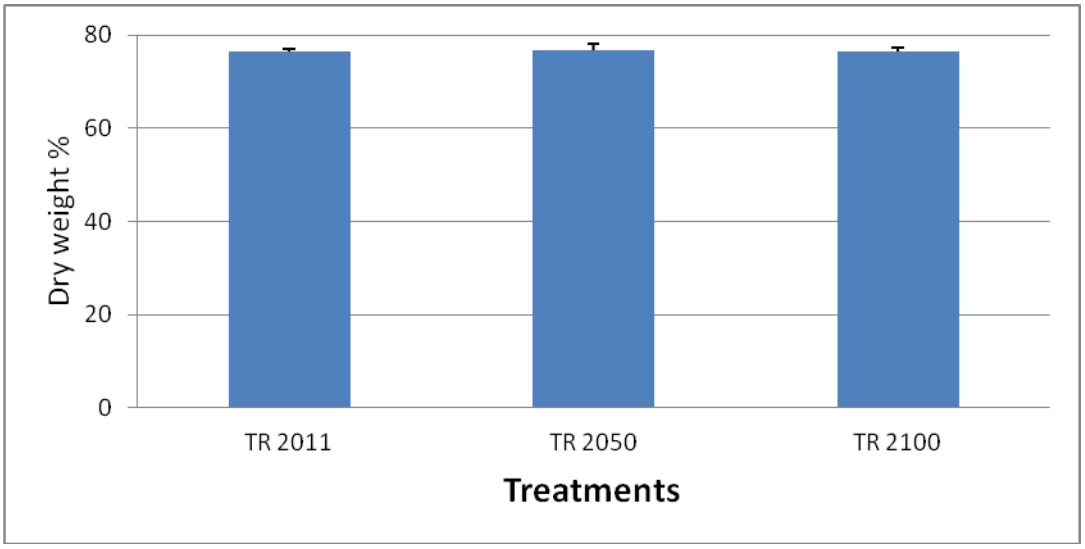

Figure 5.13 Effect of increased temperature and decreased pH on mean percentage dry weight (±SE) in *L. littorea* at the end of the experiment.

A. equine:

A Shapiro-Wilk test indicated that data for body weight change and dry weight were not normally distributed. Therefore, a non-parametric Kruskal-Wills test was used to compare treatments. The Mann-Whitney U *post-hoc* test was used to test for significant differences between treatments (Table 5.10). Buoyant weight change was found to be normally distributed, so treatments were compared using one-way ANOVA.

Table 5.11 Statistical analysis of body weight change and dry weight in *A. equina*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-square	df	*p* value
Body weight	0.889	36	0.002	19.483	2	< 0.001
Dry weight	0.840	15	0.013	1.040	2	0.595

Table 5.12 Statistical analysis of buoyant weight of *A. equina.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Buoyant weight	36	3.039	0.061	0.979	36	0.710	1.533	0.231

No significant differences in buoyant weight change or dry weight of *A. equina* were found among these three treatments (see Table 5.11 and 5.12). However, over a six-week period a significant difference in body weight change between TR 2011 compared with TR 2050 (p <0.001) and between TR 2011 compared TR 2100 (p = 0.002) was found using the Mann-Whitney U test.

Table 5.13 Mean percentage (%) ± standard error (SE) of *A. equina* for parameters measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	±SE	Mean (%)	±SE	Mean (%)	±SE
Body weight change	3.46	±5.07	-20.10	±2.23	-16.72	±4.21
Buoyant weight change	-17.88	±3.30	-5.86	±3.17	-17.09	±5.43
Dry weight	12.7	±1.68	14.15	±1.43	14.01	±1.06

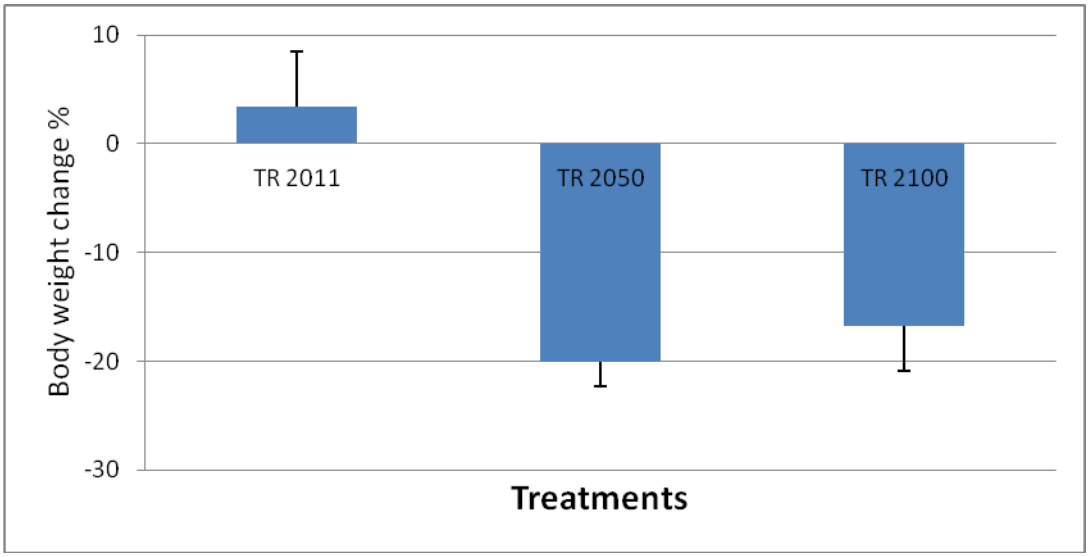

Figure 5.14 Effect of increased temperature and decreased pH on mean percentage body weight change (±SE) in *A. equina* at six weeks.

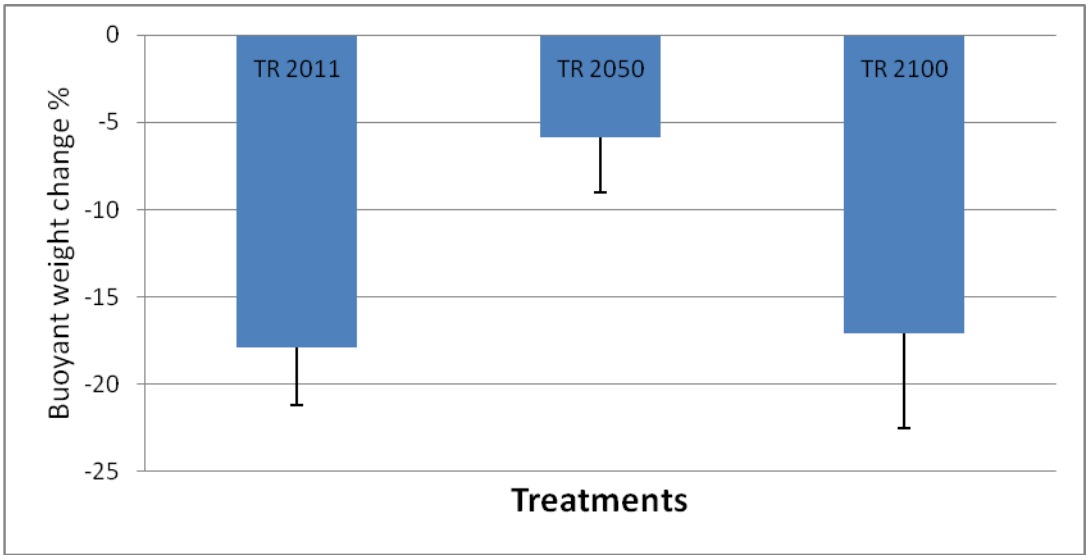

Figure 5.15 Effect of increased temperature and decreased pH on mean percentage buoyant weight change (±SE) in *A. equina* at six weeks.

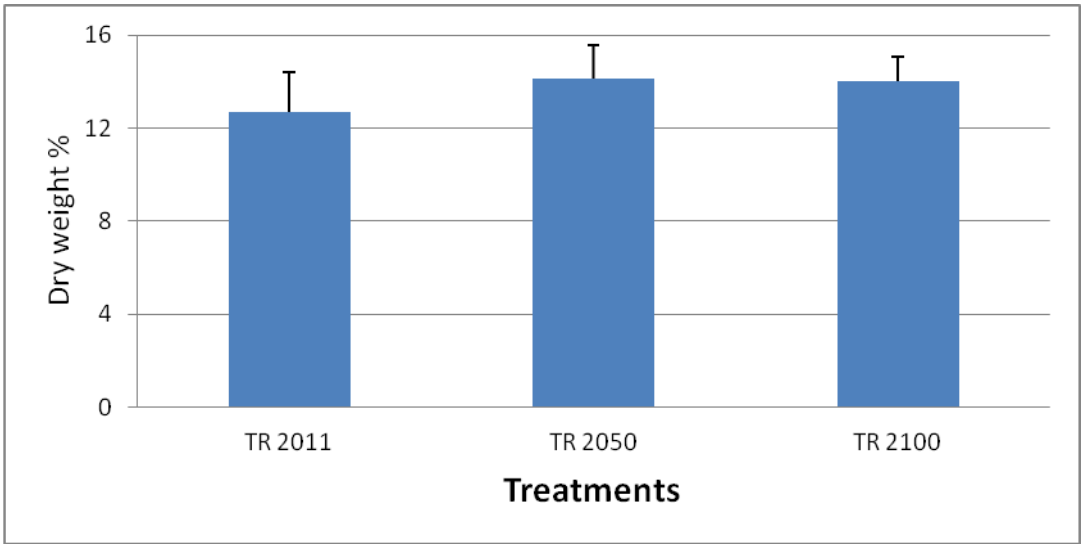

Figure 5.16 Effect of increased temperature and decreased pH on mean percentage dry weight (±SE) in *A. equina* at the end of the experiment.

A. aspersa:

Data for all parameters measured over a six-week period and at the end of the experiment were found to be normally distributed using the Shapiro-Wilk test. Therefore, treatments were compared using one-way ANOVA (see Table 5.14).

Table 5.14 Statistical analysis of body weight change, body length change, buoyant weight change, and dry weight of *A. aspersa.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	ANOVA One-way			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Body weight	9	0.196	0.827	0.951	9	0.698	2.184	0.194
Body length	9	0.053	0.949	0.912	9	0.333	2.092	0.204
Buoyant weight	9	0.532	0.613	0.944	9	0.628	1.179	0.370
Dry weight	8	2.959	0.142	0.967	8	0.870	1.064	0.412

There were no significant differences between three treatments for any of the parameters measured in *A. aspersa* over a six-week period and at the end of the experiment (see Table 5.13).

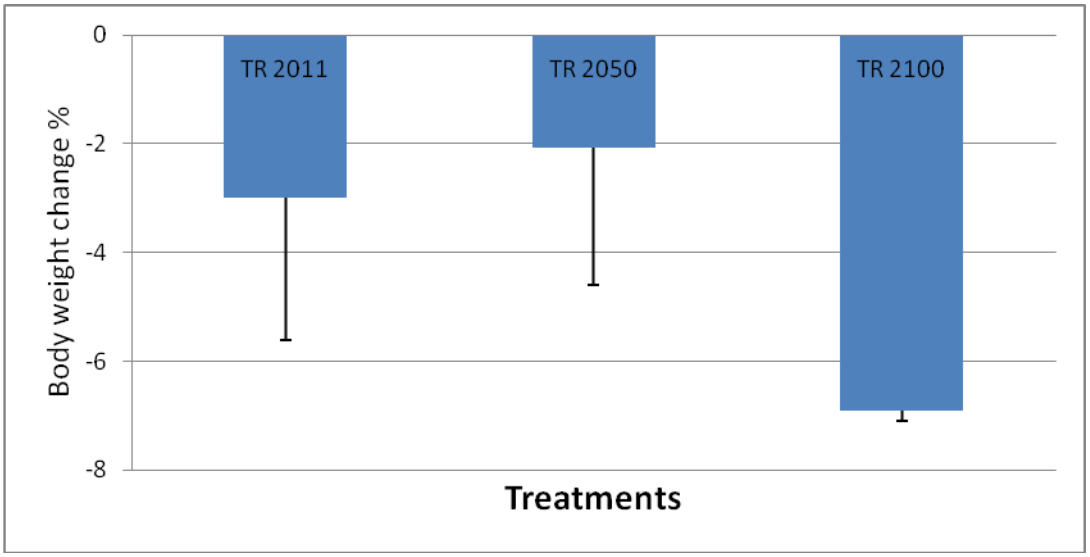

Figure 5.17 Effect of increased temperature and decreased pH on mean percentage body weight change (±SE) in *A. aspersa* at six weeks.

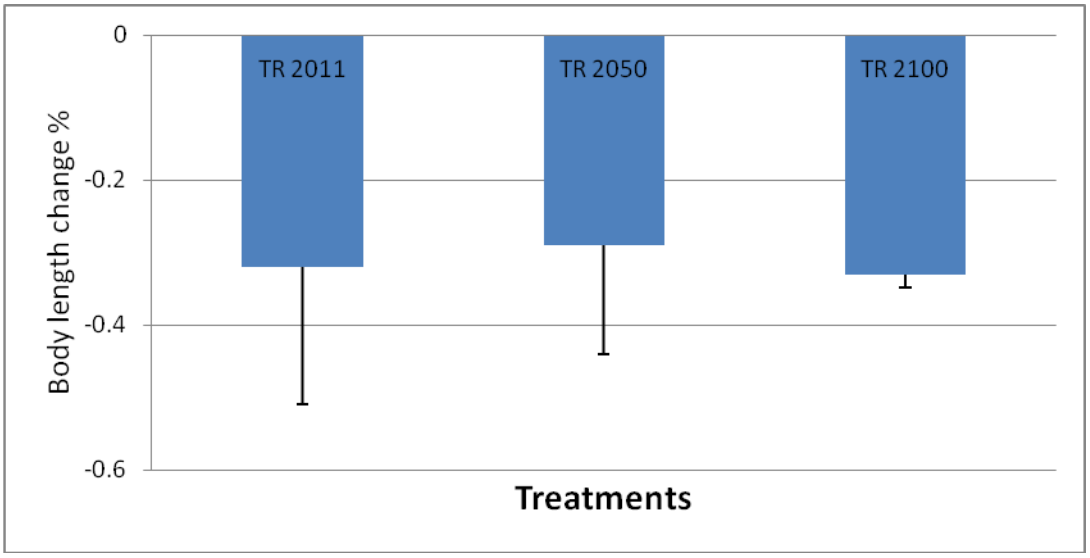

Figure 5.18 Effect of increased temperature and decreased pH on mean percentage in body length change (±SE) in *A. aspersa* at six weeks.

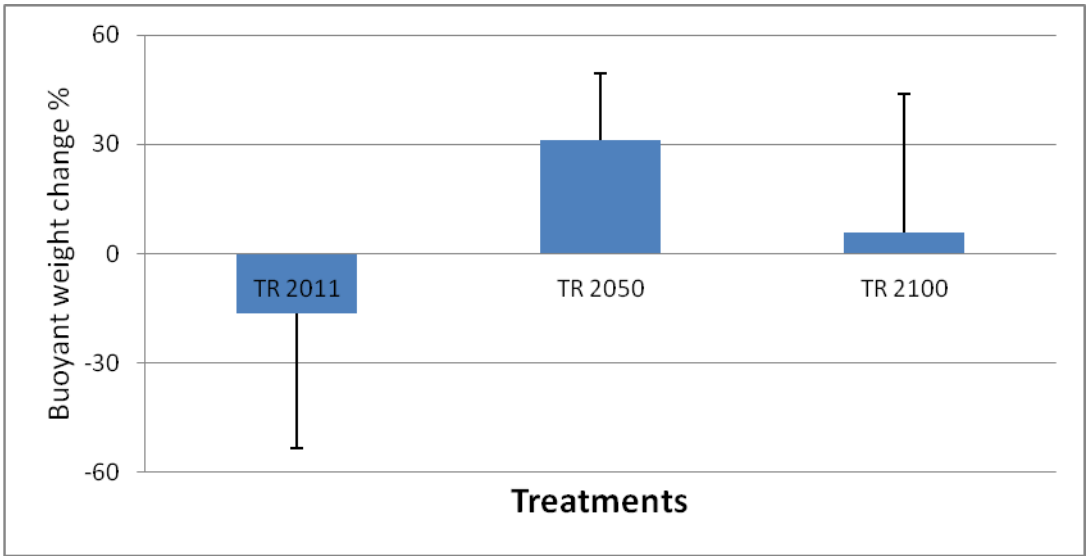

Figure 5.19 Effect of increased temperature and decreased pH on mean percentage buoyant weight change (±SE) in *A. aspersa* at six weeks.

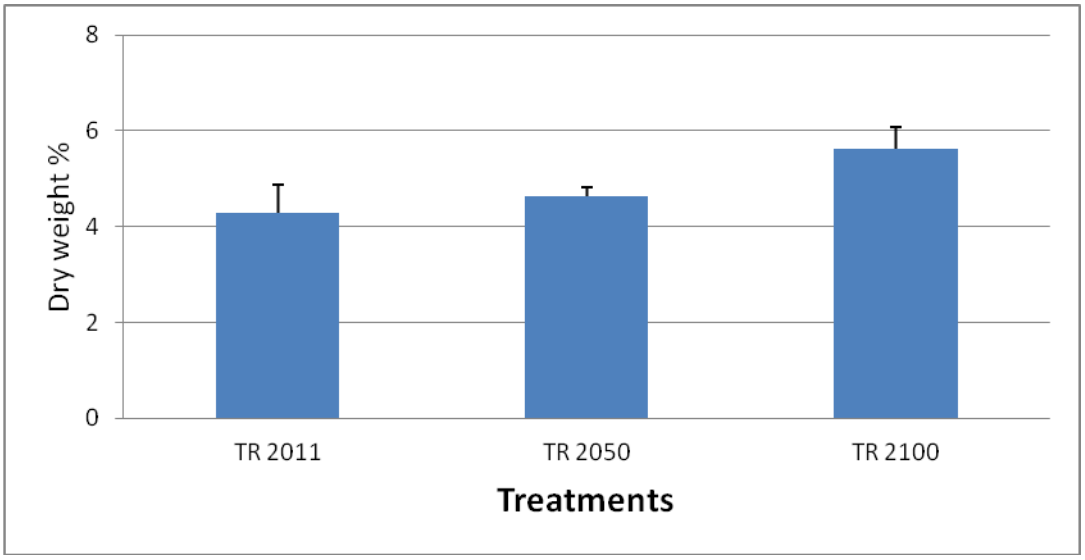

Figure 5.20 Effect of increased temperature and decreased pH on mean percentage dry weight (±SE) in *A. aspersa* at the end the of experiment.

Table 5.15 Mean percentage (%) ± standard error (SE) of parameters measured in *A. aspersa*.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Body weight change	-3.00	± 2.61	-2.08	± 2.52	-6.92	± 0.19
Body length change	-0.32	± 0.19	-0.29	± 0.15	-0.33	± 0.02
Buoyant weight change	-16.41	± 37.05	31.19	± 18.31	5.82	± 38.31
Dry weight	4.28	± 0.60	4.62	± 0.20	5.62	± 0.45

5.4 Discussion:

5.4.1 Effects of increased temperature and decreased pH on M. edulis:

All treatments had the potential to promote increases in growth with TR 2011 had the greatest effect on increasing relative body weight change (Fig. 5.5) and increasing buoyant weight change (Fig. 5.8). This was not a surprise; as the survival curve clearly indicates that the rate of survival recorded under TR 2011 was at least 10% greater than that under TR 2050 or TR 2100 (Fig. 5.1). The graph of survival also shows a slight improvement in survival, reduction of mortality rate from the fifth week of TR 2050. This slight reduction in mortality may indicate that this species was

beginning to adapt to the experimental temperature and pH conditions. Despite the increase in the proportion of body weight change (2.19 ±0.69%, Table 5.7) and the increase in buoyant weight (7.25 ±2.80%) in *M. edulis* under TR 2011, no significant differences were detected between any treatments for all measured parameters (Table 5.5 and 5.6). Increases in body length change (0.08 ±0.03%, Fig. 5.6) and body width change (0.04 ±0.04%, Fig. 5.7) were also recorded under TR 2100. These increases in growth in length and width under the TR 2100 treatment were accompanied by a decrease in buoyant weight when compared with that under the TR 2011 treatment, which may indicate an increase in calcification on the one hand or a reduction in calcification on the other hand. This may also explain how *M. edulis* use the calcification processes to maintain its internal acid-base balance to be able to cope with future seawater chemistry changes.

These results support the findings of Beesley *et al*. (2008), in which no substantial statistically significant differences in mortality appeared when testing *M. edulis* over a period of two months. Our results also support the findings of Berge *et al*. (2006), which indicated an increase in the proportion of mortality when temperatures increase. Mortality did increase in the present experiments under TR 2050 and TR 2100 treatments when compared with the TR 2011 treatment, although no significant differences were shown. The increase in the percentage of growth in length and width at pH = 7.75 under the TR 2100 treatment compared to those parameters under the TR 2011 treatment is similar an experiment (Melzner *et al.*, 2011) that showed an increase in shell growth at a pH of 7.70. The growth increases observed for *M. edulis* in all the treatments applied in this experiment have also been reported by Thomsen *et al.* (2010) at approximately the same pH. The results in growth observed in the present study also supported the findings of Appelhans *et al.* (2012) and Michaelidis *et al.* (2005), which show that there were no statistically significant differences between treatments in growth at pH levels close to those used in the present experiments. Present results for buoyant weight change in *M. edulis* (p = 0.207, Table 5.5 and 5.6, Fig. 5.8) agreed with the findings of Ries *et al.* (2009) in finding no significant difference between treatments. The results of the present experiment regarding reduced calcification also agreed those in an experiment conducted by Gazeau *et al*. (2007), which indicated significantly reduced calcification compared to the control treatment due to decreased pH. The significantly reduced

calcification in Gazeau *et al*. (2007) might have been due to the absence of an adaptation period or to the short duration of the experiment. Alternatively, it could also have been due to the use of a different method for calculating calcification. The results of the present study indicated a negative impact on mussels under increased temperatures and decreased pH, which contributed to reduced rate of survival and decreases in body weight and buoyant weight. Increases in length and width were inversely proportional to the observed decreases in weight, which may indicate an attempt by the organism to overcome chemical degradation of its shell (Findlay *et al.*, 2009b).

5.4.2 Effects of increased temperature and decreased pH on L. littorea:

At least 90% of the marine snail *L. littorea* individuals survived in all treatments (Table 5.4, Fig. 5.2), which shows that these organisms might have the ability to adapt to future conditions (mortality began to appear only after the second week of changed temperature and pH). Body weight change and body length change (Fig. 5.10 and 5.11) measurements clearly show how this organism managed to reduce mortality during the period of the experiment. Although the smallest growth measurements were recorded under TR 2050 (Table 5.10), significantly lower than the values under the TR 2011 treatment, this species still managed to maintain a 93% survival rate, only 3% less than that under the control treatment TR 2011 and 3% more than that under TR 2100 treatments (90%, Table 5.4). The responses of *L. littorea* in body weight change, body length change, and buoyant weight change (Fig. 5.12) under TR 2050 compared to those parameters under TR 2011 and TR 2100, in combination with its 93% survival rate and its improved survival curve in the fifth week, may indicate that this species could relatively successfully adjust to the changing conditions. Reduced metabolic processes, and in particular calcification processes might have favoured maintenance and repair of the body's cells so as to allow the organisms to survive and adapt to increased temperature and reduced pH in their surroundings. These results demonstrate that *L. littorea* has a strong ability to survive, as observed for some other marine snails, similar to the results of Eklof *et al*. (2012), who reported a minimum of 95% survival for all treatments (see also Cmeau *et al.*, 2009). In addition, the lack of significant differences in the dry weight under all treatments (Table 5.8, Fig. 5.13) indicated that *L. littorea* underwent decreased growth

when exposed to dual factors of high temperatures and acidity levels, as also reported by Melatunan *et al*. (2013). The results were also similar to those found by Melatunan *et al.* (2013) who found no statistically significant changes in shell length (Table 5.8, Fig 5.11). Organisms exposed to expected future temperature and pH conditions showed mixed responses in terms of the morphological characteristics of their shells (Melatunan *et al*., 2013). Under our treatment TR 2100, shells showed continued growth (0.01 ±0.01%, Table 5.10) while under treatment TR 2050, loss of shell was indicated (-0.02 ±0.013%).

5.4.3 Effects of increased temperature and decreased pH on A. equina:

Actinia equina showed great tolerance to higher temperatures and lower pH. This was clear from the high ability of individuals of this species to survive, as no mortality was observed for the duration of these experiments under all treatments (Table 5.4, Fig. 5.3). However, there was significant reduction in body weight change under the TR 2050 (-0.03 ±0.25%, Table 5.13) and TR 2100 (0.21 ±0.30%, Table 5.13) treatments ($F = 19.483$, $p < 0.001$, Table 5.11, Fig 5.10) compared to the control treatment TR 2011 (1.42 ±0.23%, Table 5.13). This might indicate a decrease in metabolic activity in organisms subjected to expected future treatment conditions (TR 2050 and TR 2100) as a way to maintain the integrity of the body's cells and to survive. The high percentage of survival in this species was not surprising, as some other species of Cnidaria have been observed in more acidic environments that led to an increase in their abundance and size (Suggett *et al*., 2012). Such an observation for *Cnidaria* may indicate that this invertebrate species may have a high degree of tolerance to higher temperatures and reduced pH in the oceans.

5.4.4 Effects of increased temperature and decreased pH on A. aspersa:

High rates of mortality in all treatments were recorded for *A. aspersa* (Table 5.4, Fig 5.4). This was not surprising as very few samples of adults can be obtained at the time of year these collections were made. The large increases in the number of deaths under all treatments decreases confidence in the results of body weight change, body length change, buoyant weight change, and dry weight measurements. Nevertheless, a slight improvement in survival under increased temperatures and decreased pH was noted, as well as a decrease in the number of individual deaths

under the TR 2050 and 2100 treatments in the fifth week. This might indicate an improvement in the ability of this species to cope with such conditions. In particular, the percentage of survival in TR 2100 reach to 23% by increase 8% than TR 2011 (15%, Table 5.4), that reinforce what Dupont and Thorndyke (2009) said that a decrease in pH level of -0.4 units led to an increase in the rates of survival in *A. aspersa*. However, Dupont and Thorndyke (2009) did not give any more information about how, when and where they conducted the experiment. These results of the present study might be a warning for the possible extinction of this species of marine invertebrate under higher temperatures and lower pH because their presence and survival in the future will depend on their ability to adapt to changes in temperature and pH in the oceans during the early stages of growth in summer. There has been little research on the physiological responses of sessile invertebrate filter feeder species in the Ascidiacea class, to projected future environmental conditions, so hopefully, the present study may provide some initial insights into the responses of such species to climate change.

5.5 Conclusion:

This study of four species of coastal marine invertebrate showed that the sedentary non-calcified invertebrate *A. equina* has a strong ability to cope with predicted future climatic conditions including higher temperatures and increased acidification in the oceans. This adaptability is shared to some extent by the mobile calcified invertebrate *L. littorea*, which is clearly superior in terms of adaptability when compared to its sedentary counterpart *M. edulis*. However, these results showed a significant increase in the proportion of deaths in *A. aspersa* under all treatment conditions even without any significant differences among treatments for all other parameters. This suggests that such dramatic mortality might have been caused by seasonal effects or was due to the life cycle of the organism, but not necessarily due to the environmental effects expected under climate change in these experiments.

Chapter 6. The Effects of Global Climate Change on Body Composition of Common British Marine Invertebrates in summer

Abstract:

It is expected that the increase in temperature and decrease in pH levels predicted globally for the middle and at the end of this century will have an impact on the coastal marine organisms of invertebrates. Therefore, for this study, we first exposed four species of calcified marine invertebrates and non-calcified marine invertebrates to the climatic conditions of the 2011/2012 summer. Subsequently, the animals under study were given two weeks to adapt and then exposed to future levels of acidity and warmth for a period of six weeks. At the end of the study period, the content of water, somatic weight, shell weight (for calcified marine invertebrates), content of lipid, C:N ratios and metabolic rates were measured. It was observed that for the majority of the measured calcified and non-calcified invertebrates, no statistical significance was recorded. However, the study found significant positive results in the metabolic rate for *M. edulis* at levels expected to occur in 2100. Also, numbers of positive statistical significance were observed for the same species at levels expected in 2050 according to C:N ratios. In *L. littorea*, positive significant results were recorded for content of fat, C:N ratio and increased metabolic rates for TR 2100 with acceptable numbers for mortality. These results were replicated for *A. equina* in the form of increased metabolic rates for TR 2100 with good amounts of lipid content which indicates health results that reflected positively on survival. The study showed an improvement in survival for TR 2100 of *A. aspersa* due to higher metabolism to levels that reflected significant differences in comparison with other treatments. These results indicate that each animal has a special response and potential to adapt or not to adapt to the climatic conditions expected in the near future. These findings highlight the need for further study of the expected effects of these climatic changes on the non-calcified invertebrates.

6.1 Introduction:

In ocean ectothermic species most of the physiological processes are optimum at narrow thermal range (Bolton and Havenhand, 2005). The metabolic rate is directly proportional to the temperature rise due to increased burning of carbohydrates for energy production (Levinton, 2009) which leads to increased consumption of oxygen (Zainal *et al.*, 1992; Walther *et al.*, 2009). A continuation of this rise over a long period of time will reduce energy reserves and animals will not be able to regulate their metabolic processes above a certain temperature (Levinton, 2009). This could have a negative impact on the biological processes and survival (Harley *et al*., 2009). An expected increase in temperature in the coming years of this century is expected that goes along with a decrease in pH. This decrease in pH is caused by the increased continuous rise in the amount of CO_2 in the atmosphere and oceans (see Chapter 1). The increase in CO_2 has led to a reduction in the metabolism of marine invertebrates due to acidification of body fluids (Langenbuch *et al.,* 2006) and a reduction of energy transduction (Melatyunan *et al.,* 2013). Molluscs, crustaceans and sea urchins which have structures of calcium carbonate will particularly be affected by ocean acidification (Wood *et al.*, 2008). Various marine species differ in their ability to deal with low pH level (Wood *et al*., 2008). For example, Michaelidis *et al*. (2005) reported a decreased metabolic rate in *Mytilus galloprovincialis*, however, Thomsen and Melzner (2010) observed increased metabolism in *Mytilus edulis* and *L. littorea* demonstrated by a significant drop in metabolic rates (Melatunan *et al*., 2011). On the other hand, many of the studies included that most of the biological processes had non-significant impact with regard to near-future ocean acidification on non-calcified invertebrate (Kroeker *et al.,* 2010). High temperatures and low pH interact synergistically to have a positive effect on the abundance of algal turfs (Connell and Russell, 2010). *Nereis virens* showed a non-significant effect on metabolic rate to decrease pH (Widdichombe and Needham, 2007). Chapter 5 of this study showed that there were non-significant effects on calcified marine invertebrate responses to high temperature and low pH levels expected during the current century. On the other hand, this study recorded a significant fall in body weight in *A. equina*. However, there were no significant effects of various treatments in the *A. aspersa*. This chapter will try to help to clarify the impact of climate change on the invertebrates under

study and the ability of these organisms to adapt to near-future conditions in terms of survival and growth.

6.2 Methods:

The methods in this chapter are similar to those previously mentioned in Chapter 5, but the measurements in this chapter deal with the water content, somatic weight (include gonads), lipid content, C:N ratio and metabolic rate. Table 6.1 explains the numbers of samples used in the measurements of the two experiments together of each species (for more see Chapters 2 and 5).

Table 6.1 Numbers of samples used for summer condition the measurements of parameters for the species in this study.

Parameters	Treatments											
	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
Water content	5	5	5	2	5	5	5	3	5	5	5	3
Somatic weight	5	5	5	2	5	5	5	3	5	5	5	3
Shell weight	5	5	---	---	5	5	---	---	5	5	---	---
Lipid content	5	5	5	3	5	5	5	3	5	5	5	2
C:N ratio	5	5	5	3	5	5	5	3	5	5	5	4
Metabolic rate	12	12	12	7	12	12	12	10	12	12	12	10

Where *M.e.* refer to *M. edulis*, *L.l.* refer to *L. littorea*, *A.e.* refer to *A. equina* and *A.a.* refer to *A. aspersa*.

6.3 Results:

6.3.1 Body composition analysis and metabolic rates:

M. edulis:

A Shapiro-Wilk test indicated that the distribution of content of water and C:N ratio were not normal. The non-parametric Kruskal-Wallis test has been used (Table 6.2). Soft tissue weight, shell weight, lipid content and metabolic rate were normally distributed and treatments were carried out as comparisons with one-way ANOVA (Table 6.3). Scheffe's method was used *post hoc* to test the significance between treatments.

Table 6.2 Statistical analysis of content of water and C:N ratio of *M. edulis*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameters	Statistics	df	*p* value	Chi-square	df	*p* value
Content of water	0.843	15	0.014	0.260	2	0.878
C:N ratio [a]	0.946	15	0.467	0.860	2	0.651

[a] C:N ratio tested by Kruskal-Wallis test because test of homogeneity of variances was significant ($p = 0.004$).

Table 6.3 Statistical analysis of soft tissue weight, shell weight, lipid content and metabolic rate of *M. edulis.*

	Treatment comparison			Normality test			Homogeneity test	
Parameters	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*P*	Statistic	df	*p*	Statistic	*P*
Soft tissue weight	15	0.171	0.845	0.928	15	0.253	2.839	0.098
Shell weight	15	0.171	0.845	0.928	15	0.253	2.839	0.098
Lipid content	15	4.065	0.045	0.965	15	0.783	0.761	0.488
Metabolic rate	15	5.845	0.007	0.978	34	0.722	0.624	0.542

There were no significant differences in the content of water, soft tissue weight, shell weight and C:N ratio of *M. edulis* at the end of the experiments (see Table 6.2 and Table 6.3). However, there were significant differences in lipid content between TR 2011 and TR 2050 ($p = 0.048$), in the metabolic rate between TR 2011 and TR 2100 ($p = 0.013$) and between TR 2050 and TR 2100 ($p = 0.042$) when using Scheffe's method.

Table 6.4 Mean percentage (%) ± standard error (SE) of *M. edulis* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	51.50	± 4.49	51.49	± 6.00	52.60	± 5.38
Soft tissue weight	10.20	± 2.94	8.56	± 1.14	10.38	± 2.13
Shell weight	89.80	± 2.43	91.44	± 1.14	89.62	± 2.13
Lipid content	7.20	± 1.05	11.48	± 1.32	9.72	± 0.76
C:N ratio	4.48	± 0.24	4.31	± 0.13	4.46	± 0.03
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.59	± 0.13	0.48	± 0.08	0.88	± 0.12

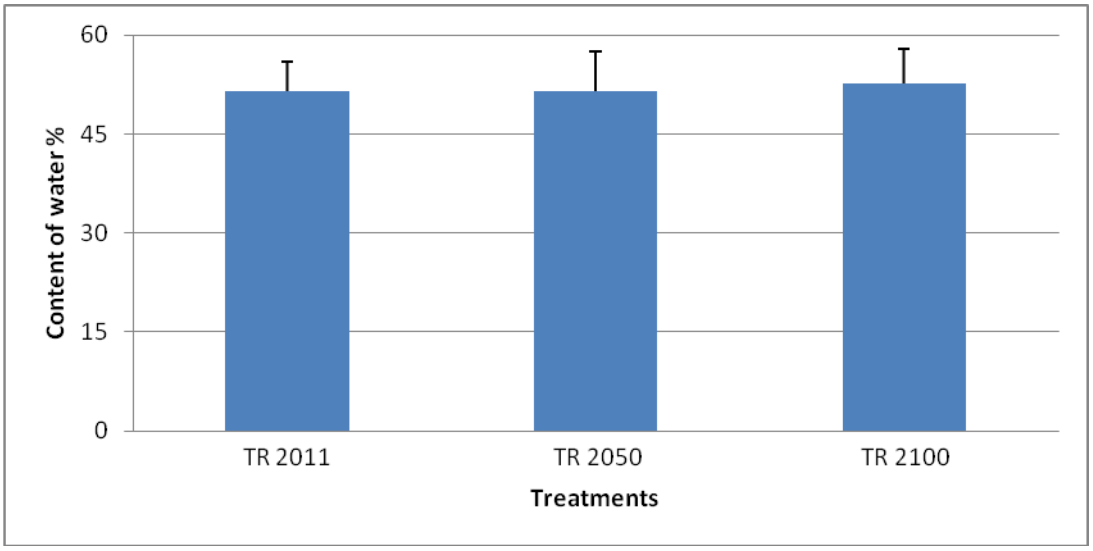

Figure 6.1 Effect of increased temperature and decreased pH level on mean percentage content of water (±SE) in *M. edulis* at the end of experiment.

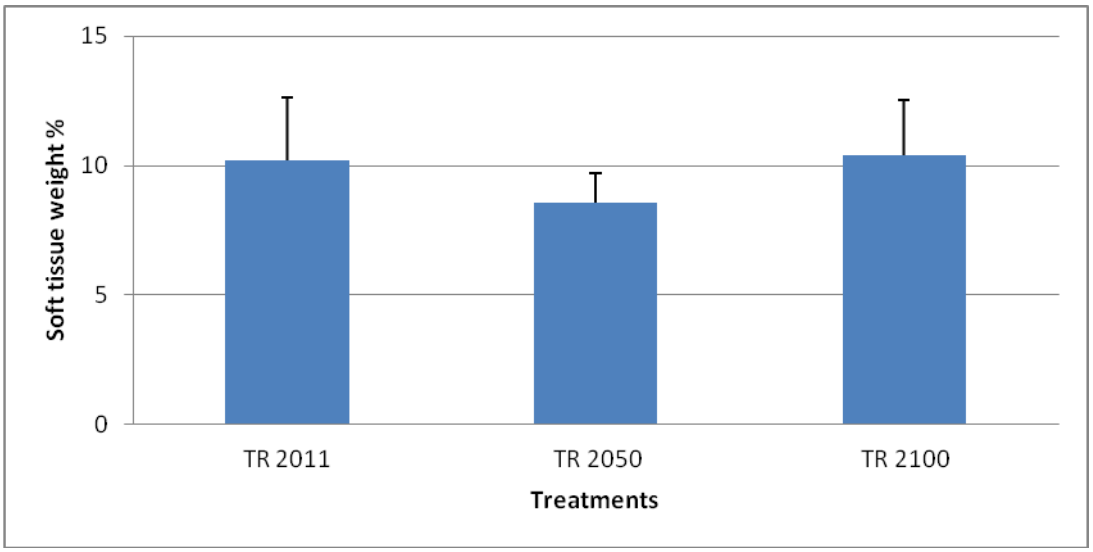

Figure 6.2 Effect of increased temperature and decreased pH level on mean percentage soft tissue weight (±SE) in *M. edulis* at the end of experiment.

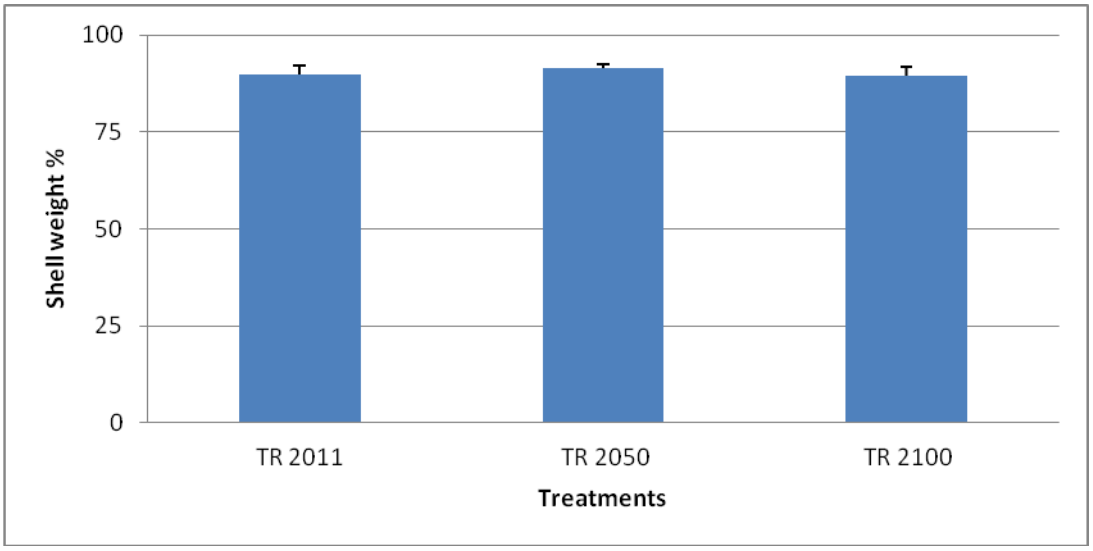

Figure 6.3 Effect of increased temperature and decreased pH level on mean percentage shell weight (±SE) in *M. edulis* at the end of experiment.

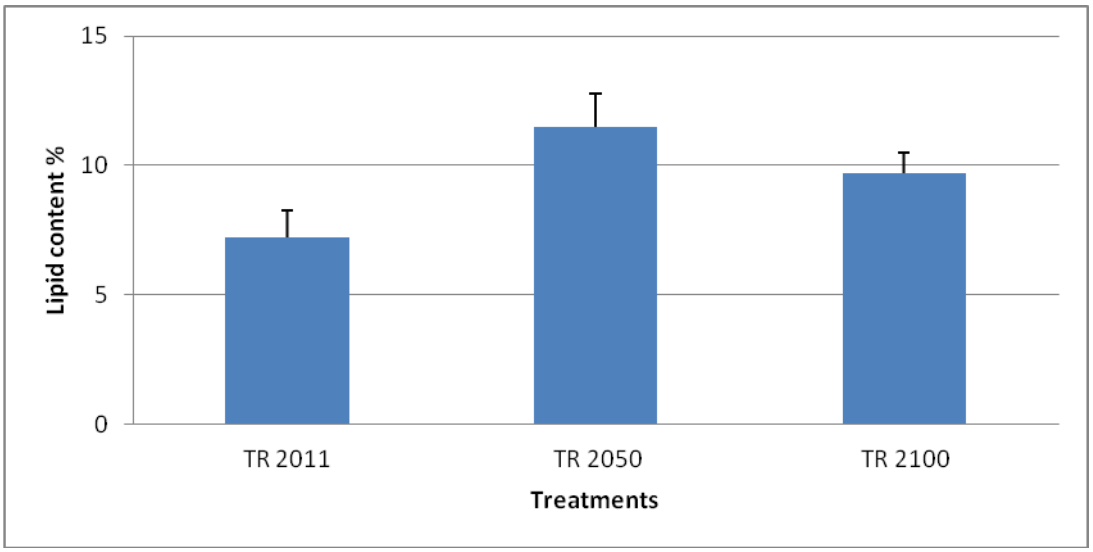

Figure 6.4 Effect of increased temperature and decreased pH level on mean percentage lipid content (±SE) in *M. edulis* at the end of experiment.

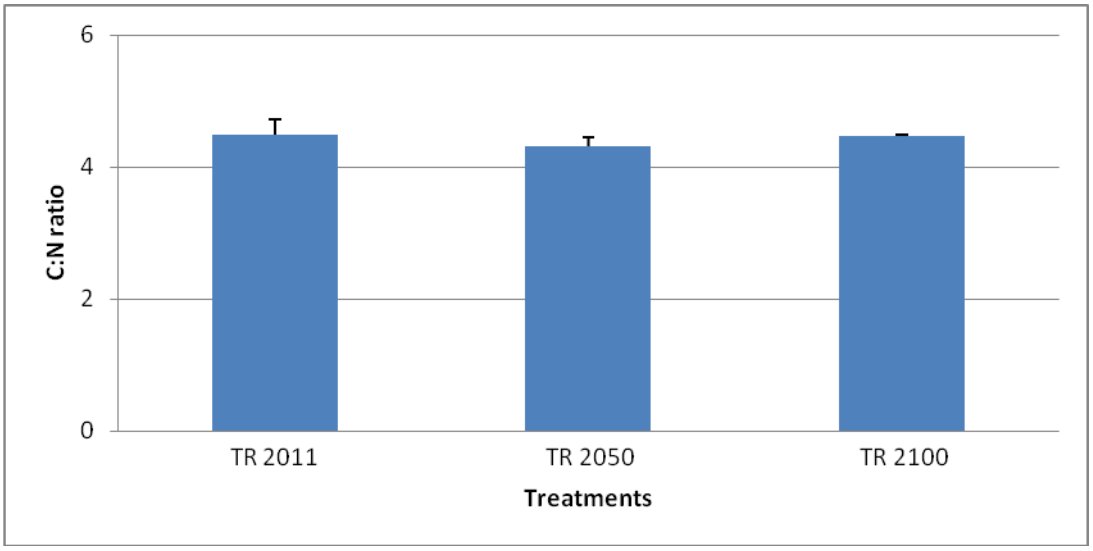

Figure 6.5 Effect of increased temperature and decreased pH level on mean percentage C:N ratio (±SE) in *M. edulis* at the end of experiment.

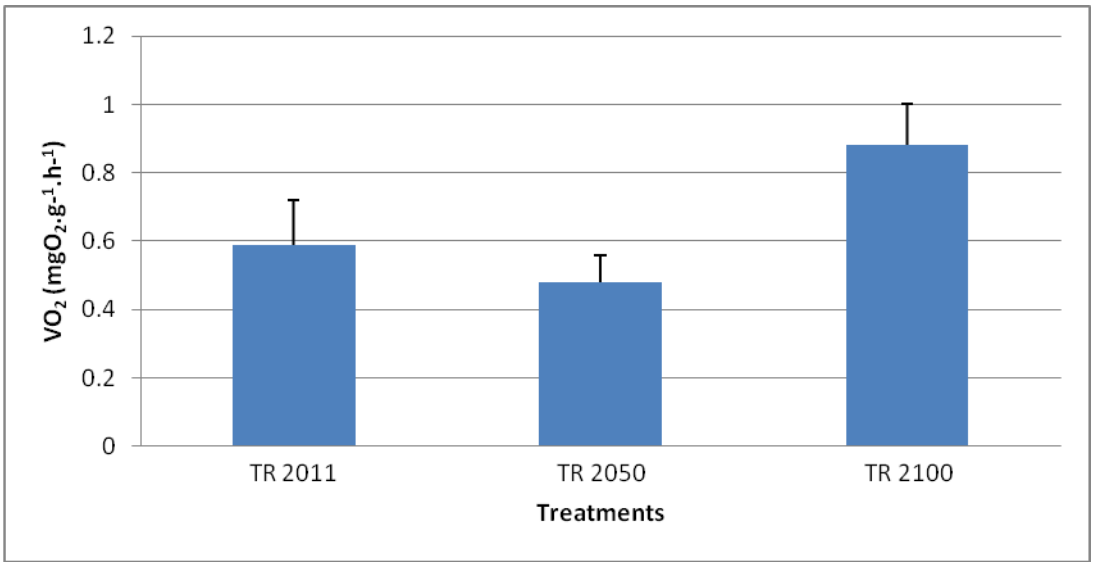

Figure 6.6 Effect of increased temperature and decreased pH level on mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) in *M. edulis* at the end of experiment.

6.3.1.2 L. littorea:

A Shapiro-Wilk tests indicated that the distribution of all measurements were normal. Treatments were carried out as comparisons with one-way ANOVA (Table 6.5). Scheffe's method was used *post hoc* to test the significance between treatments.

Table 6.5 Statistical analysis of content of water, soft tissue weight, shell weight, lipid content, C:N ratio and metabolic rate of *L. littorea.*

	Treatment comparison			Normality test			Homogeneity test	
Parameters	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Content of water	15	0.020	0.980	0.956	15	0.627	3.134	0.080
Soft tissue weight	15	0.905	0.431	0.979	15	0.962	2.055	0.171
Shell weight	15	0.905	0.431	0.979	15	0.962	2.055	0.171
Lipid content	15	8.284	0.005	0.968	15	0.821	0.030	0.970
C:N ratio	15	7.640	0.007	0.932	15	0.293	3.328	0.071
Metabolic rate	36	18.926	<0.001	0.955	36	0.145	2.839	0.073

There were no significant differences in the content of water, soft tissue weight and shell weight of *L. littorea* at the end of experiments (see Table 6.5). However, there were significant differences in lipid content between TR 2011 and TR 2100 ($p = 0.006$), in C:N ratio between TR 2011 and TR 2100 ($p = 0.013$),between TR 2050 and TR 2100 ($p = 0.027$), in the metabolic rate between TR 2011and TR 2050 ($p = 0.018$), between TR 2011 and TR 2100 ($p = 0.014$) and between TR 2050 and TR 2100 ($p < 0.001$) when using Scheffe's method.

Table 6.6 Mean percentage (%) ± standard error (SE) of *L. littorea* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	23.54	± 0.65	23.35	± 1.55	23.56	± 0.77
Soft tissue weight	4.91	± 0.38	4.38	± 0.49	4.22	± 0.20
Shell weight	95.09	± 0.38	95.62	± 0.49	95.78	± 0.20
Lipid content	4.58	± 0.61	7.03	± 0.78	8.97	± 0.92
C:N ratio	3.90	± 0.04	3.94	± 0.08	4.25	± 0.08
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.77	± 0.06	0.52	± 0.04	1.03	± 0.08

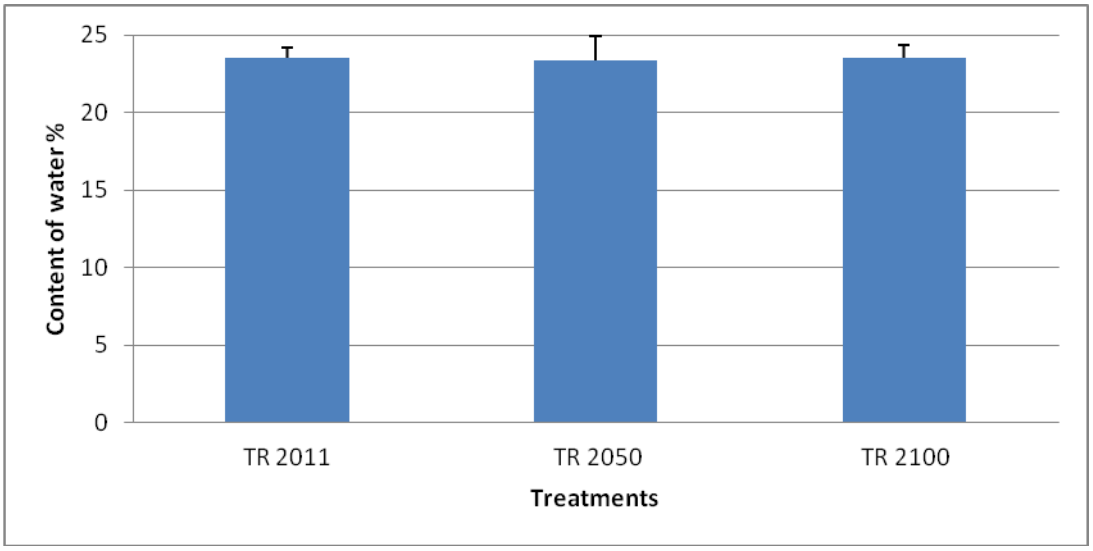

Figure 6.7 Effect of increased temperature and decreased pH level on mean percentage content of water (±SE) in *L. littorea* at the end of experiment.

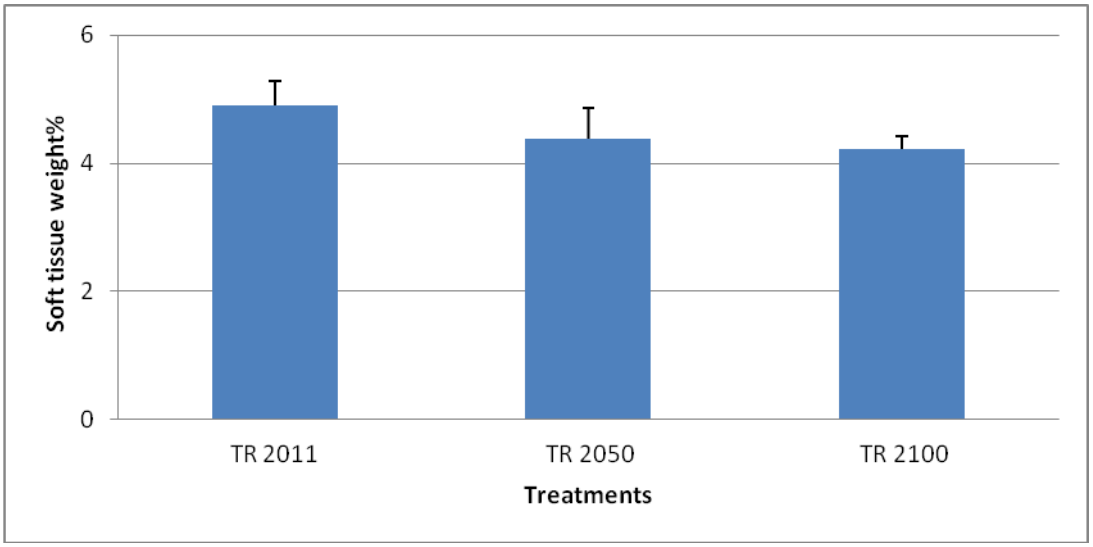

Figure 6.8 Effect of increased temperature and decreased pH level on mean percentage soft tissue weight (±SE) in *L. littorea* at the end of experiment.

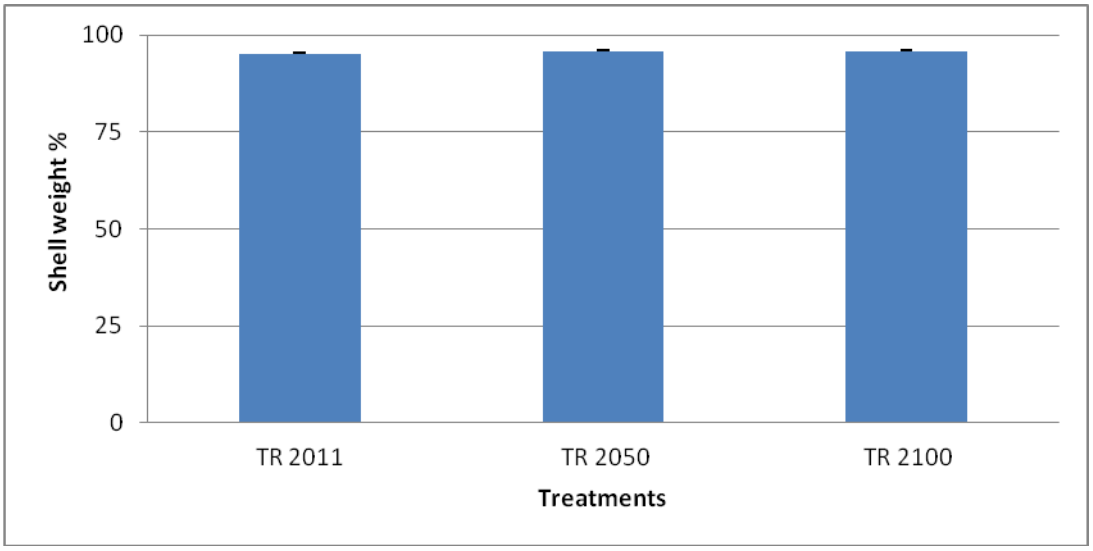

Figure 6.9 Effect of increased temperature and decreased pH level on mean percentage shell weight (±SE) in *L. littorea* at the end of experiment.

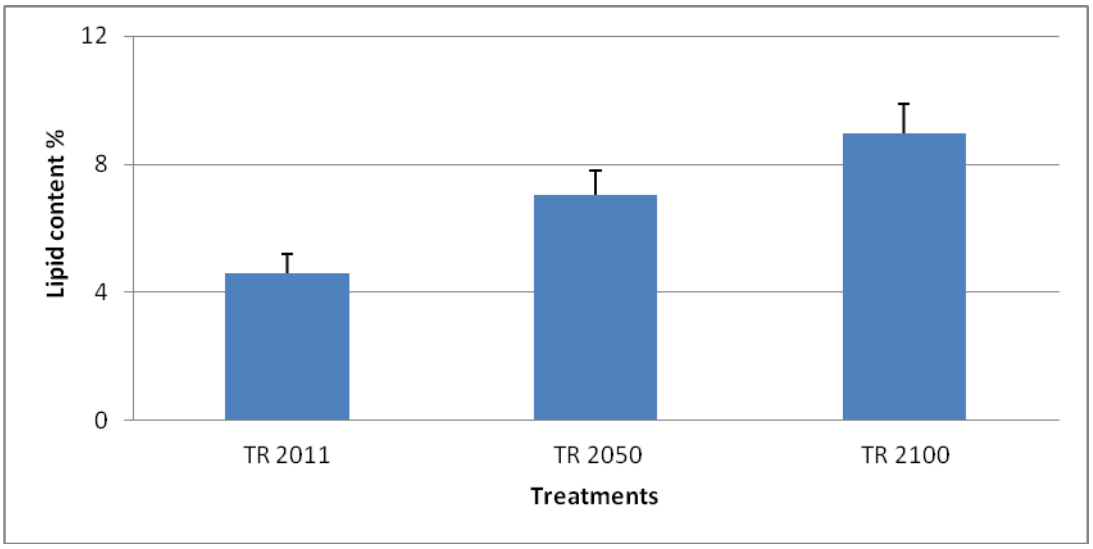

Figure 6.10 Effect of increased temperature and decreased pH level on mean percentage lipid content (±SE) in *L. littorea* at the end of experiment.

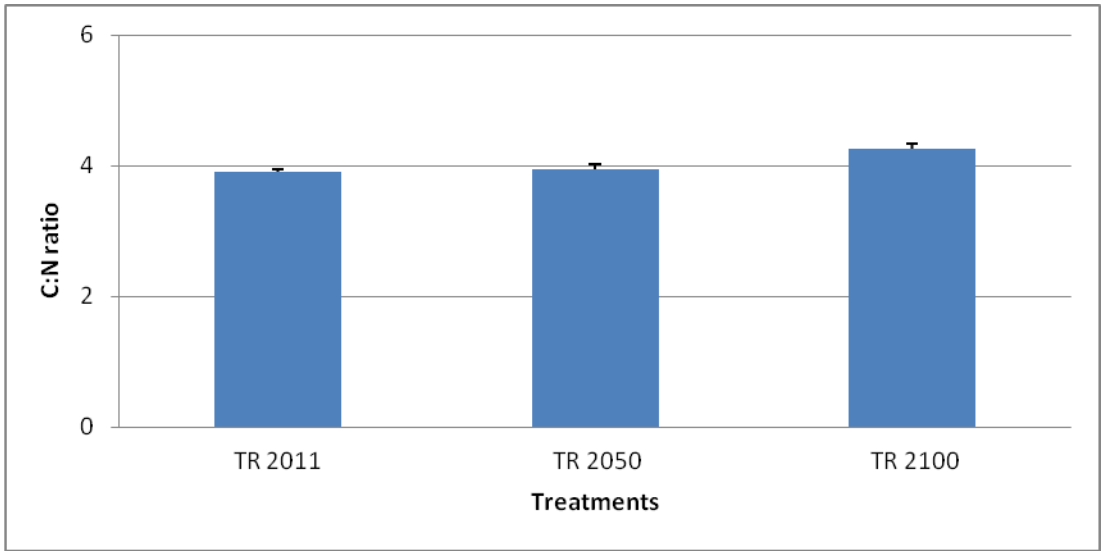

Figure 6.11 Effect of increased temperature and decreased pH level on mean percentage C:N ratio (±SE) in *L. littorea* at the end of experiment.

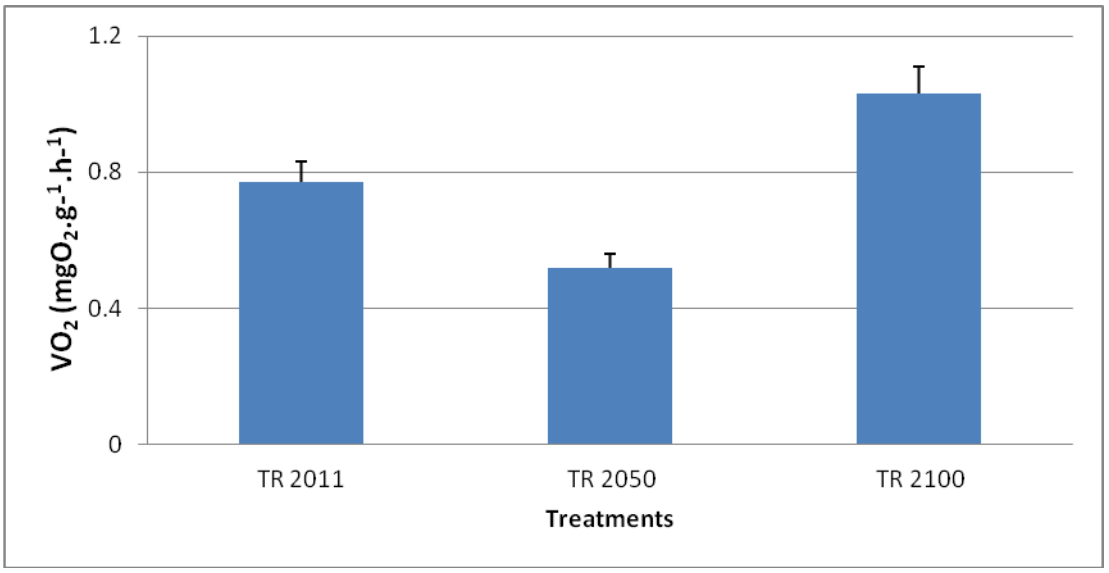

Figure 6.12 Effect of increased temperature and decreased pH level on mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *L. littorea* at the end of experiment.

A. equina:

A Shapiro-Wilk tests indicated that the distribution of content of water and soft tissue weight were not normal. A non-parametric Kruskal-Wallis test has been used (Table 6.7). However, lipid content was normally distributed and treatments were carried out as comparisons with one-way ANOVA (Table 6.8). Scheffe's method was used post hoc to test the significance between treatments.

Table 6.7 Statistical analysis of content of water and soft tissue weight of *A. equina.*

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameters	Statistics	df	*p* value	Chi-Square	df	*p* value
Content of water	0.840	15	0.013	1.040	2	0.595
Soft tissue weight	0.840	15	0.013	1.040	2	0.595

Table 6.8 Statistical analysis of lipid content, C:N ratio and metabolic rate of *A. equina.*

	Treatment comparison			Normality test			Homogeneity test	
Parameters	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Lipid content	15	15.476	<0.001	0.941	15	0.401	0.510	0.613
C.N ratio	15	1.943	0.186	0.967	15	0.816	0.820	0.464
Metabolic rate	36	8.837	0.001	0.946	36	0.77	0.781	0.466

Table 6.9: Mean percentage (%) ± standard error (SE) of *A. equina* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	87.30	± 1.68	85.86	± 1.43	85.99	± 1.06
Soft tissue weight	12.70	± 1.68	14.15	± 1.43	14.01	± 1.06
Lipid content	12.08	± 0.73	17.77	± 0.56	14.91	± 0.80
C:N ratio	4.15	± 0.10	4.25	± 0.10	4.41	± 0.07
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.36	± 0.08	0.47	± 0.08	1.08	± 0.21

There were no significant differences in the content of water, soft tissue weight, shell weight and C:N ratio of *A. equina* at the end of the experiments (see Table 6.7 and Table 6.8). However, there were significant differences in lipid content between TR 2011 and TR 2050 ($p < 0.001$), between TR 2011 and TR 2100 ($p =$

0.043), in the metabolic rate between TR 2011 and TR 2100 (p = 0.001) and between TR 2050 and TR 2100 (p = 0.014) when using Scheffe's method.

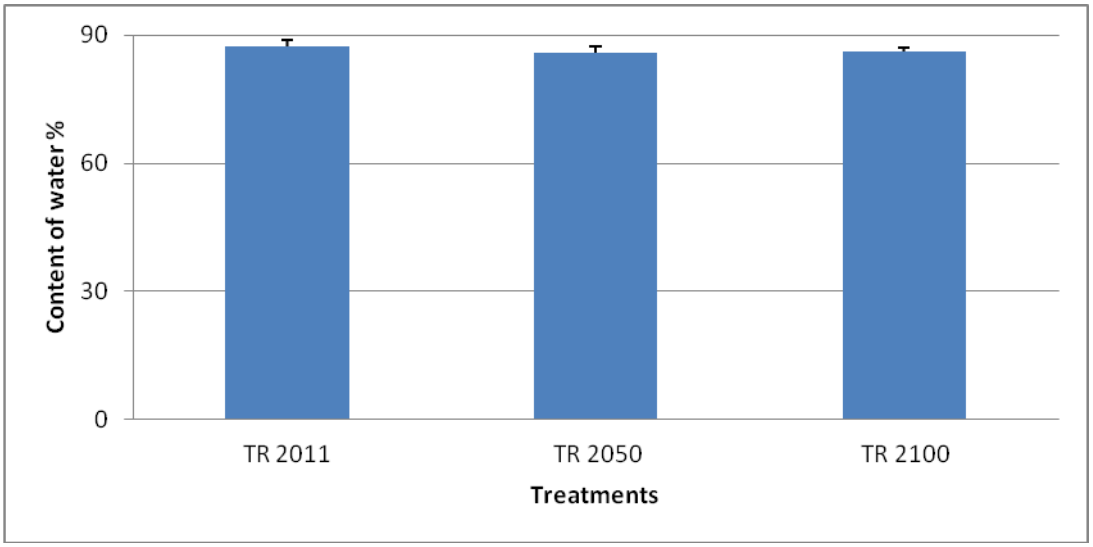

Figure 6.13 Effect of increased temperature and decreased pH level on mean percentage content of water (±SE) in *A. equina* at the end of experiment.

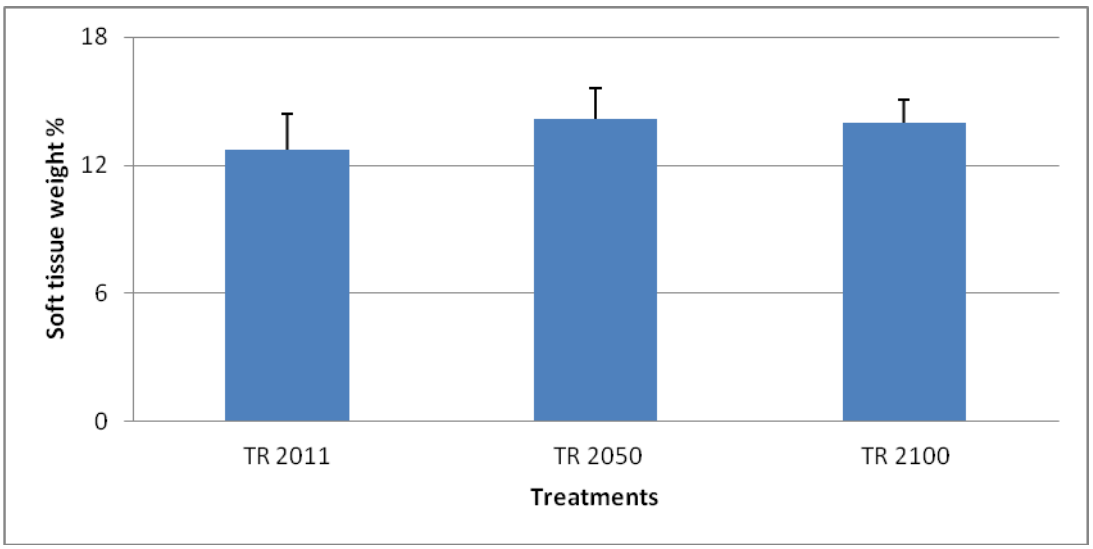

Figure 6.14 Effect of increased temperature and decreased pH level on mean percentage soft tissue weight (±SE) in *A. equina* at the end of experiment.

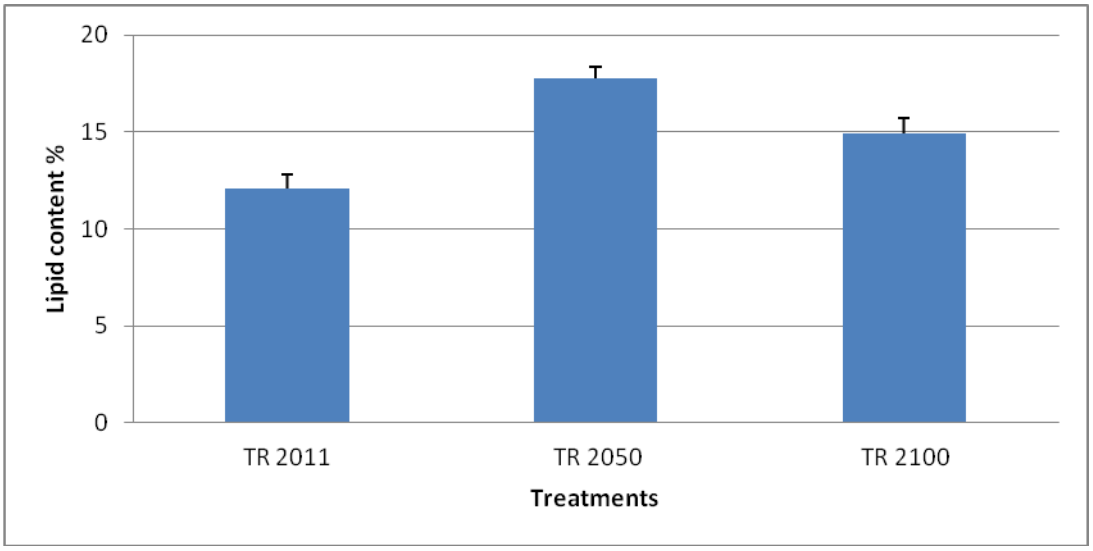

Figure 6.15 Effect of increased temperature and decreased pH level on mean percentage lipid content (±SE) in *A. equina* at the end of experiment.

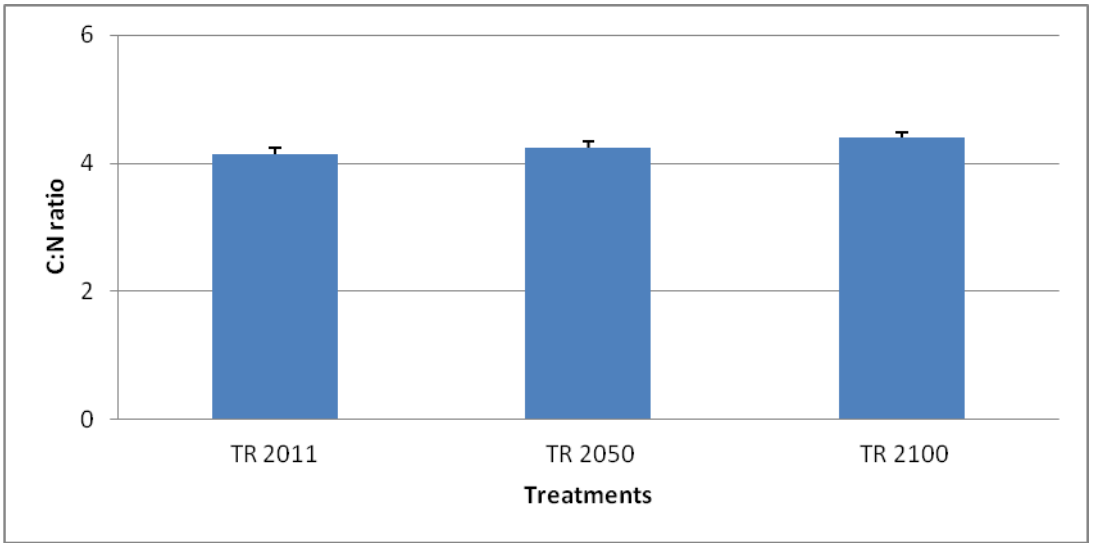

Figure 6.16 Effect of increased temperature and decreased pH level on mean percentage C:N ratio (±SE) in *A. equina* at the end of experiment.

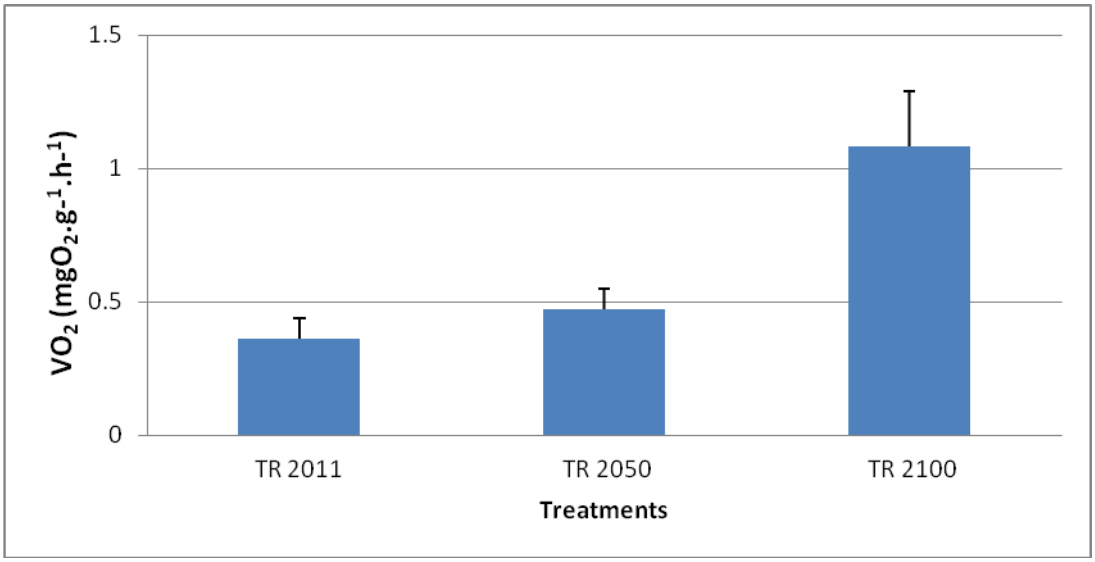

Figure 6.17 Effect of increased temperature and decreased pH level on mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *A. equina* at the end of experiment.

A. aspersa:

A Shapiro-Wilk tests were indicated that all measurements were normally distributed (Tables 6.10 and 6.11). The content of water, soft tissue weight, lipid content and metabolic rate were normally distributed, then the treatments were carried out as comparisons with one-way ANOVA (Table 6.11). Scheffe's method was used *post hoc* to test the significance between treatments.

Table 6.10 Statistical analysis of C:N ratio of *A. aspersa*.

	Test of normality			Treatment comparison		
	Shapiro-Wilk test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-Square	df	*p* value
C:N ratio[a]	0.925	18	0.161	0.364	2	0.834

[a] C:N ratio tested by Kruskal-Wallis test because test of homogeneity of variances was significant ($p = 0.004$).

There were no significant differences in the content of water, soft tissue weight, lipid content and C:N ratio of *A. aspersa* at the end of experiments (see Table 6.10 and Table 6.11). However, there was a significant difference in the metabolic rate between TR 2011 and TR 2100 ($p < 0.001$) and between TR 2050 and TR 2100 ($p < 0.001$) when using the Scheffe's method.

Table 6.11 Statistical analysis of content of water, soft tissue weight, lipid content and metabolic rate of *A. aspersa.*

	Treatment comparison			Normality test			Homogeneity test	
Parameter	One-way ANOVA			Shapiro-Wilk test			Levene's test	
	n	F	*p*	Statistic	df	*p*	Statistic	*p*
Content of water	8	2.938	0.143	0.975	8	0.933	1.309	0.349
Soft tissue weight	8	2.938	0.143	0.975	8	0.933	1.309	0.349
Lipid content	8	1.480	0.313	0.941	8	0.623	1.406	0.328
Metabolic rate	15	56.762	<0.001	0.890	15	0.066	1.543	0.253

Table 6.12 Mean percentage (%) ± standard error (SE) of *A. aspersa* for each parameter measured.

Parameter	TR 2011		TR 2050		TR 2100	
	Mean (%)	± SE	Mean (%)	± SE	Mean (%)	± SE
Content of water	95.72	± 0.60	95.39	± 0.20	94.38	± 0.45
Soft tissue weight	4.28	± 0.60	4.62	± 0.20	5.62	± 0.45
Lipid content	9.89	± 0.52	8.50	± 0.35	7.89	± 1.04
C:N ratio	5.73	± 0.55	5.42	± 0.18	5.12	± 0.21
	Mean	± SE	Mean	± SE	Mean	± SE
Metabolic rate	0.69	± 0.26	0.15	± 0.02	1.06	± 0.11

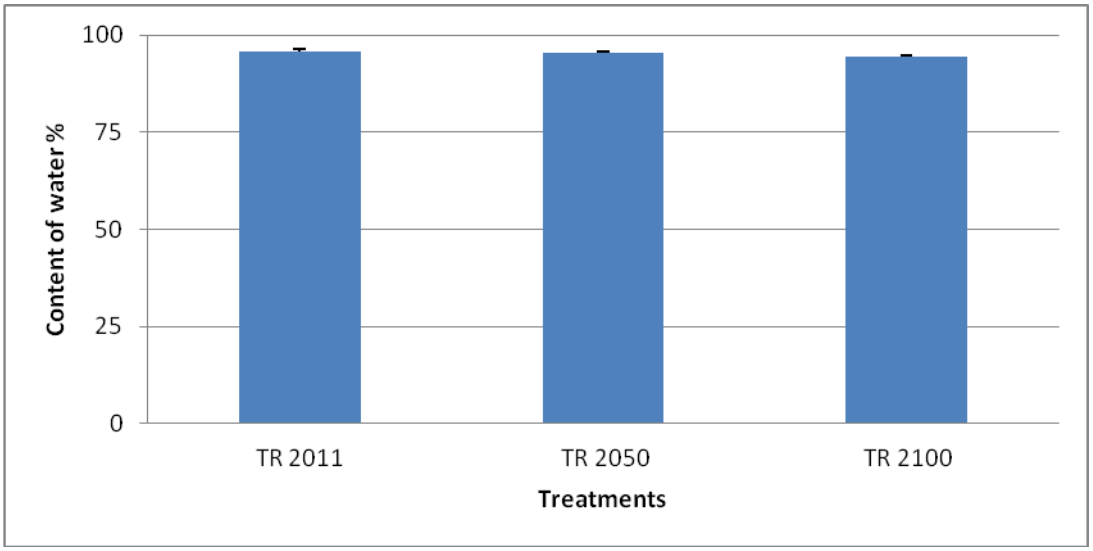

Figure 6.18 Effect of increased temperature and decreased pH level on mean percentage content of water (±SE) in *A. aspersa* at the end of experiment.

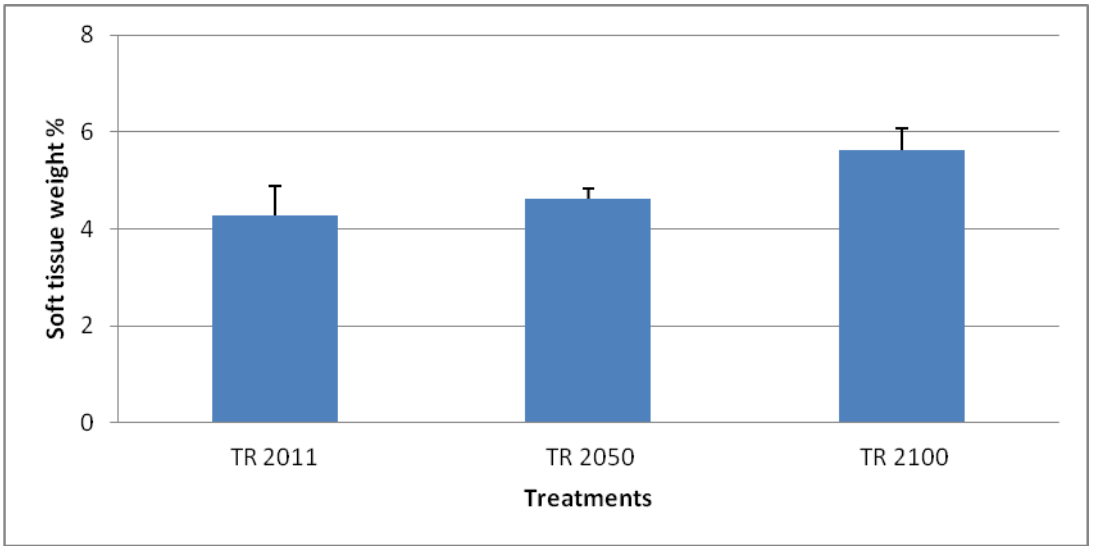

Figure 6.19 Effect of increased temperature and decreased pH level on mean percentage soft tissue weight (±SE) in *A. aspersa* at the end of experiment.

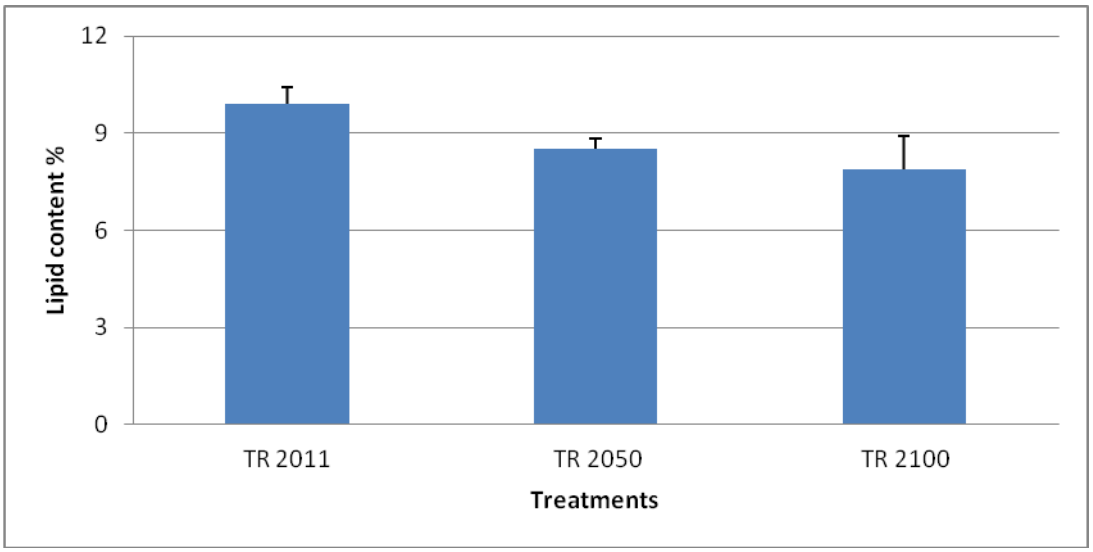

Figure 6.20 Effect of increased temperature and decreased pH level on mean percentage lipid content (±SE) in *A. aspersa* at the end of experiment.

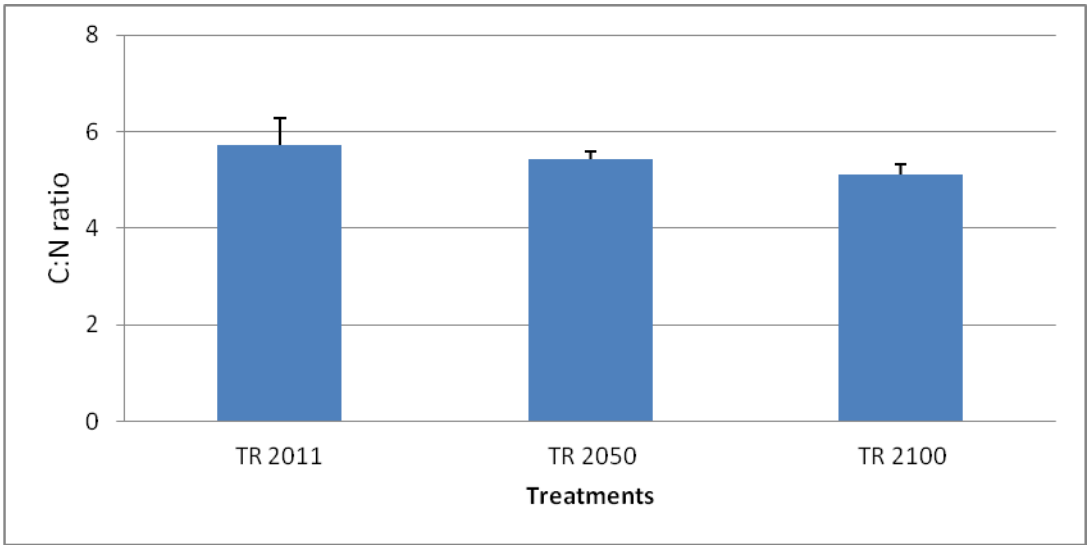

Figure 6.21 Effect of increased temperature and decreased pH level on mean percentage C:N ratio (±SE) in *A. aspersa* at the end of experiment.

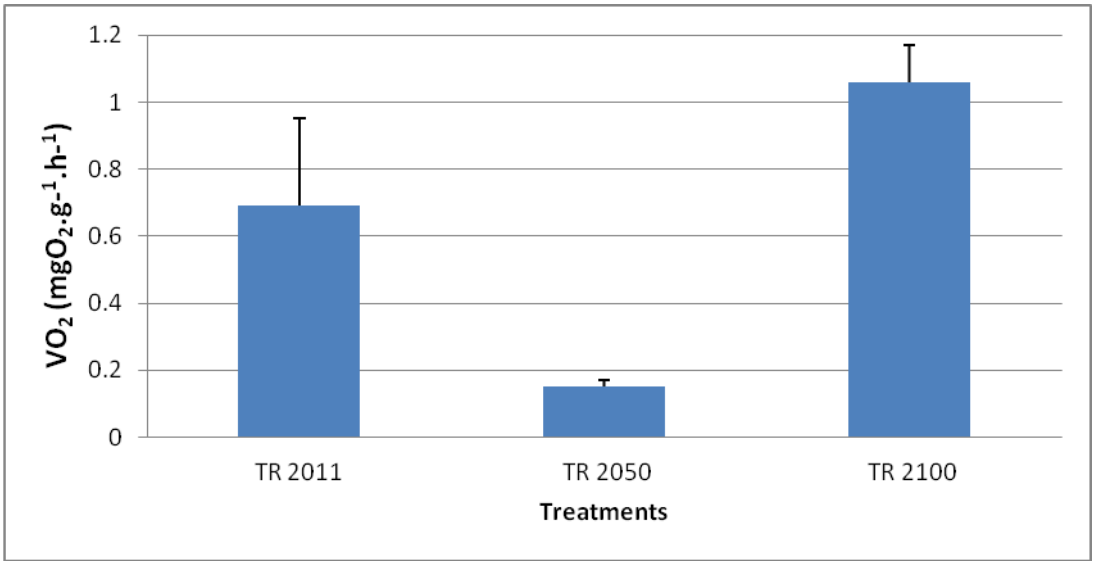

Figure 6.22 Effect of increased temperature and decreased pH level on mean oxygen consumption (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) (±SE) in *A. aspersa* at the end of experiment.

6.4 Discussion:

6.4.1 Effects on M. edulis

Most measurements of *M. edulis* were not significantly different (Tables 6.2 and 6.3). However, there was a significant rise in oxygen consumption (F = 5.845, p = 0.007) in TR 2100 compared with TR 2011 (p = 0.013) and TR 2050 (p = 0.042), which means an increase in the metabolic rate (Fig. 6.5). Referring back to the results of Chapter 5, we found that TR 2100 recorded the lowest growth of body weight and buoyant weight changes. With rising rates of metabolism and a lack of growth, this may be an indication that future conditions of high temperatures and low pH levels did not disturb metabolisms, but led to the conversion of energy consumption used in the growth to be used in the maintenance and repair for maintaining survival. In this way the organisms were able to resist and reduce the mortality rate to reach about 20% only compared to 10% for the control treatment. Soft tissue weight did not record a significant change in all the measurements (Table 6.3, Fig. 6.2), which are similar to the results obtained by Thomsen and Melzner (2010) and Thomsen *et al*. (2010). This study has also seen an increase in the rates of metabolism in treatment TR 2100 at pH (7.65) and 19.2 ^{o}C which are similar to the same rates of Thomsen and Melzner (2010). This increase in metabolic rate may indicate the probability of increasing the cost of cellular calcification and balance at low pH levels, combined with high temperatures (Thomsen *et al*., 2010). It should be noted here that there had been a decrease in metabolism in TR 2050 (0.48 ± 0.08, Table 6.4) compared to TR 2011 (0.59 ± 0.13, Table 6.4), although not at significant levels, but it was accompanied by a slight reduction in dry soft tissue weight compared to the control treatment (Fig. 6.2). A significant rise in the proportion of the lipid content demonstrated (F = 4.071 ± 0.045%, Table 6.3, Fig 6.4) when compared to TR 2011 (p = 0.046). This may indicate that medium treatment may be affected by exposure to the temperature and pH levels expected in 2050, which led to changes in metabolism resulting in an increase in lipid content with a decrease in soft tissue weight. This may be a kind of resistance to survive, especially when compared to the results of Chapter 5 which indicate an increase in the body and buoyant weight changes in TR 2050 compared to TR 2100 and a decrease in growth when compared with the control treatment (TR 2011). Results in the previous chapter showed improvement in the

mortality rate in the fifth week which may indicate the development of a kind of ability to cope with the conditions and the surrounding variables, represented by dissolution of the shell and reduction of the metabolism as a means to maintain the stability of pH in the cells at TR 2050 (see Gazeau *et al*., 2007). This may be due to the exposure of mussels *M. galloprovincialis* to high levels of acidity up to pH 7.3. It is thus clear that the increased temperatures and decreased pH levels, as expected during the middle and end of this century in the summer months, had an impact on calcified organisms and led to a decrease in growth and an increase in mortality, however these organisms have managed to reduce this effect and continue to live. Those changes were not a significant danger that could threaten these organisms in the near-future.

6.4.2 Effects on L. littorea

The marine snail (*L. littorea*), which was exposed to the expected conditions in the middle of this century, provides a typical example for survival by reducing growth calcification rates (see Chapter 5). The organism showed a significant reduction in the rates of oxygen consumption in TR 2050 (p = 0.018, Fig. 6.6), resulting in a decrease in the rates of metabolism and therefore the reduction of total growth rates. All of this for the sake of survival by up to 93%, which is only less by 3% compared to TR 2011 (see Chapter 5 and Gazeau *et al*., 2007). This was accomplished by exposing the marine snail animals under study to the expected rises in temperature at the end of this century by as much as 19.2 ^{o} C and in pH levels by 7.65 (TR 2100), which led to a significant increase in metabolic rate (p = 0.014). Also, there was a significant rise in the lipid content of TR 2100 (p = 0.006, Fig. 6.10). These are in comparison with the results obtained in Chapter 5 which indicated a reduction in the body weight change and buoyant weight change compared with the control treatment, while maintaining a mortality rate at under 10%, which is only 6% in TR 2100 less than the control treatment. It is clear from all of the above that exposure to the double factors expected in 2100 has had a negative impact on the *L. littorea* caused by the increase in the cost of producing the energy needed for the restoration, maintenance and preservation of the vitality of the cells, especially in case of the availability of food.

6.4.3 Effects on A. equina

At the same time, it was clear that there was a positive impact for future levels of temperature and pH levels on *A. equina*. This study found higher rates of oxygen consumption ($F = 8.837$, $p = 0.001$, Table 6.7, Fig. 6.17) for TR 2050 and TR 2100 associated with high levels of lipid content ($F = 15.476$, $p < 0.001$, Table 6.8, Fig. 6.15) in the dry soft tissue weight, which in turn was high when compared to the control treatment. This explains the ability of these organisms to survive by 100% during the experiments if we go back to the results of Chapter 5. These results also indicate an increase in the body weight change of the control treatment accompanied by a decrease in buoyant weight change and this may be due to the increased water content and not due to increased soft tissue weight, which is a very slight increase. These results of the dry weight gain (Table 6.7, Fig 6.13), with increasing temperatures and decreasing pH levels, appeared in algal turfs (see Connell and Russell, 2010).

6.4.4 Effects on A. aspersa

In *A. aspersa* exposed to the climatic conditions expected at the end of this century (TR 2100), metabolism was not disrupted due to these changes; on the contrary, there was a significant rise in oxygen consumption ($p < 0.001$, Fig. 6.22) concurrent with a decrease in lipid content (non-significant) and an increase in the percentage of soft tissues compared with the other two treatments (TR 2011 and TR 2050) strengthen the results obtained when studying some non-calcified organisms (Attrill *et al.*, 2007; Richardson and Gibbons, 2008; Connell and Russell, 2010). This may also strengthen the results of Chapter 5 that treatment TR 2100 obtained the highest percentage of survival, which was 23%, an increase of 8% compared to the control treatment. TR 2050 showed a significant drop in metabolic rates compared with TR 2100 ($p < 0.001$, Fig. 6.22) but with a rise in buoyant weight change (Chapter 5). This indicates that it has been able to adapt to changing conditions to continue the physiological processes even with a decrease as an expected reaction to resist these changes in ocean temperature and pH.

6.5. Conclusion:

This study conducted on adult members of the four species of coastal marine invertebrates for six weeks under estimated future climatic conditions of high temperatures and low pH in the middle and at the end of the twenty-first century. Our results showed a significant rise in the metabolic rates in all species when exposed to the climatic conditions expected at the end of this century. This indicates increased cost of energy for their growth and survival (biological processes) (Thomsen *et al.*, 2010). While the medium treatment TR 2050 recorded a significant decrease in only one case, in *L. littorea*, which did not affect the general functionality significantly but was one of the methods used to survive by lowering metabolic rates (Michaelidis *et al.*, 2005), this is a well-known strategy for adaptation (Widdicombe and Needham, 2007). These results dramatically demonstrate that the fears of climate impact threatening marine invertebrates at the temperatures expected during this century are grounded in reality, however may be exaggerated to some extent. Moreover, the disruption in the vital functions that may occur when exposed to climatic conditions is greater than expected during this century (Thomsen and Melzner, 2010). On the other hand, even the unexpected positive changes may be evidence of disorders in the ecosystems (Connell and Russell, 2010), which may have a negative effect over the long term. These results show complex reactions to future climate changes not only for the different species of marine invertebrates, but also for the observed difference in reactions between the same species on the expected changes during the middle and end of this century. It is also noted here that the decrease in metabolic rate does not depend on the increase in the reduced levels of pH that could occur within the next 50–100 years but it might be true in the high levels of acidification (Thomsen and Melzner, 2010).

Chapter 7. Seasonal Effects of Temperature and pH on four Common British Marine Invertebrates and General Discussion

Abstract:

Climate change is likely to have profound effects on marine animals due to the predicted increases in seawater temperature and acidity. Many studies have examined the effects on marine animals of climate change at the extreme temperatures expected for the summer season, but few studies have investigated how altered temperature profiles may affect these animals in the winter. This study conducted two series of laboratory experiments and investigated the effect of both winter and summer temperatures on the metabolic rate of intertidal marine invertebrate species: two calcified (*Mytilus edulis* and *Littorina littorea*) and two non-calcified (*Actinia equina* and *Ascidiella aspersa*). Following a period of acclimatisation during which the temperature was gradually increased and pH decreased, animals were exposed to the climatic conditions predicted for 2050 (TR 2050) and 2100 (TR 2100) over a six-week period. During these experiments, survival, body weight change, and buoyant weigh change were measured. At the end of the experiments, metabolic rates, and lipid content were measured. The present study revealed a contrast between calcified and non-calcified marine organisms as to the manner in which each deals with the high temperatures and high acidity in different seasons. These distinctions became even clearer the variation in how the same species dealt with the different levels of acidity and different temperature regimes. Calcified animals showed positive growth and survival responses in all seasons in comparison with the non-calcified animals, although they were in their best condition in the winter season. There were no statistically significant differences in growth in any of the species used in this study due to season. There was a significant decrease in lipid content in *L. littorea* under the summer treatment (TR 2011S) compared with the winter treatment (TR 2011W). Metabolic rates showed a noticeable increase in all species of summer treatment (TR 2100S). The summer treatment results in a significant increase in metabolic rates when compared with winter treatments for *M. edulis*, *L. littorea*, and *A. aspersa*. In this study two separate series of experiments were performed, but conducting experiments on the same sample animals over an entire year might allow more

comprehensive findings on the ability of these organisms to adapt to variable seasons and long-term exposure to high temperature and low pH.

7.1 Introduction:

A review of the research on the impact of climate change on marine invertebrates reveals a scarcity of studies concerned with seasonal influences or comparisons between winter and summer, despite the importance of seasonality in pH changes (Findlay *et al*., 2009a). During the summer, pH can drop to 7.5 and during the winter increase again by 1.0 unit up to 8.5 (Findlay *et al.*, 2008). In addition to the fact that temperatures reach maxima in the summer and minima in the winter, rising temperatures could lead to maximally increased rates of calcification (Marshall & Clode, 2004; Rodrigues & Grottoli, 2006) in summer (Rodolfo-Metalpa *et al.*, 2009; Rodolfo-Metalpa *et al.*, 2010). However, any temperature decrease may increase dissolution of $CaCO^3$ from shells (McClintock *et al.*, 2009) and thus decrease calcification rates in winter (Rodolfo-Metalpa *et al*., 2009; Rodolfo-Metalpa *et al*., 2010). For example, we find that more than 90% of the growth of mussel shells was recorded in the period between April and September (Boyden, 1971; Nagarajan *et al.*, 2006). At the same time, a decrease in temperature in winter is accompanied by a decrease in phytoplankton abundance (Bayne and Worrall, 1980; Nagarajan *et al.*, 2006) and thus a decrease in the growth of invertebrates. When surrounding temperatures are less than 10 °C in winter and greater than 20 °C in summer, growth in the mussel decreases (Bayne and Worrall, 1980; Nagarajan *et al*., 2006). High temperatures within the optimal limits, together with abundant food increases observed growth. For example when temperatures are at their highest summer average, conditions are suitable for the highest observed rates of calcification in corals (Marshall & Clode, 2004; Rodrigues & Grottoli, 2006). However, when summer high temperature tend to be above average, lower rates of calcification are observed (Clausen and Roth, 1975; Rodrigues and Grottoli, 2006). Low winter water temperatures near 5 °C have been shown to affect *Cerastoderma edule*, snails that are also known as cockles, and led to decreased growth (Jones, 1979; Nagarajan *et al.*, 2006). Mussels also display the same growth inhibition at 5 °C (Melzner *et al*., 2011),

which indicates the effect of seasonality on variation in shell weight (De Moel *et al.*, 2009).

In any case, semi-fatal effects such as low growth may arise due to prolonged exposure to extreme temperatures, whether high in summer or low in winter. Furthermore, increased warming during the seasons that are supposed to be relatively cool can send early signals of season change and cause animals to respond to such signals at seasonally inappropriate times (known as a shift in phenology) (Noone *et al.*, 2013).

In this chapter, results of the studies comparing changes in body weight, buoyant weight, lipid content, and metabolism between winter treatments and corresponding summer treatments are presented. Data from the winter and summer control treatments were compared; then the TR 2050 winter treatment, TR 2050W was compared with the TR 2050 summer treatment, TR 2050S; and finally the TR 2100 winter treatment, TR 2011W, was compared with its TR 2100 counterpart in the summer, TR 2100S.

7.2 Methods:

The methods of this chapter were described in detail in Chapter 2, and methods specific to the winter and summer seasons were described in Chapters 3 and 5, respectively. Tables 7.1, 7.2, and 7.3 give information about dates of animal collections and experimental periods, numbers of animals used for each measurement, and seawater parameters measured during these experiments (for more see Chapters 2, 3, and 5).

Table 7.1 Dates of collections and experimental periods of winter and summer experiments.

Season	Experiment No.	Collection date	Experiment period
Winter	1	01 February 2012	01 February–28 March 2012
	2	24 September 2012	24 September–22 November 2012
Summer	1	24 May 2012	24 May–17 July 2012
	2	19 July 2012	19 July–13 September 2012

Table 7.2 Numbers of samples used to measure parameters in these study species for the winter and summer experiments.

Parameters	Treatments											
measured	TR 2011				TR 2050				TR 2100			
	M.e.	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*	*M.e.*	*L.l.*	*A.e.*	*A.a.*
No.[a] at start [w]	84	66	53	102	81	69	46	97	78	63	49	89
No.[a] at start [s]	71	102	53	33	71	108	50	37	78	106	46	40
No.[a] at end [w]	84	66	34	98	68	67	34	63	71	62	35	16
No.a at end [s]	64	98	53	5	56	100	50	6	61	95	46	9
No.[a] surviving[w]	84	66	53	102	81	69	46	97	78	63	49	89
No.[a] surviving[s]	71	102	53	33	71	108	50	37	78	106	46	40
Body weight[w]	12	12	12	14	12	12	12	9	12	12	11	4
Body weight[s]	12	12	12	4	11	11	12	3	12	11	12	2
Buoyant weight[w]	12	12	12	14	12	12	12	9	12	12	11	4
Buoyant weight[s]	12	12	12	4	11	11	12	3	12	11	12	2
Lipid content[w]	6	5	6	6	6	5	5	5	6	6	6	2
Lipid content[s]	5	5	5	3	5	5	5	3	5	5	5	2
Metabolic rate[w]	10	12	12	12	12	12	12	12	12	12	11	8
Metabolic rate[s]	12	12	12	7	12	12	12	10	12	12	12	10

[a] refers to number of animals used to calculate survival; [w] refers to the winter season; [s] refers to the summer season; *M.e.* refers to *M. edulis*; *L.l.* refers to *L. littorea*; *A.e.* refers to *A. equina*; and *A.a.* refers to *A. aspersa*.

Table 7.3 Parameters of seawater chemistry measured during the summer and winter experiments in the control the treatment and future condition treatments TR 2050 and TR 2011 (Mean ± S.E.).

Season	Treatment	Temperature (°C)	Salinity (ppt)	pH	O_2 (%)
Winter	TR 2011	4.7 (± 0.20)	36.1 (± 1.08)	8.05 (± 0.07)	97.4 (± 1.40)
	TR 2050	6.4 (± 0.20)	36.0 (± 1.18)	7.90 (± 0.06)	97.3 (± 1.60)
	TR 2100	8.7 (± 0.24)	35.9 (± 1.11)	7.75 (± 0.07)	96.8 (± 1.74)
Summer	TR 2011	14.8 (± 0.13)	36.3 (± 1.23)	8.05 (± 0.14)	96.6 (± 1.45)
	TR 2050	17.0 (± 0.20)	36.6 (± 1.20)	7.84 (± 0.11)	97.0 (± 1.45)
	TR 2100	19.2 (± 0.20)	36.6 (± 1.22)	7.65 (± 0.08)	96.8 (± 1.46)

7.3 Results:

7.3.1 Effect of Seasonality on Survival:

Mytilus edulis:

The results of survival analysis revealed an increase in mortality in the summer in comparison with the corresponding treatments in the winter (Table 7.4,

Fig. 7.1). The largest difference (13%) was between TR 2100W (91%) compared with TR 2100S (78%), although this value was very close to the difference in mortality observed between the summer and winter controls (10%).

Littorina littorea:

The results of survival analysis showed an increase in mortality in the summer in comparison with the corresponding treatments during the winter. Generally, the mortality rate is very low; the lowest value was recorded in TR 2100S and did not exceed 10% (Table 7.4, Fig. 7.2). However, the difference in mortality between TR 2100W compared with TR 2100S was 8%, while the difference in mortality between the control treatments was 4%.

Actinia equina:

The results showed that the preponderance of individuals survive the summer in comparison with the results for winter in all treatments. No deaths were recorded in the summer, while the highest winter mortality rate of 36% recorded the control treatment TR 2011W (Table 7.4, Fig. 7.3).

Ascidiella aspersa:

Summer mortality rates were very high for all treatments and resemble rates observed in the winter treatment at the highest temperature and acidity levels (TR 2100W). The percentage survival under the control treatment in the winter (TR 2011W) reached 96% (Table 7.4, Fig. 7.4).

Table 7.4 Percentage survival of the four study species under different treatments over a six-week period in the winter and summer treatments.

Season	Species	TR 2011		TR 2050		TR 2100	
		n	Survival %	n	Survival %	n	Survival %
Winter	*M. edulis*	84	100	81	84	78	91
Summer		71	90	71	79	78	78
Winter	*L. littorea*	66	100	69	97	63	98
Summer		102	96	108	93	106	90
Winter	*A. equina*	53	64	46	74	49	71
Summer		53	100	50	100	46	100
Winter	*A. aspersa*	102	96	97	65	89	18
Summer		33	15	37	16	40	23

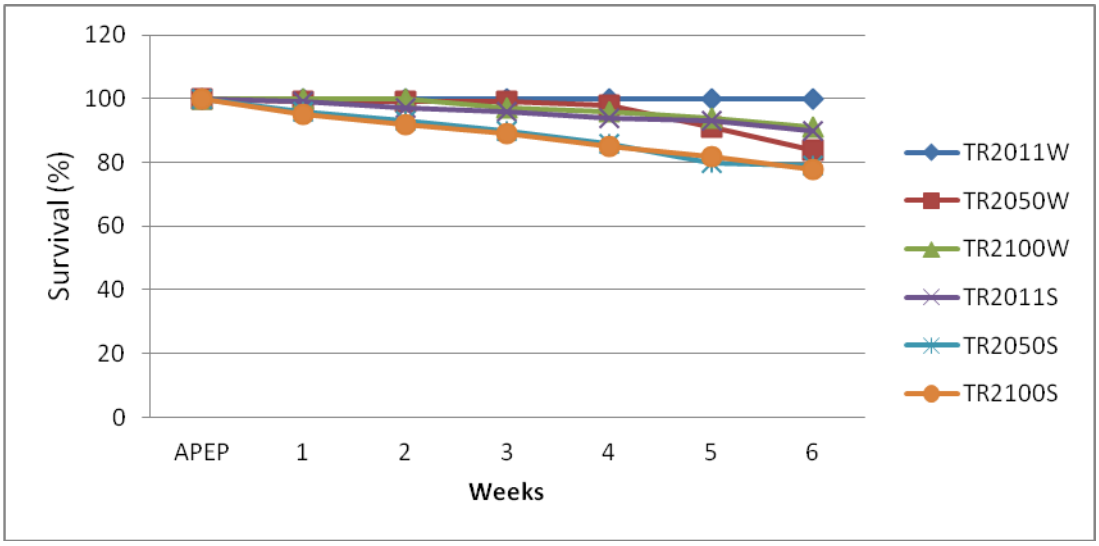

Figure 7.1 Percentage survival of *M. edulis* over a six-week experimental period for all treatments under winter and summer conditions. In the treatment labels, "W" represents winter and "S" represents summer. The experimental winter treatment TR 2011W is represented by blue diamonds, TR 2050W is represented by red squares, and TR 2100W is represented by green triangles. The experimental summer treatment TR 2011S is represented by purple Xs, TR 2050S is represented by cyan asterisks, and TR 2100S is represented by orange circles. APEP represents the acclimatization period end points.

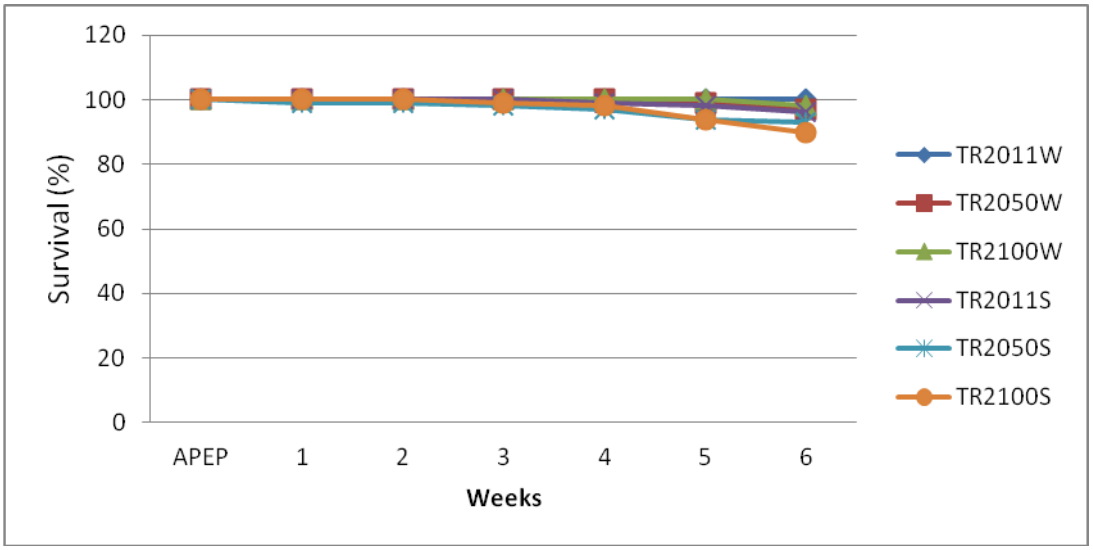

Figure 7.2 Percentage survival of *L. littorea* over a six-week experimental period for all treatments under winter and summer conditions. In the treatment labels, "W" represents winter and "S" represents summer. The experimental winter treatment TR 2011W is represented by blue diamonds, TR 2050W is represented by red squares, and TR 2100W is represented by green triangles. The experimental summer treatment TR 2011S is represented by purple Xs, TR 2050S is represented by cyan asterisks, and TR 2100S is represented by orange circles. APEP represents the acclimatization period end points.

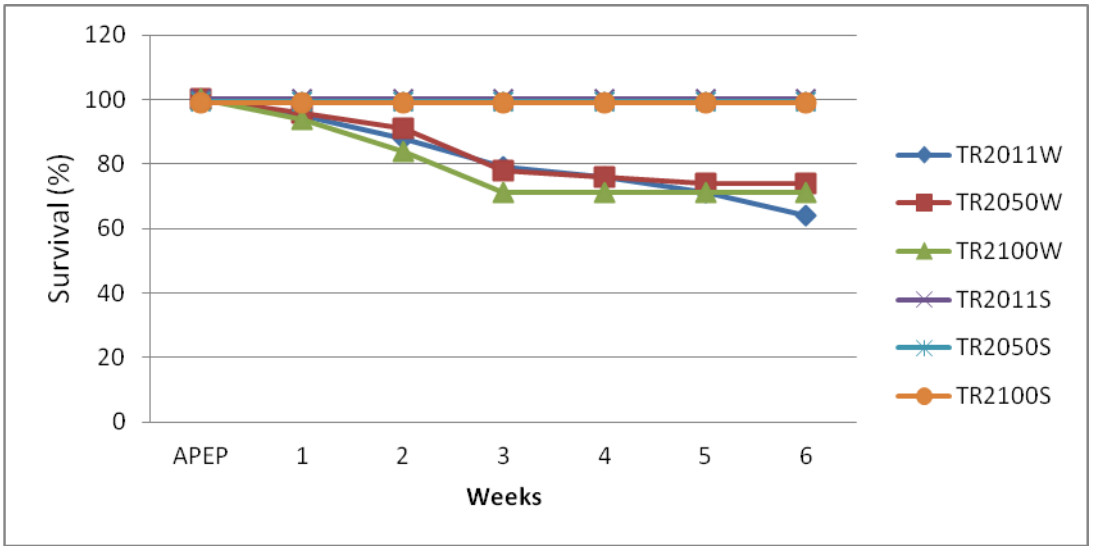

Figure 7.3 Percentage survival of *A. equina* over a six-week experimental period for all treatments under winter and summer conditions. In the treatment labels, "W" represents winter and "S" represents summer. The experimental winter treatment TR 2011W is represented by blue diamonds, TR 2050W is represented by red squares, and TR 2100W is represented by green triangles. The experimental summer treatment TR 2011S is represented by purple Xs, TR 2050S is represented by cyan asterisks, and TR 2100S is represented by orange circles. APEP represents the acclimatization period end points.

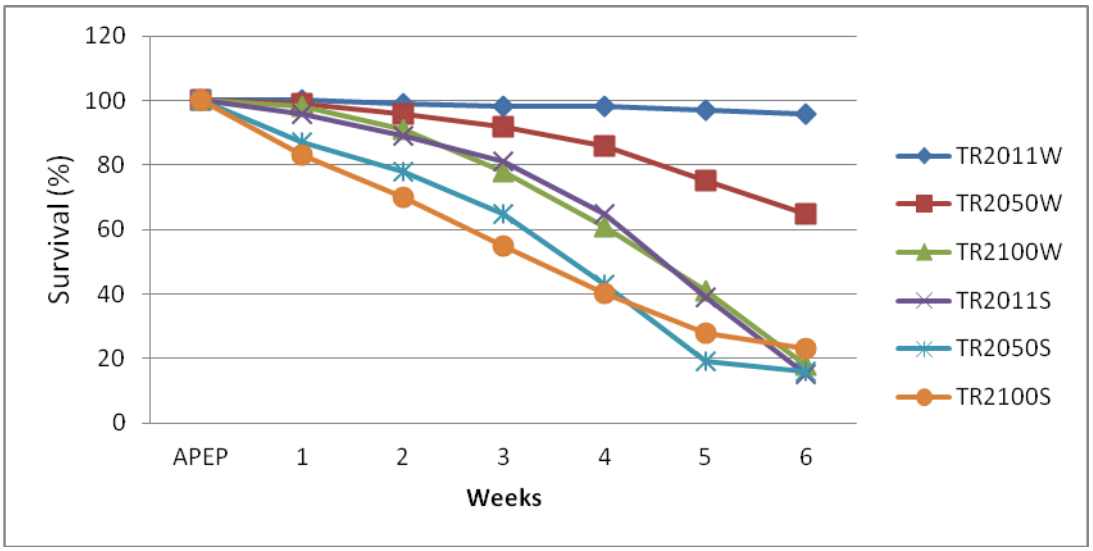

Figure 7.4 Percentage survival of *A. aspersa* over a six-week experimental period for all treatments under winter and summer conditions. In the treatment labels, "W" represents winter and "S" represents summer. The experimental winter treatment TR 2011W is represented by blue diamonds, TR 2050W is represented by red squares, and TR 2100W is represented by green triangles. The experimental summer treatment TR 2011S is represented by purple Xs, TR 2050S is represented by cyan asterisks, and TR 2100S is represented by orange circles. APEP represents the acclimatization period end points.

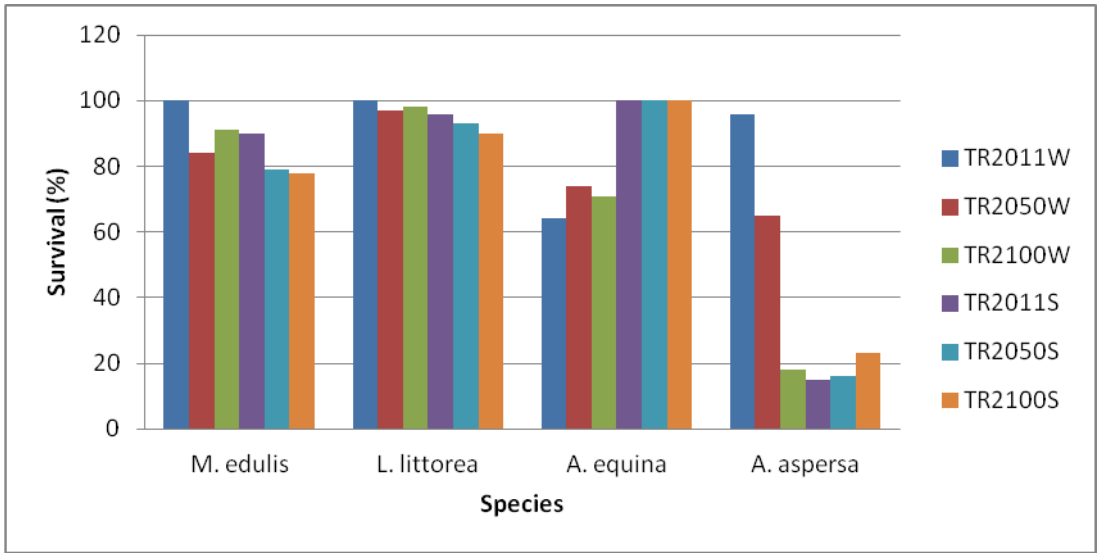

Figure 7.5 The chart compares survival rates for all species and treatments under study. Seasonal effects were observed more for non-calcified organisms than for calcified marine invertebrates. In the treatment labels, "W" represents winter and "S" represents summer.

7.3.2 Effect of Seasonality on Growth and Body Composition:

Mytilus edulis:

Results of Shapiro-Wilk's tests indicated that data for body weight change, buoyant weight change, and dry weight in *M. edulis* were not normally distributed, so the non-parametric Kruskal-Wallis test was used to compare treatment effects (Table 7.5). The Mann-Whitney U test was then used *post hoc* to test for significant differences between specific treatments. However, metabolic rate was normally distributed, so treatments were compared with one-way ANOVA (Table 7.6) and Scheffe's method was then used *post hoc* to test for differences between treatments.

There were no significant differences in body weight change or lipid content in *M. edulis* between the corresponding treatments under winter and summer conditions (see Tables 7.5 and 7.6, and Fig. 7.6 and Fig. 7.8). Although there was a significant difference in buoyant weight change between some treatments, there was no significant difference between corresponding treatments in winter and summer conditions (Table 7.5, Fig. 7.7). Also, a significant difference in metabolic rate (Table

7.6, Fig. 7.9) between TR 2100W compared with TR 2100S (p = 0.032), was identified using the Mann-Whitney U test

Table 7.5 Statistical analysis of body weight change, buoyant weight change, and lipid content of *M. edulis*.

	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-square	df	*p* value
Body weight	0.770	71	< 0.001	4.050	5	0.542
Buoyant weight	0.886	71	< 0.001	11.374	5	0.044
Lipid content[a]	0.960	33	0.261	7.196	5	0.206

[a] Treatment effects for lipid content were analysed using the Kruskal-Wallis test because its variance was not homogeneous; the Levene's test for homogeneity of variances was significant (p = 0.003).

Table 7.6 Statistical analysis of metabolic rate of *M. edulis*.

		ANOVA test		Normality test			Homogeneity test	
Parameter		One-way		Shapiro-Wilk's test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Metabolic rate	72	3.448	0.008	0.979	72	0.259	1.663	0.156

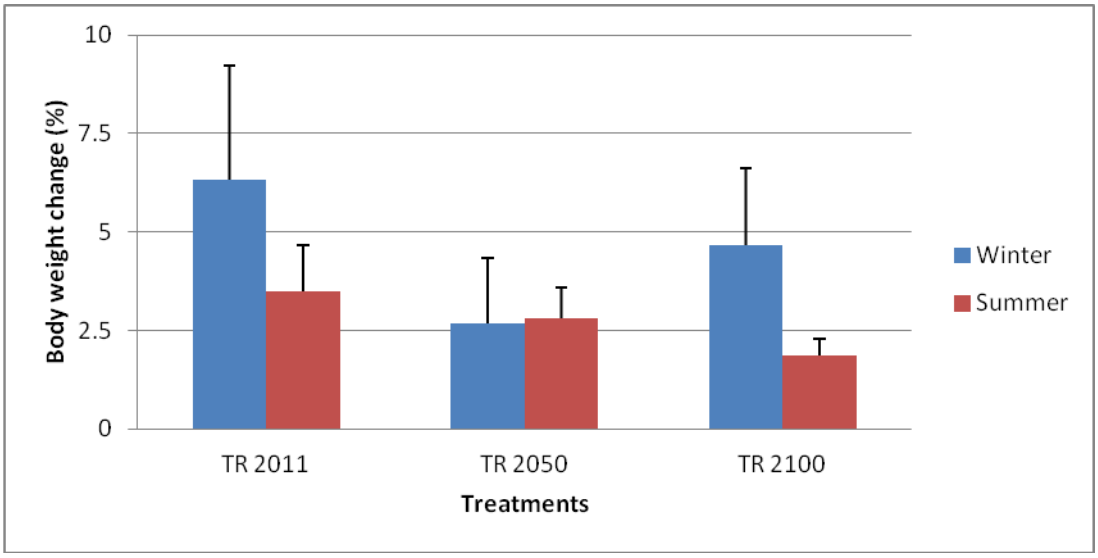

Figure 7.6 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) body weight change in *M. edulis* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

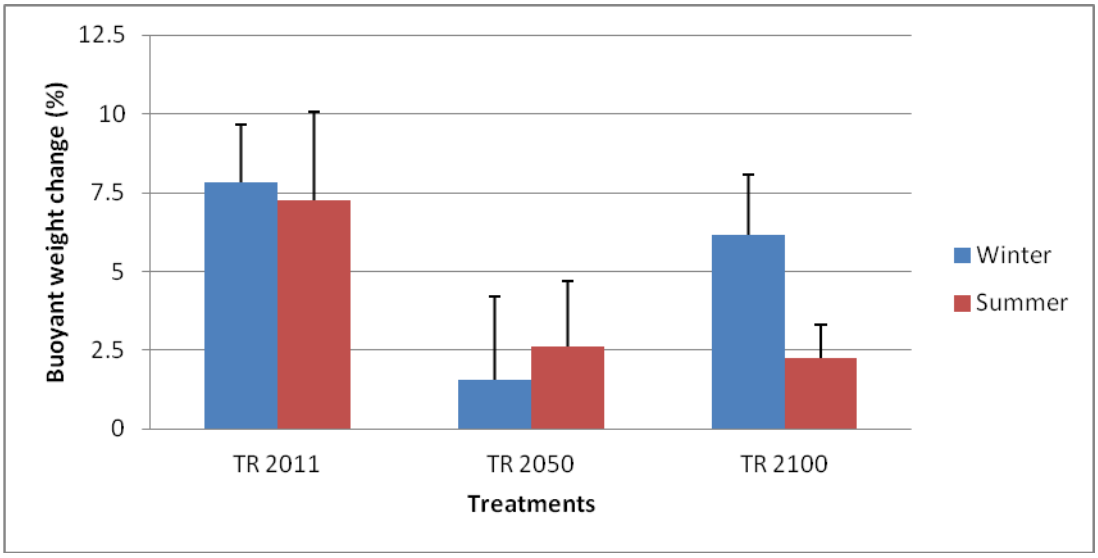

Figure 7.7 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) buoyant weight change in *M. edulis* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

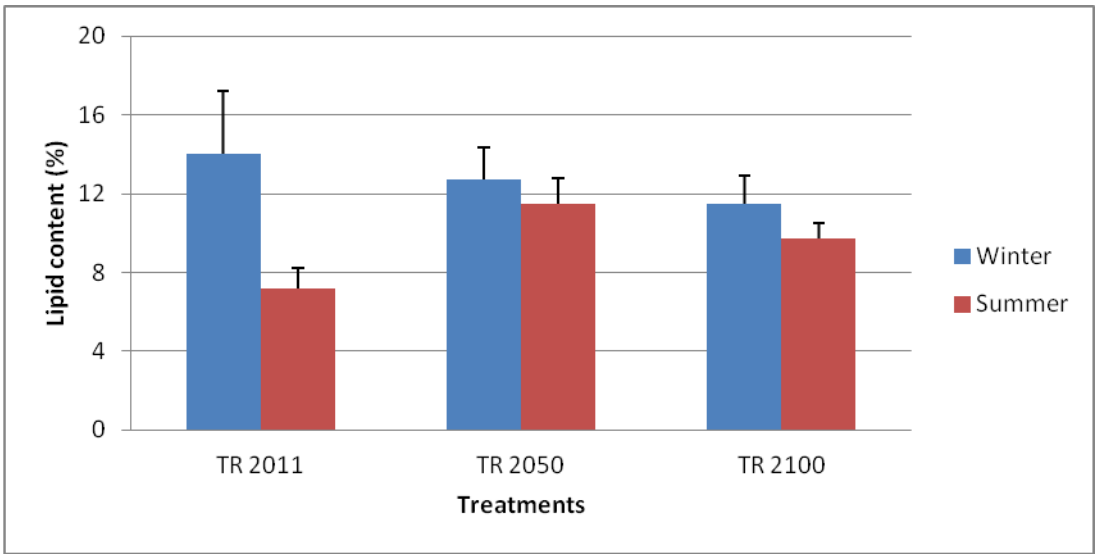

Figure 7.8 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) lipid content in *M. edulis* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

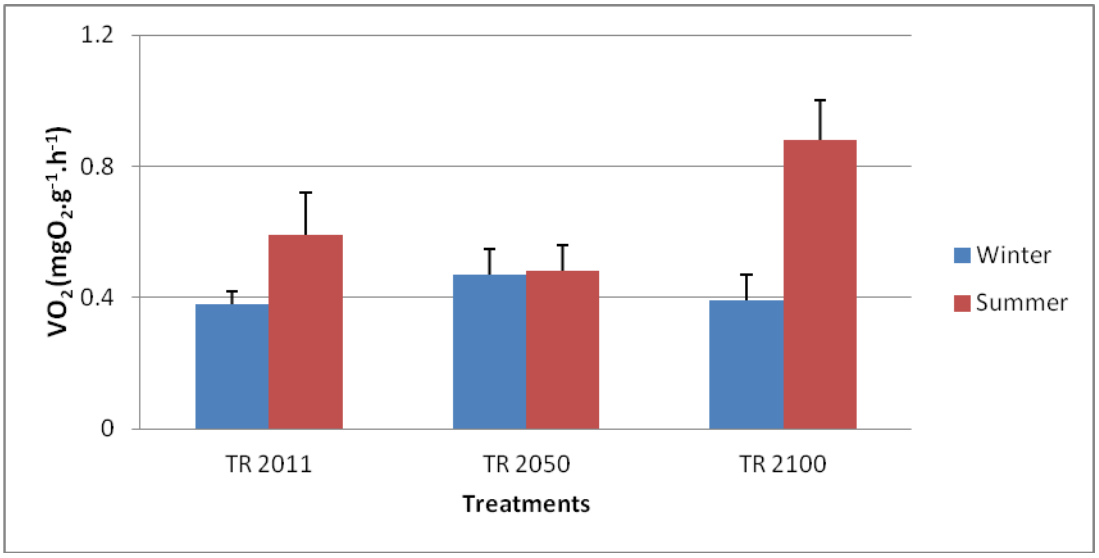

Figure 7.9 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) metabolic rate (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) of *M. edulis* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

Littorina littorea:

The Shapiro-Wilk's test indicated that data for body weight change was not normally distributed. Therefore, the non-parametric Kruskal-Wallis test was used (Table 7.7) with a *post hoc* Mann-Whitney U test to detect significant differences between treatments. However, lipid content and metabolic rate were normally distributed and treatments effects for those parameters were compared using one-way ANOVA (Table 7.8). Scheffe's method was then used *post hoc* to test for significant differences between treatments.

Table 7.7 Statistical analysis of body weight change and buoyant weight change in *L. littorea*.

	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
Parameter	Statistics	df	*p* value	Chi-square	df	*p* value
Body weight	0.931	70	0.001	13.917	5	0.016
Buoyant weight[a]	0.975	70	0.171	11.126	5	0.050

[a] Buoyant weight change was analysed using the Kruskal-Wallis test because the Levene's test for homogeneity of variances was significant ($p = 0.020$).

Table 7.8 Statistical analysis of lipid content and metabolic rate in *L. littorea.*

		ANOVA test		Normality test			Homogeneity test	
Parameter		One-way		Shapiro-Wilk's test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Lipid content	31	5.360	0.002	0.963	31	0.351	0.396	0.847
Metabolic rate	72	9.244	<0.001	0.970	72	0.088	1.853	0.115

There was no significant difference in buoyant weight change in *L. littorea* between the corresponding treatments in winter and summer (see Table 7.7, Fig. 7.11). However, there was a significant difference in body weight change between some treatments, but not between corresponding treatments in winter and summer (Table 7.7, Fig. 7.10). Also, significant differences were found in lipid content (Table 7.8, Fig. 7.12) between TR 2011W and TR 2011S (p = 0.016), and in metabolic rate (Table 7.8, Fig. 7.13) between TR 2100W and TR 2100S (p = 0.029) using the Mann-Whitney U test.

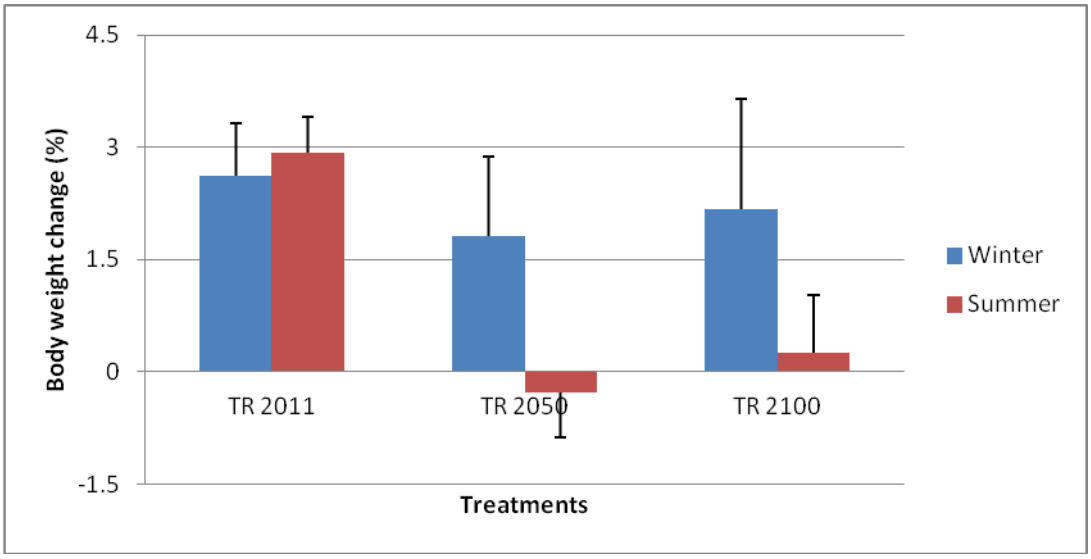

Figure 7.10 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) body weight change in *L. littorea* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

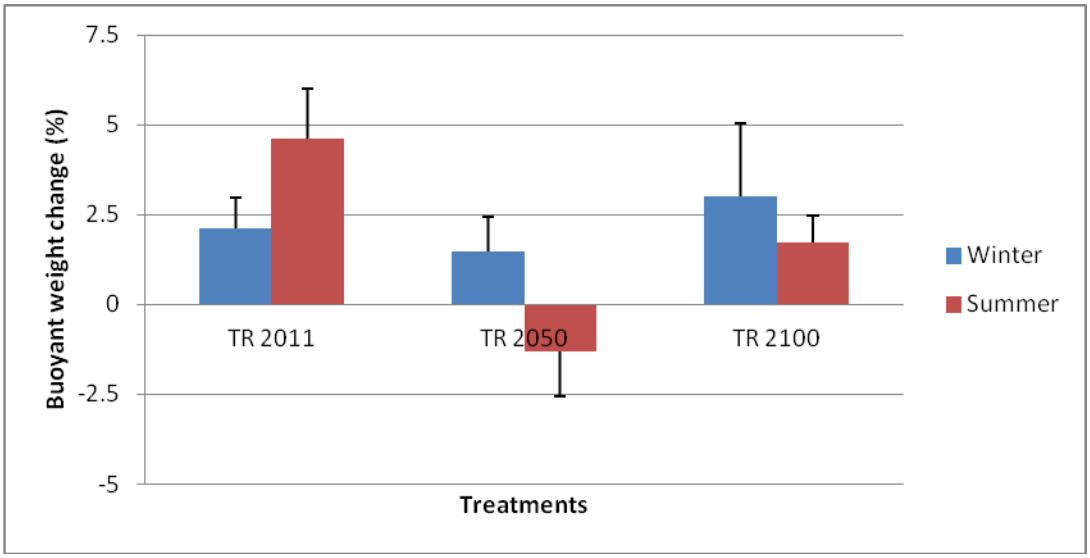

Figure 7.11 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) buoyant weight change in *L. littorea* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

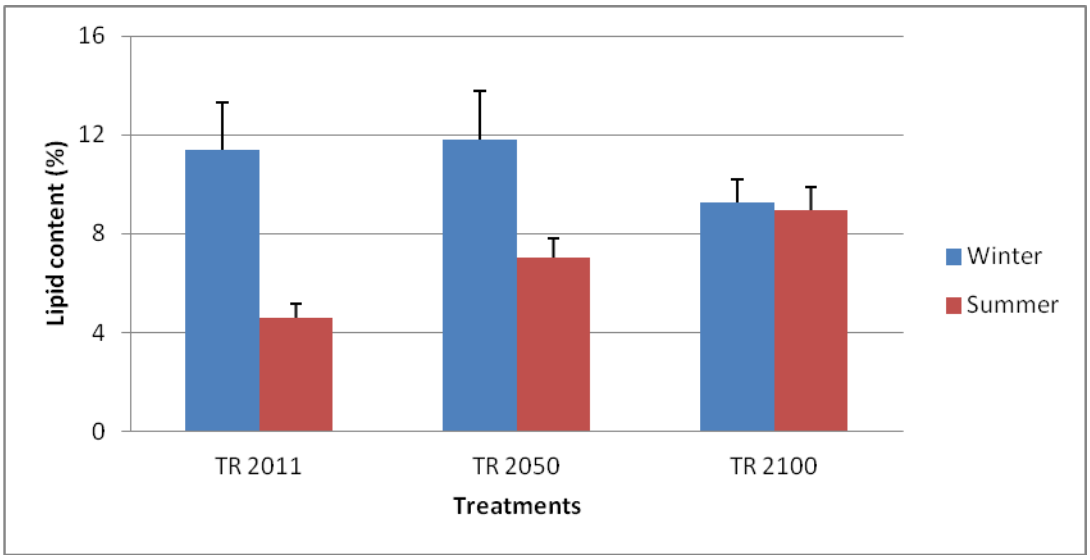

Figure 7.12 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) lipid content in *L. littorea* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

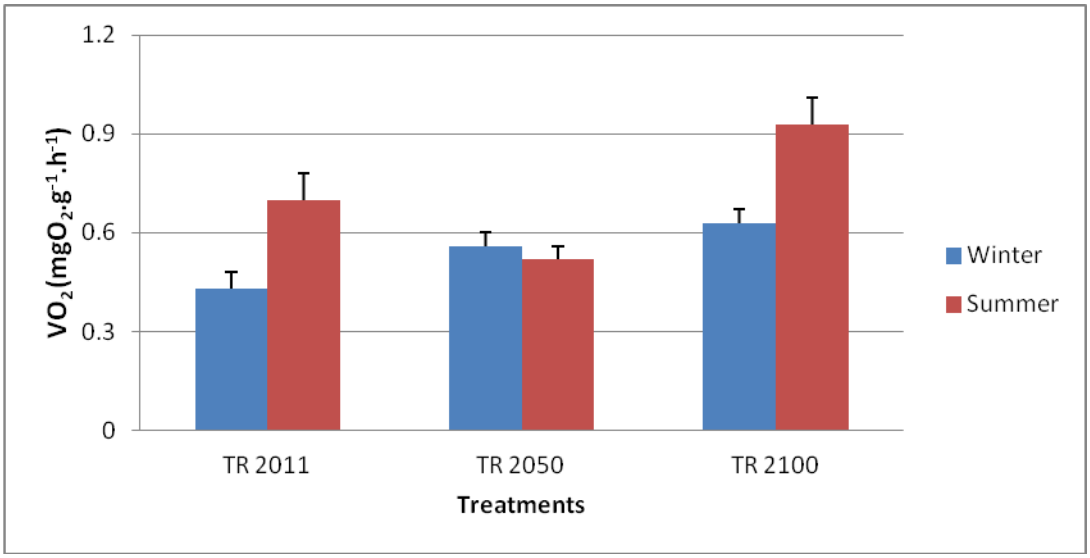

Figure 7.13 Effect of increased temperature and decreased pH in winter and summer on mean (with SE bar) metabolic rate (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) in *L. littorea* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

Actinia equina:

Data for lipid content change were not normally distributed according to a Shapiro-Wilk's test, therefore, the non-parametric Kruskal-Wallis test was used to compare treatments (Table 7.9). The Mann-Whitney U test was then used *post hoc* to test for significant differences between specific treatments. However, data for body weight change, buoyant weight change, and metabolic rate were normally distributed, so treatments were compared by one-way ANOVA (Table 7.10). Scheffe's method was then used as a *post hoc* test for significant differences between treatments.

Table 7.9 Statistical analysis of lipid content in *A equina*.

	Normality test			Treatment comparison		
Parameter	Shapiro-Wilk's test			Kruskal-Wallis test		
Measurement	Statistics	df	*p* value	Chi-Square	df	*p* value
Lipid content[a]	0.968	32	0.439	12.109	5	0.033

[a] Lipid content analysed using the Kruskal-Wallis test because according to the Levene's test there was significant ($p < 0.001$) for homogeneity of variances, that mean the variance of this lipid content was not homogeneous .

Table 7.10 Statistical analysis of body weight change, buoyant weight change, and metabolic rate in *A. equina*.

		One-way		Normality test			Homogeneity test	
Parameter		ANOVA		Shapiro-Wilk's test			Levene's test	
	n	F	*p*	Statistics	df	*p*	Statistics	*p*
Body weight	67	8.469	<0.001	0.973	67	0.155	1.241	0.301
Buoyant weight	70	1.618	0.168	0.980	70	0.307	1.462	0.215
Metabolic rate	71	3.970	0.003	0.991	71	0.876	0.400	0.847

There was no significant difference in buoyant weight change in *A. equina* between the corresponding treatments in winter and summer (see Table 7.10, Fig. 7.15). However, there were significant differences in body weight change, lipid content, and metabolic rate between some treatments, but not between corresponding treatments in winter and summer (Table 7.10, Fig. 7.14, Fig. 7.16, and Fig. 7.17).

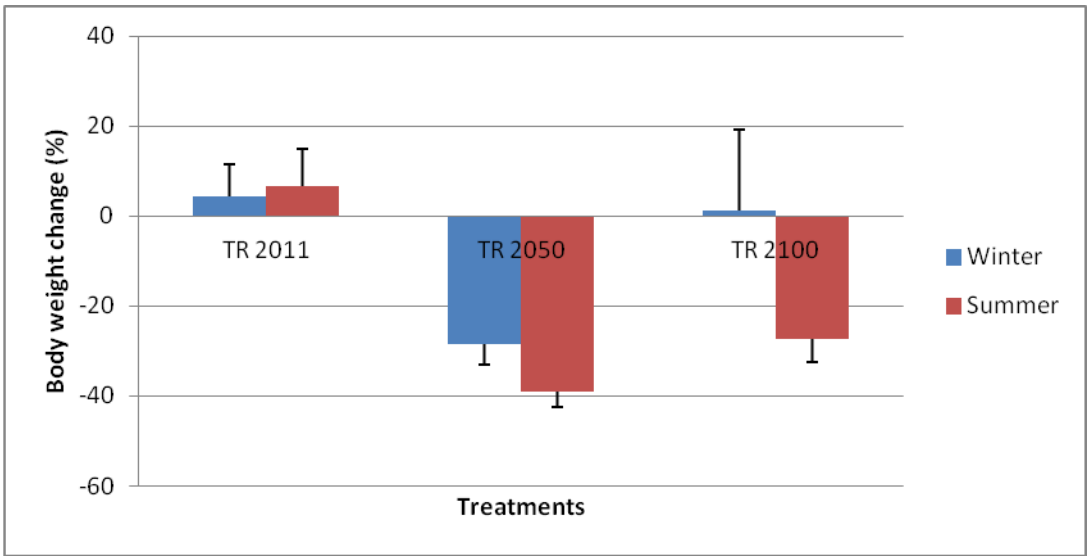

Figure 7.14 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) body weight change in *A. equina* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

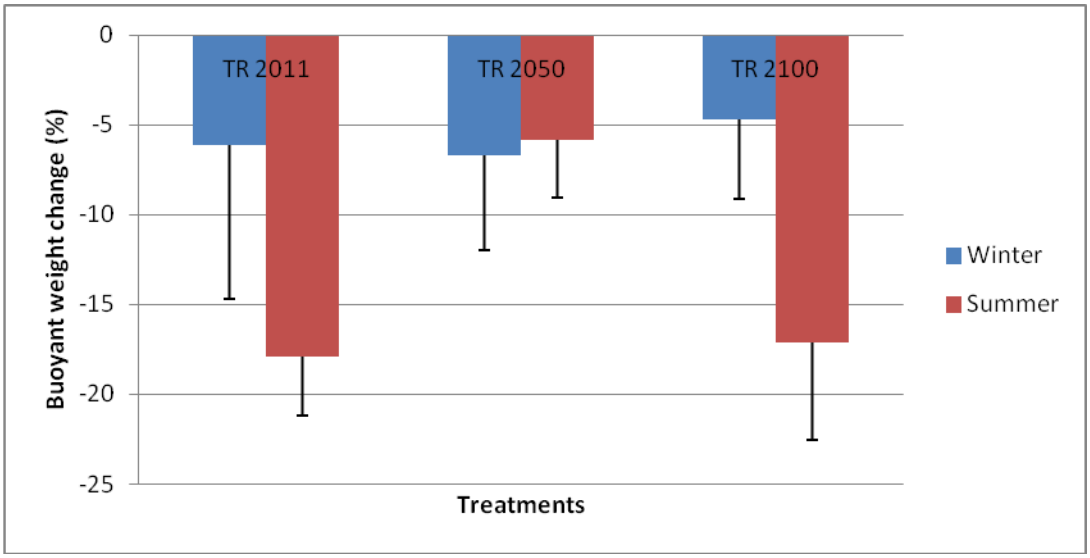

Figure 7.15 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) buoyant weight change in *A. equina* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

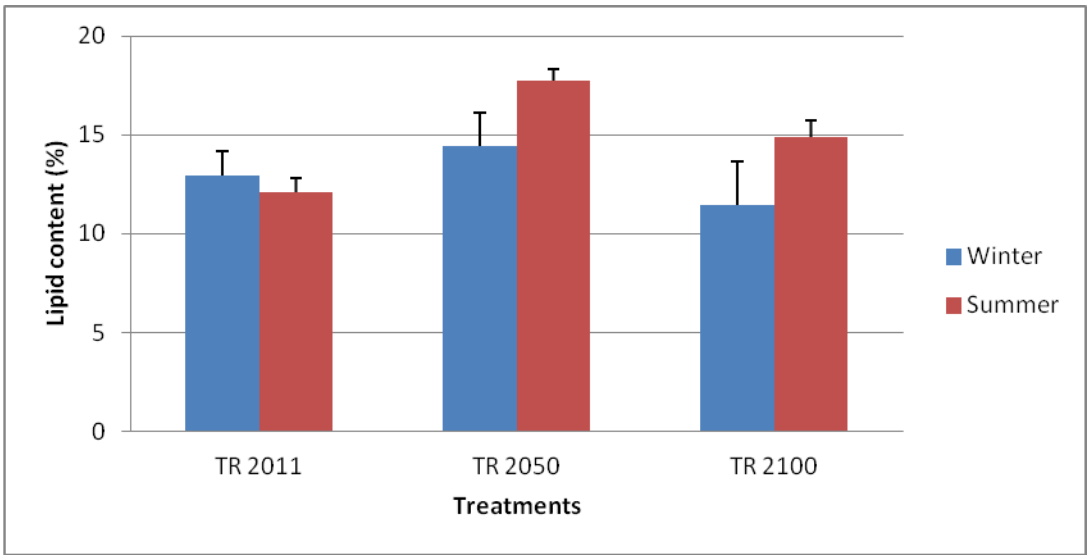

Figure 7.16 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) lipid content in *A. equina* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

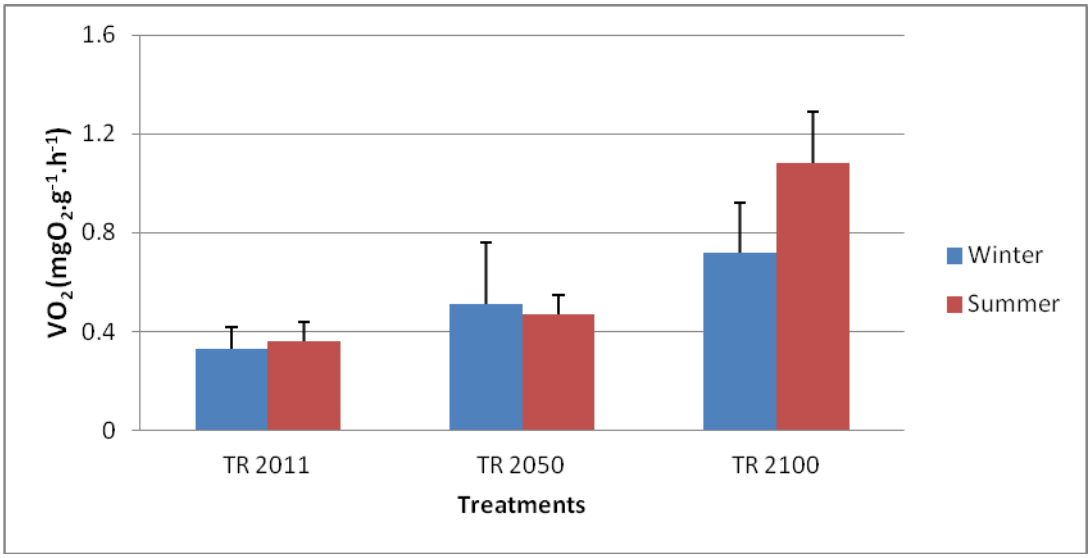

Figure 7.17 Effect of increased temperature and decreased pH in winter and summer on mean (with SE bar) metabolic rate (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) in *A. equina* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

Ascidiella aspersa:

The Shapiro-Wilk's test indicated that metabolic rate data were not normally distributed. However, although body weight change, buoyant weight change, and lipid content were normally distributed, variances for those parameters were not homogeneous (Table 7.11). Therefore, the non-parametric Kruskal-Wallis test was used to compare treatment effects for body weight change, buoyant weight change, and lipid content. Then the Mann-Whitney U test was used *post hoc* to test for significant differences between treatments.

There were no significant differences in buoyant weight change and lipid content of *A. aspersa* between the corresponding treatments in winter and summer (see Table 7.11, Fig. 7.19 and Fig. 7.20). However, there was a significant difference in body weight change between some treatments, but not between corresponding treatments in winter and summer (Table 7.11, Fig. 7.18). Also, there was significant difference in metabolic rate (Table 7.11, Fig. 7.21) between TR 2011W compared with TR 2011S (p = 0.019), between TR 2050W compared with TR 2050S (p =

0.008) and between TR 2100W compared with TR 2100S ($p < 0.001$), using the Mann-Whitney U test.

Table 7.11 Statistical analyses for body weight change, buoyant weight change, lipid content, and metabolic rate in *A aspersa*.

Parameter	Normality test			Treatment comparison		
	Shapiro-Wilk's test			Kruskal-Wallis test		
	Statistics	df	*p* value	Chi-Square	df	*p* value
Body weight[a]	0.958	35	0.206	12.533	5	0.028
Buoyant weight[b]	0.942	31	0.096	8.233	5	0.144
Lipid content[c]	0.991	21	0.999	8.506	5	0.130
Metabolic rate	0.374	61	<0.001	35.758	5	<0.001

[a] Body weight change was analysed using the Kruskal-Wallis test because the variance for this parameter was not homogeneous (Levene's test was significant (p = 0.018)). [b] Buoyant weight change tested using the Kruskal-Wallis test because its variance was not homogeneous; the Levene's test for homogeneity of variances was significant (p = 0.005). [c] Lipid content tested using the Kruskal-Wallis test because the significant (p = 0.034) Levene's test for homogeneity of variances indicated that its variance was not homogeneous.

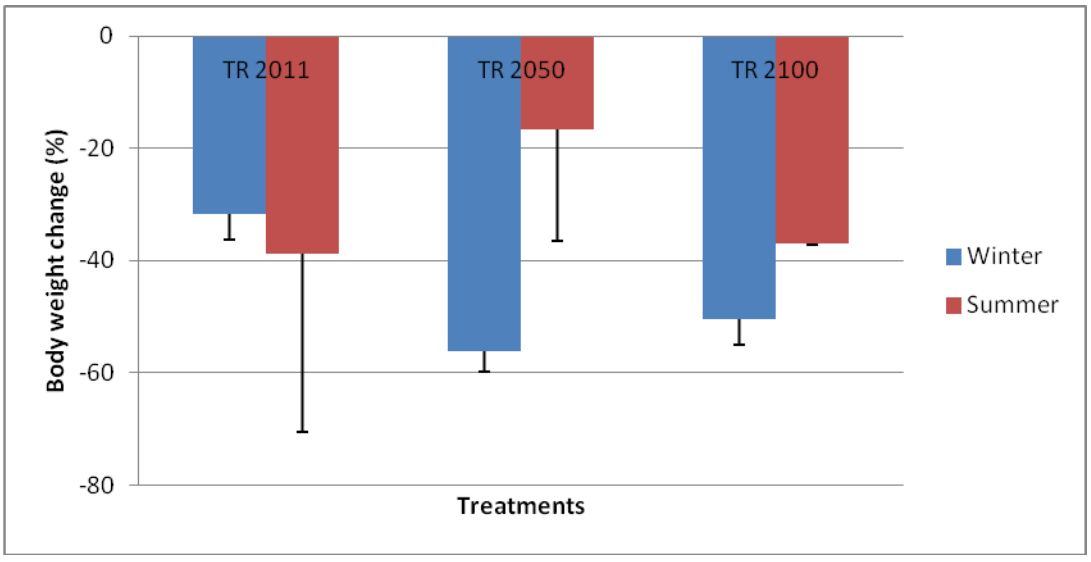

Figure 7.18 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) body weight change in *A. aspersa* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

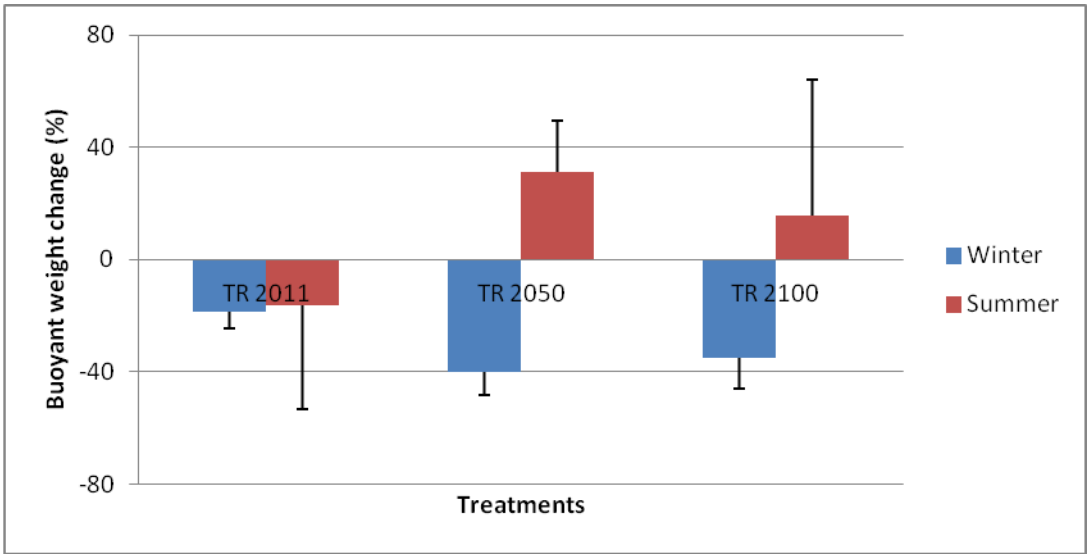

Figure 7.19 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) buoyant weight change in *A. aspersa* over a six-week period. Winter treatments represented by blue columns and summer treatments represented by red columns.

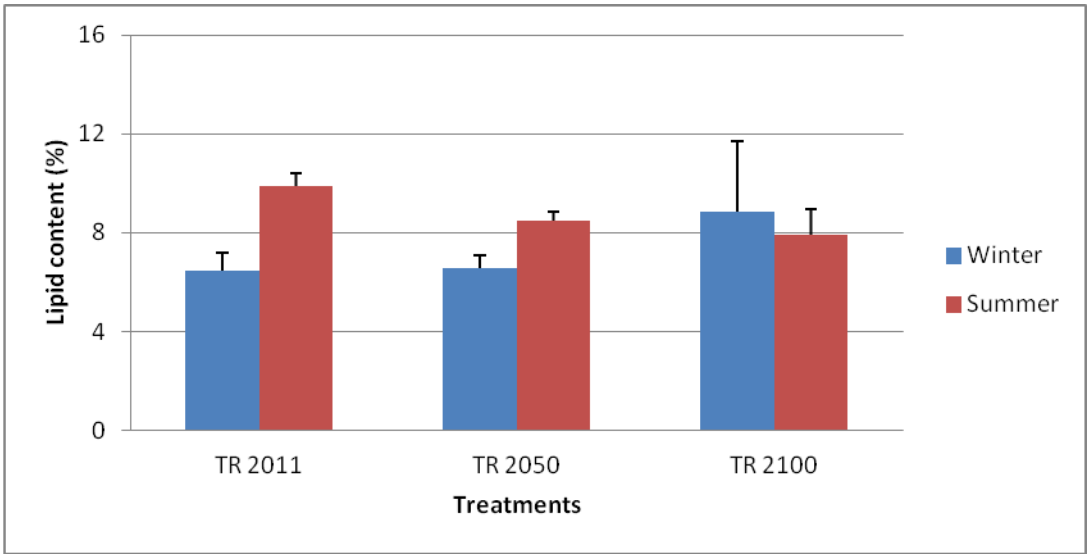

Figure 7.20 Effect of increased temperature and decreased pH in winter and summer on mean percentage (with SE bar) lipid content in *A. aspersa* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

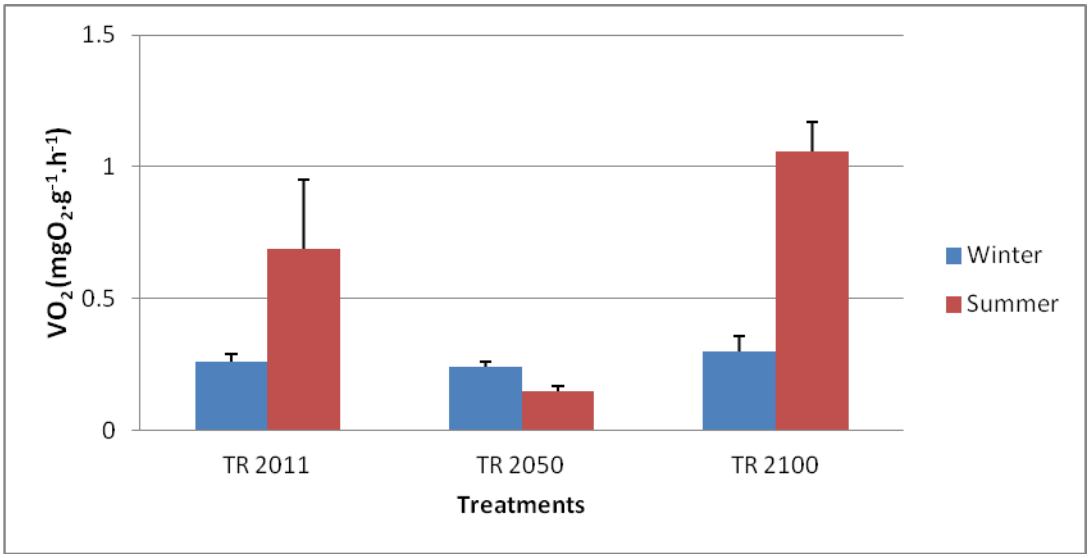

Figure 7.21 Effect of increased temperature and decreased pH in winter and summer on mean (with SE bar) metabolic rate (VO_2 (mg $O_2.g^{-1}.h^{-1}$)) in *A. aspersa* at the end of these experiments. Winter treatments represented by blue columns and summer treatments represented by red columns.

7.4 Seasonal and General Discussion:

7.4.1 Effects on M. edulis:

No significant differences in growth parameter changes between the corresponding treatments in winter and summer were observed in *M. edulis* (Table 7.5) (Thomsen *et al*., 2010). However, an increase in growth in TR 2011W and TR 2100W was apparent in comparison to corresponding treatments in summer (Fig. 7.6 and Fig. 7.7), with a slight increase in TR 2050S compared to TR 2050W (Fig. 7.6 and Fig. 7.7). These findings support the notion that some marine invertebrates require an optimal temperature range for the optimal growth (Levinton, 2009) and that deviation from this range, whether lower or higher, can lead to reduced growth (Rodrigues and Grottoli, 2006). Non-significant differences in lipid content were recorded for winter and summer treatments (Table 7.5, Fig 7.8). However, an otherwise expected (Nagarajan *et al*., 2006) increase in lipid content for *M. edulis* in winter (Fig. 7.8) was observed. A significant increase in metabolic rate was recorded for TR 2100S compared to TR 2100W (p = 0.032, Fig. 7.9), which was expected because metabolic rates normally increase due to increased temperatures in the

summer. The increased growth observed in winter may be attributable to greater abundance of food combined with reduced energy requirements (Findlay *et al.*, 2009a). The trends for all parameters measured in the summer and winter TR 2100 treatments track those in the TR 2011 treatments, except that the increased metabolic rates observed in the summer were significant at the high temperature limits. This increase did not reflect a significant impact on the growth in summer when compared to winter, perhaps due to the inability of the organism to synchronize energy expenditure to match their rate of food consumption (Nagarajan et al., 2006). The results showed an increase in mortality (22%), by difference of 13% compared to winter treatment (9%) (Table 7.4, Fig. 7.1, and Fig 7.5). There were strong similarities in survival data for TR 2050 between summer and winter (79% and 84%, respectively, Table 7.4, Fig 7.1)). Overall survival rates were best in the winter for all treatments, but were not high. Generally, these results indicate that the ability of these organisms to grow and carry on necessary physiological processes reflected their ability to survive.

Under exposure to the higher temperatures and seawater acidity expected in winter at the end of this century, *M. edulis* experienced reduced survival and slower growth in the present study. Together, these outcomes reflect conflicting results regarding the sensitivity of *M. edulis* to predicted future higher temperatures and lower pH (Landes and Zimmer, 2012). On the other hand, their results were similar to those in the present study for TR 2050, which revealed no significant effects on metabolic rates, with low differences in growth recorded for all measurements. In summer, *M. edulis* showed an increase in mortality rates directly proportional to the increase in temperature and acidity levels. In TR 2100, there was also a decrease in wet weight and the buoyant weight, and an increase in the size of the shell with an increase in the metabolic rate, which may reflect responses to resist shell dissolution in this treatment (Findlay *et al*., 2009b). Although there were different responses in TR 2050, *M. edulis* showed evidence of short-term adaptation through significantly reduced rates of metabolism. Despite the fact that most of the differences in parameters under future conditions were not statistically significantly different from controls, the present results indicated that the predicted future adverse effects of higher temperature and lower pH on *M. edulis* can lead to increased mortality,

reduced body weight, and reduced buoyant weight, but were not critical (Appelhans *et al*., 2012).

7.4.2 Effects on L. littorea:

The growth changes observed in corresponding treatments were not significantly different in summer and winter in *L. littorea*. Decreased growth in winter experiments and increased growth in summer were expected, especially as reflected in buoyant weight measurements (Rodolfo-Metalpa *et al.*, 2010) but these expectations were not met in the future treatments (Fig 7.10 and Fig. 7.11). However, the effects of concurrent increased availability of food, with low metabolic rates and low energy expenditure on other physiological processes may lead to increased growth in all winter treatments. The irregular responses were noted in the summer treatments when the temperature rose above the seasonal average (Godbold and Solan, 2013), while an increase in the metabolic rate under TR 2011S (Fig. 7.13) occurred in tandem with a non-significant increase in growth under the same treatment in winter (Fig. 7.10 and Fig. 7.11). In TR 2050, a non-significant decrease in growth in the summer was observed. Differences in metabolic rates and lipid content were also non-significant between corresponding treatments under winter and summer conditions, except for the increased metabolic rate recorded for treatment TR 2100S (p = 0.029. Table 7.8, Fig. 7.13). As seen in *M. edulis*, and as expected, metabolic rate increased under TR 2100S in *L. littorea* in the summer and rose significantly under TR 2100W. Such an increase in metabolic rate, combined with lower growth and intermediate lipid content indicates increased energy consumption directed to the restoration and maintenance of tissues, and increased cost of growth and calcification to compensate for dissolved shell components (Fig 7.11 and Fig. 7.13). The increase in metabolic rate observed in TR 2100S coincided with decreased summer survival (90%) compared with the winter treatment (98%). The highest difference in mortality observed was between corresponding treatments (8%), which was no more than 4% in the other treatments (Table 7.4, Fig. 7.2, and Fig. 7.5). Generally, winter treatments showed better growth and survival parameters when compared to the summer treatments, except that the largest change in growth was observed under TR 2011S compared with TR 2011W.

L. littorea showed a high capacity for survival in winter, while exhibiting a decrease in body growth offset by an increase in the growth of the shell. This

coincided with an increase in metabolic rate. Therefore, it is clear that increasing the acidity levels and temperatures did not disrupt metabolism or decrease calcification, although deficits in length and weight change may indicate a reciprocal relationship between the dissolution of old structures and the formation of new structures (Findlay *et al*., 2009b). However, none of these effects were statistically significant, possibly due to the ability of *L. littorea* to convert energy and thereby reduce growth and increase calcification to cope with the new environmental conditions and survive at higher rates. The present results showed decreases in the growth of *L. littorea* in 2050 and in 2100 due to elevated temperature and decresed pH, as shown decreases in body weight under the higher temperature and decreased pH (Melatunan *et al*., 2013) in the summer. The lower growth together with high metabolism might be a response to the TR 2100 treatment that would improve individual animals' ability to survive. Otherwise, growth would have increased, boosted by the animals' high fat content as compared to that of animals under the control treatment. This response also reveals shell dissolution under the TR 2050, which indicates the occurrence of divergent responses to the phenotypic characteristics of the shell between treatments (Melatunan *et al.,* 2013). These results show that higher temperature and lower pH have a small but negative impact on *L. littorea* that may be due to the increased cost of producing the energy needed for the restoration, maintenance, and preservation of cell viability and shell integrity.

7.4.3 Effects on A. equina:

A. equina as a species tends to tolerate a broad range of thermal conditions, because its tidal habitat tends to exhibit a wide range of seasonal and daily temperatures (Levinton, 2009). The findings in this study supported the hypothesis that an increase in temperature may affect the abundance of some non-calcified marine invertebrate species. Mortality was zero in the summer season (0%), while the mortality rate ranged from 26% to 36% under all winter treatments and was highest under TR 2011W (64%, Table 7.4, Fig 7.3 and Fig 7.5). However, there were no significant responses for any measurements under corresponding treatments under winter and summer conditions. Decreased pH under future condition treatments in winter might have played a role in reducing mortality and reducing the difference between winter and summer treatments, unlike the effects observed on calcified invertebrates in the present study (Fig, 7.5). *A. equina* might have a strong ability to

adapt to future environmental conditions, via energy conversion and reduced growth to increase its ability to survive. Under summer conditions, these animals were highly successful, but the winter usually tough on *A. equina*. Elevated temperatures and low pH of the predicted future conditions might have improved the ability of these organisms to seasonally adapt.

The ability of *A. equina* to survive in winter were similar under future treatment conditions, with a slight non-significant increase in survival compared to control, which indicates that some non-calcified invertebrates may become more abundant in a more acidic environment. Suggett *et al.* (2012), for example, suggested that the abundance of Cnidaria would increase. In addition, these results showed an increase in metabolic rates directly proportional to the temperature and acidity increases, together with decreased growth change. This may indicate that reduced growth and higher rates of metabolism allowed the animals to cope with the more difficult environment and maintain acceptable levels of survival. Under summer conditions, there were no surprises for any of the responses measured in *A. equina*, which were within expectations. Results for *A. equina* demonstrated that these animals tolerated increased temperatures and acidity, and have a high capacity to adapt, which led to an absence of mortality in all treatments.

7.4.4 Effects on A. aspersa:

A. aspersa is a species with seasonal mortality and a short life cycle (Schmidt, 1983), These animals are typically quite abundant when animals are collected at the beginning of winter, but are less abundant in the middle of winter. Adult animals greater than 25 mm in size were rarely observed in the summer. Hence, most adults might reach the end of their life cycle in the summer while others do so in the middle of winter. Percentage mortality in the summer for all treatments ranging from 77% to 85% (Table 7.4), approaching the mortality rate of 82% observed in TR 2100W (Fig. 7.3 and Fig. 7.5). The lowest mortality rate of 4% under winter treatments was observed in TR 2011W, while 35% mortality was recorded for TR 2050W. These results showed that mortality increased significantly in winter treatments under predicted future conditions, while there was slight improvement in survival in the summer treatments under predicted future conditions (TR 2050S and TR 2100S). Despite the impact of a short life cycle (18 months) (Morton and Dinesen, 2011) on

its mortality rates, temperature increases of more than 7 °C may have led these animals to sense a change of season. That may lead to a phenology shift so that behavioral responses that are expected to begin in one season start to occur earlier or later (Godbold and Solan, 2013). Here, responses expected to occur in the summer start to occur late in the winter and the animals inappropriately transition to the next phase, resulting in a higher mortality rate. *A. aspersa* showed responses different to some extent from those for other marine invertebrate species, when comparing the corresponding treatments in summer and winter. In this species, there were non-significant responses in growth and lipid content, while all corresponding treatments showed significant treatment responses in metabolic parameters during the winter and summer seasons (Table 7.11, Fig. 7.21). Hence, the present study found that elevated temperature had a negative effect on impact on this species of marine invertebrate, which is affected by seasonality. The winter season launches the animals into readiness for the beginning of a new life cycle in the spring, so increased temperature and decreased pH may both reduce viability and the chances for reproductive success for these marine organisms.

The results of the present study of predicted future climate effects on *A. aspersa* in winter were in stark contrast to positive responses to decreased pH land increased temperature on non-calcified marine invertebrates reported in other studies (Connell & Russell, 2010, Kroeker *et al.,* 2010). The present study results indicated higher and statistically significant percent mortality in direct proportion to the higher temperature and acidity levels in winter. Furthermore, generally lower growth (body weight change and dry weight), with increased metabolic rates under TR 2100 conditions, and lower metabolic rates under TR 2050 conditions were recorded. For *A. aspersa* summer responses, factors related to these organisms' short life cycle might have led to very high percentage mortality under all treatments, including the control treatment. However, the level of improvement in survival rates when temperature and acidity levels increased to their treatment maxima, supports what Dupont and Thorndyke (2009) noted that a lower pH of -0.4 units led to increases in the rate of survival. Slight increases appeared in buoyant weight, body weight, and lipid content. Metabolic rate showed variable responses; a significant increase was observed under TR 2100 conditions, but a significant decrease was observed under

TR 2050 conditions. These results illustrate the variability of responses to different pH and temperature levels by one species in the same season.

8.4 Future Work:

Further tests based on the results obtained so far will be performed, including calorimetric energy calculations and protein content determinations in dried tissues. These tests will be integrated with the present results partially by scanning electron microscopy of tissues from the marine invertebrate species to determine whether environmental conditions resulting from climate change have any effects on any structures or tissues of the study species, specifically on mitochondria in the epithelial layer (see Appendices).

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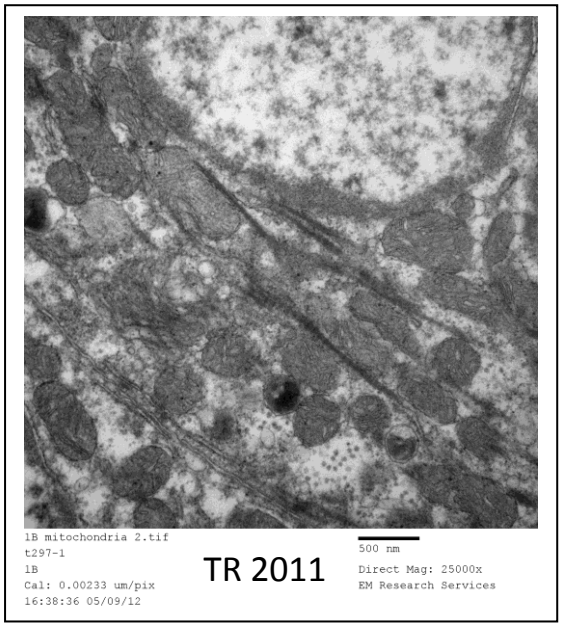
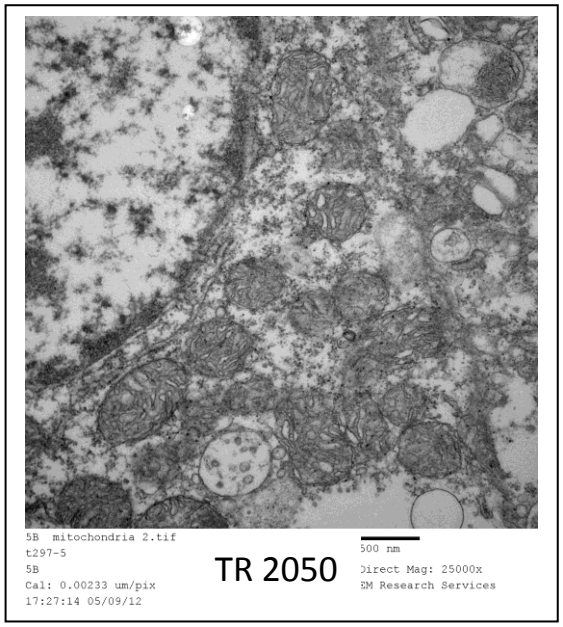
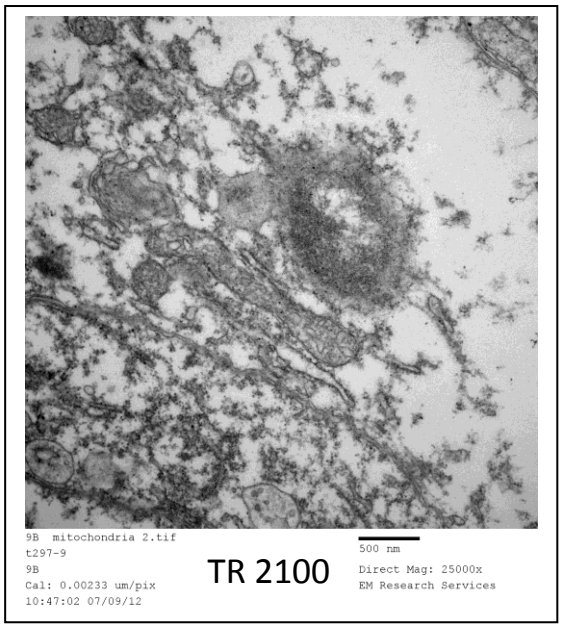
Appendices:

Appendix I: Histology of *M. edulis.*

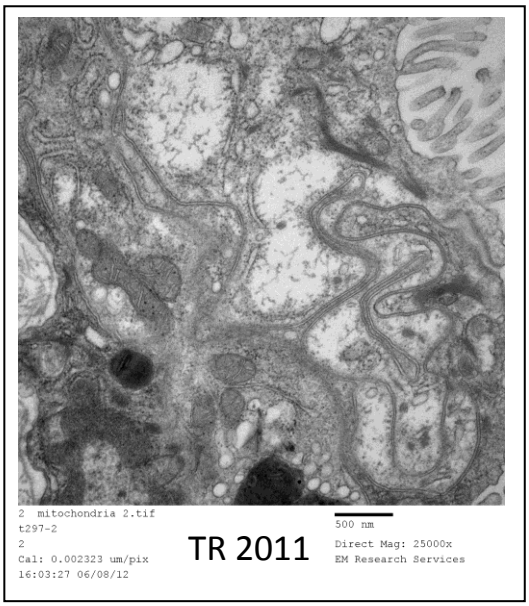


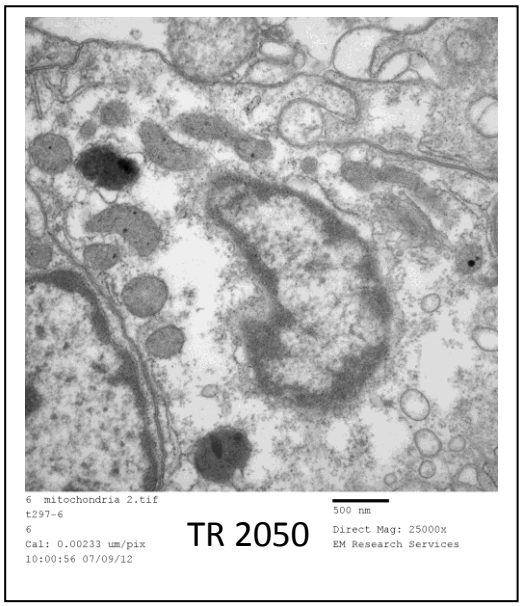


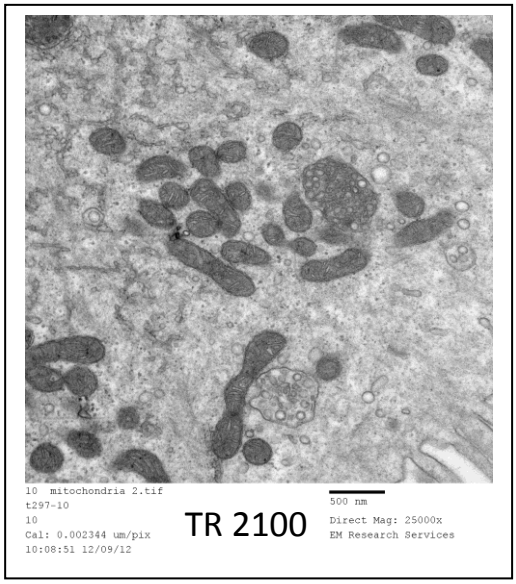


Transmission Electron Microscopy (TEM) sections of *M. edulis* tissues showing effects of experimental conditions on mitochondria in the epithelial layer of the gills to all treatments (TR 2011, TR 2050 and TR 2100) in summer.

Appendix II: Histology of *L. littorea.*

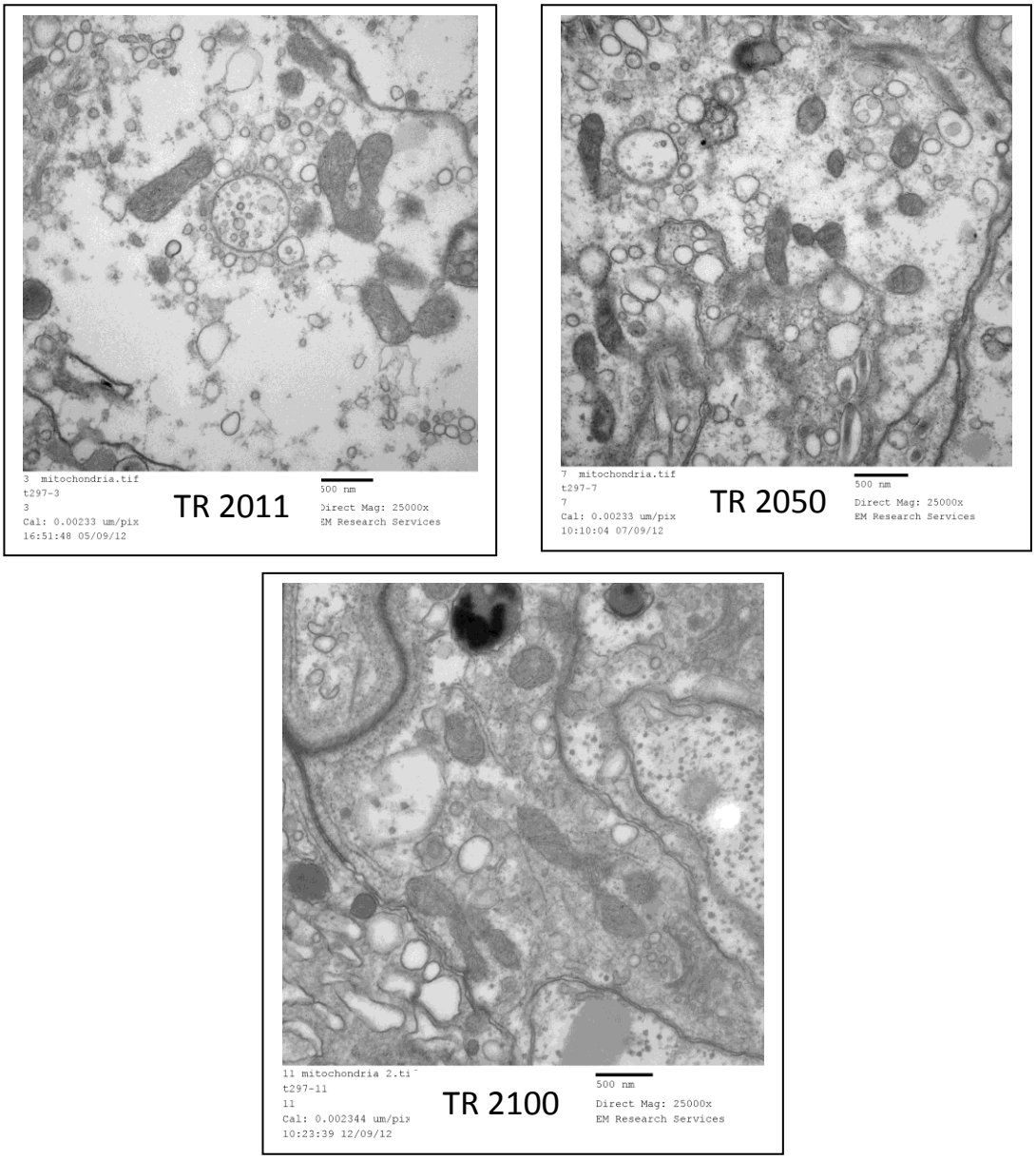






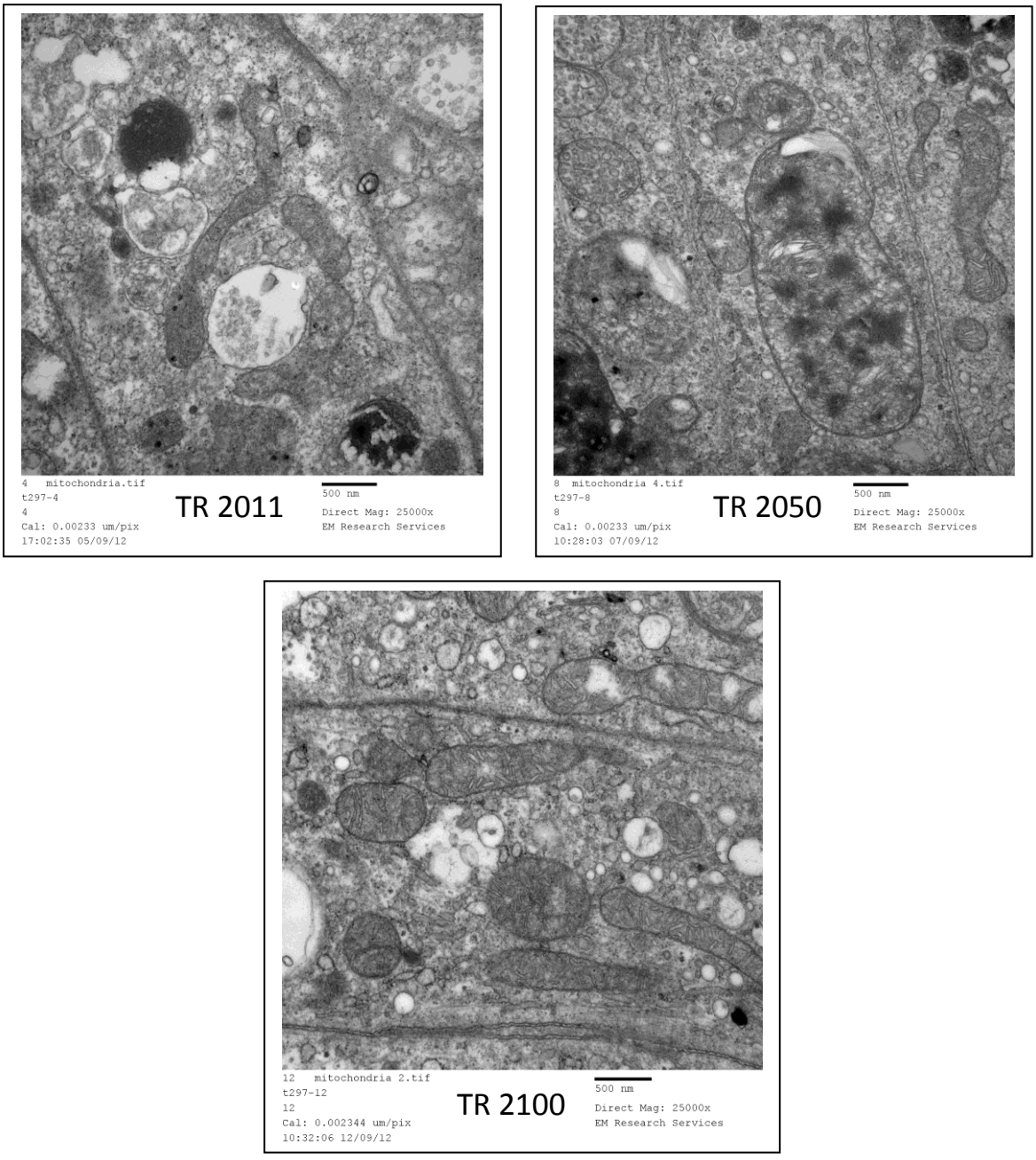
Transmission Electron Microscopy (TEM) sections of *L. littorea* tissues showing effects of experimental conditions on mitochondria in the epithelial layer of the midgut to all treatments (TR 2011, TR 2050 and TR 2100) in summer.

Appendix III: Histology of *A. equina.*



Transmission Electron Microscopy (TEM) sections of *A. equina* tissues showing effects of experimental conditions on mitochondria in the epithelial layer of the tentacle to all treatments (TR 2011, TR 2050 and TR 2100) in summer.

Appendix IV: Histology of *A. aspersa.*



Transmission Electron Microscopy (TEM) sections of *A. aspersa* tissues showing effects of experimental conditions on mitochondria in the epithelial layer of the gut to all treatments (TR 2011, TR 2050 and TR 2100) in summer.