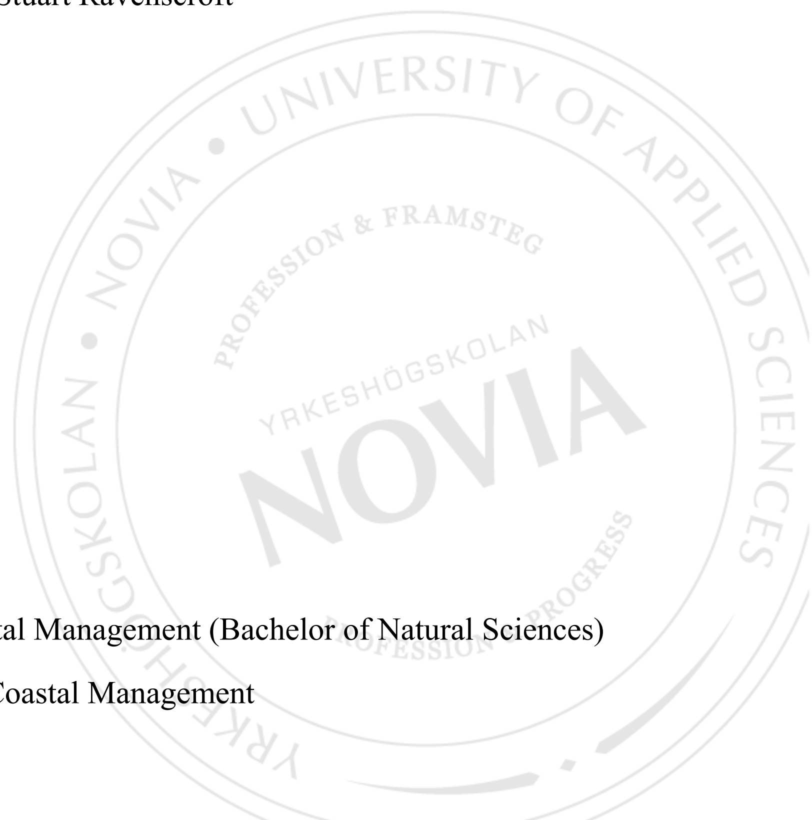
Oxidative stress as an indicator for environmental factors in *Crassostrea gigas* summer mortality events

A toxicological study of *Crassostrea gigas* samples taken from the west coast of Sweden during the summer mortality event of 2014

Stuart Ravenscroft



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Summary

Crassostera gigas (Thunberg 1793), commonly known as the Pacific oyster originates from Japan but can now be found in many parts of the world due to it's extensive use in aquaculture. *C. gigas* was introduced to Europe in the 60's and 70's for aquaculture but has spread into the wild and is now considered a bio-invader in many parts for Europe. For the past 20 years, mass mortality events have been recorded in *C. gigas* populations globally that have become known as 'summer mortality' events. Most studies have focused on Herpes virus OsHV-1 and *Vibrio* bacteria, however the latest research indicates that other factors may be involved (Petton *et al*. 2015).

Using samples taken from wild *C. gigas* populations affected by high mortalities during autumn 2014, this thesis uses oxidative stress as an indicator of environmental factors in summer mortality events. As little previous research exists on measuring for oxidative stress in *C. gigas*, the first objective was to optimize pH antioxidant enzyme measurement assays for glutathione S-transferase (GST), glutathione reductase (GR) and Catalase. The second objective was to determine whether environmental factors are contributing to summer mortality events by measuring for oxidative stress in the form of antioxidant activity measurements for GST, GR, Catalase and Superoxide dismutase (SOD). As oxidative stress can be caused by stressors other than environmental, these were also evaluated in this thesis.

Results for the pH optimisation measurements showed that for GST and GR, the standard pH level of 7.5 is unsuitable for *C. gigas* with 7.2 being optimal. For Catalase the result was inconclusive and the standard pH 6.5 was used in this thesis. It should be noted that not all pH levels were measured for catalase and possible future pH optimisation cannot be excluded. Results from measuring the samples for oxidative stress, although varying across the samples, showed a general trend confirming oxidative stress. This, in combination with a review of previous research on the causes of oxidative stress, supports the second hypotheses that environmental factors as measured by oxidative stress are contributing to *C. gigas* summer mortality events.

Language: English Key words: *Crassostrea gigas*, oxidative stress, summer mortality, environmental factors

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1. Introduction

Originating from Japan and Southeast Asia, the Pacific oyster (*Crassostrea gigas,* Thunberg, 1793) can now be found in many regions of the world (Ruesink *et al.* 2005) and is popular for use in aquaculture due to its resilience and high growth rate. Like in many regions of the world, *C.gigas* was introduced in the 1960's to Europe for aquaculture, beginning in Osstershelde, Zeeland the Netherlands in 1964 (Drinkwaard 1999) and has become wide spread (Fig. 1). The industry started using *C. gigas* on a large scale in France during the 1970's due to mass deaths of *Crassostrea angulata* from an Iridovirus (Grizel, 1991).

Figure 1 Occurrence of C. gigas in European and adjacent coastal waters (Nehring, 2011)

At the time of introduction, it was thought that sea temperatures in Europe and specifically the North Sea were too cold for *C. gigas* to reproduce (Drinkwaard 1999). This assumption has been shown to be incorrect, and *C. gigas* is now seen to cause a bio-invasion in the Netherlands, Germany, Denmark (Andrews 1980, Diederich 2005) and since 2007 on a large scale in Sweden and southern Norway (Wrange *et al*. 2010). *C. gigas* has proven to be resilient and adaptable, and although it has been seen in Northern Europe that they cannot withstand ice coverage (Büttger *et al*. 2011), studies carried out by Strand *et al*. (2011) have shown that if they are acclimatized to cold conditions they can withstand -22°C with a 50% survival rate after 72 hours. There is speculation that global warming could be a contributing factor to *C. gigas's* spread north to higher latitudes such as Sweden and Norway (Hobbs and Mooney 2005, Troost 2010, Laugen *et al.* 2015).

For many years, mass mortality events have been recorded in *C. gigas* populations globally and have become known as "summer mortality" events (Samain and McCombie 2008). Scandinavian populations remained unaffected until the autumn of 2014, where mass mortalities were observed in both wild and cultivated populations of *C. gigas* in the northern part of the Swedish west coast and the southern coast of Norway, with recorded deaths reaching 90% (Mortensen et al 2016). For some time, studies from many countries have focused on investigating the significance of the Herpes Virus OsHV-1 (Jouaux *et al.* 2013) and also *Vibrio* bacteria (Lacoste *et al.* 2001) in these summer mortality events. Further studies suggest that Herpes OsHV-1 replication and/or the *Vibrio* pathogenic effect related to a summer mortality event depends on the oysters susceptibility and may result from environmental factors and/or genetic traits (Petton *et al*. 2015). This is corroborated by another OsHV-1 study which found that when oysters are introduced to an area where summer mortality occurs, they suffer a lower mortality rate than oysters already established in that site (Jouaux *et al.* 2013). One method to assess whether an environmental factor such as pollution or other water born toxin could be involved, is to measure for oxidative stress (Hellou *et al.* 2012, Regoli and Principato 1995, Béguel *et al.* 2013 , Cossu *et al.* 1997).

Late in 2014 a student of Göteborg University (Agnes Faxén) took samples from the wild of sick and dying *C. gigas* from sites in Tjärnö, Koster, Lysekil and samples from the native flat oyster (*Ostrea edulis*) in Tjärnö as well as control samples from Tjärnö. These samples were taken during the 2014 summer mortality event. To assess whether there is an environmental factor as part of summer mortality events, these samples were used in this thesis to measure for oxidative stress.

2. Terminology

Many terms used in this thesis come from both the marine biology and molecular biology fields, but also have relevance in general biology and medicine. This section introduces some important terms that relate to the work carried out in this thesis.

2.1 Aerobic organisms

During earth's history, we have seen varying oxygen levels in the atmosphere that differ from the current 21% we have today. When the first organisms (anaerobic organisms) appeared on earth they split hydrogen from water to drive their metabolic reductions ($2H_2O \rightarrow 4H + O_2$). By splitting oxygen from water anaerobic organisms released oxygen into the atmosphere, slowly increasing over millions of years. This not only increased the oxygen percentage in the atmosphere but also enabled the formation of the ozone layer, creating an environment in which multicellular organisms can exist. Oxygen being a very efficient way of energy creation in a cell, and with the increased oxygen levels in the atmosphere and oceans, it became possible for multicellular aerobic organisms to evolve. Today a majority of animals, plants and bacteria are obligate or facultative aerobic organisms and use an electron transport chain to create energy, with anaerobic organisms only found in places where no oxygen is present (Halliwell & Gutteridge, 1999).

2.2 Electron transport chain

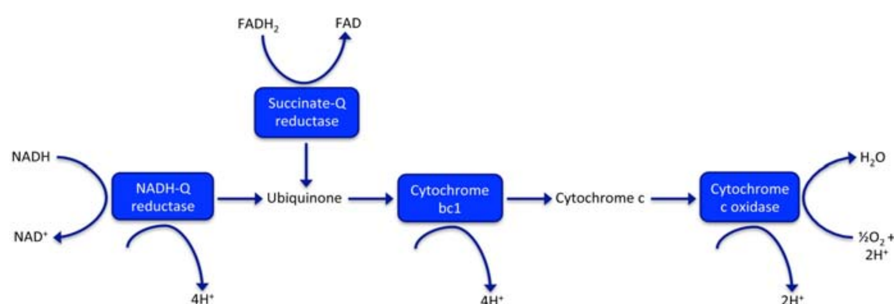


Figure 2 Electron transport chain

The electron transport chain (Fig 2) is the process within a cell whereby electrons are transferred between a series of enzymes in the form of redox reactions (transfer of electrons between chemical species) beginning with a redox reaction between a biological substance called NADH to non-haem iron proteins. As energy is released through the electron transport chain process, it enables synthesis of *Adenosine triphosphate* (ATP) a high-energy molecule that stores the energy for use by the cell (Halliwell & Gutteridge, 1999).

2.3 Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) is the collective term used to describe substances such as O_2^-, H_2O_2 , $OH^\cdot$, that cause damage when present in a cell. As will be discussed further in the following section, ROS originates from several sources including as leakage from the electron transport chain (Fig. 2) and when not being efficiently combated by antioxidants, cause a prooxidant-antioxidant imbalance and therefore oxidative stress (Halliwell & Gutteridge 1999, Almroth 2010).

2.4 Antioxidant Enzymes

The role of antioxidant enzymes in a cell is to manage levels of ROS, and they are the cells main defence against an imbalance of prooxidants (oxidative stress). This is done by managing oxidation within the cell, oxidation being the loss of an electron from a molecule and not only is caused by external factors but as natural leakage from for example the electron transport chain (Fig 2). In addition, leakage can come from CYP reactions, mitochondria, lysosomes and other sources. By measuring antioxidant enzymes, it is possible to assess the prooxidant-antioxidant balance and therein oxidative stress. Several enzymes are commonly measured; glutathione S-transferase (GST), glutathione reductase (GR), catalase, and superoxide dismutase (SOD). (Halliwell & Gutteridge, 1999, Almroth, 2010). Glutathione a low molecular weight antioxidant has multiple functions, the most important in relation to oxidative stress being the ability to donate electrons while in its reduced state (GSH) to ROS molecules, with GR then converting oxidized state (GSSG) back to reduced (GSH) (Fig. 4). In addition, glutathione helps in DNA synthesis/repair, and is important in iron metabolism.

3. *Crassostrea gigas*: an introduction

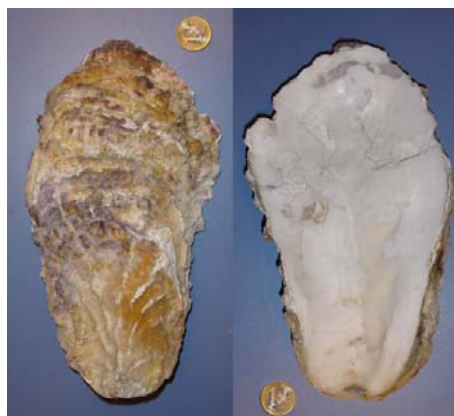


Figure 3 Crassostrea gigas (Nehring, 2011)

***C**rassostrea gigas,* with the common name Pacific oyster, is an intertidal/shallow sub tidal species that is very tolerant to varying conditions and habitats. It can tolerate temperatures from sub-zero to approximately +30°C (Nehrign, 2011). *C. gigas* has been found to live up to 30 years with a shell size of 400mm, although lengths are normally between 80mm-200mm (Nehring, 2011). The shell structure (Fig. 3) varies and is irregular, with the shape being determined by the substrate on which it lives. The external colour of an adult is normally whitish with purple streaks and spots, with the internal colour white (Nehring, 2011).

C. gigas is extensively used in aquaculture due to its resilience and fast growth rate. This adaptability however, has also made *C. gigas* very successful as a bio-invader in many regions of the word and can now be found in many parts of Europe (Fig 1). The Commonwealth Scientific and Industrial Research Organisation (CSIRO) for the Department of Environment and Heritage (Australia) classes *C. gigas* as one of ten most damaging species, based on overall economic and environmental impact (Hayes *et. al.* 2005).

As a reef-building species, *C. gigas* modifies the habitat in which it settles, creating high coverage, closely packed reefs. Although this changes the environment in which *C. gigas* invades, it does help to stabilise the substrate and by removing plankton from the water column they help prevent nutrients from staying in the lower trophic levels (Norling *et. al*. 2015).

C. gigas changes it's sex several times during its lifetime, normally starting off as male. Studies indicate that the sex is determined by environmental conditions and the availability of food (Nehring, 2011). Being extremely fertile, they produce between 50 and 100 million eggs over several bursts in a single spawning and should be fertilized by the male sperm between 10 to 15 hours after spawning. The resulting larvae can survive 3-4 weeks and travel up to 1,300km before recruitment. Although they are susceptible to pollutants at the egg and larvae stages, juveniles and adults are known to be very resistant. This being said, it is the juveniles and smaller adults that seem most susceptible in summer mortality events. Although they are very effective in their ability to bioaccumulate pollutants and survive in environments that other species could not, they are not suitable for human consumption in these circumstances (Nehring, 2011).

4. Background

4.1 Theoretical starting point

For some time, studies have focused on the presence of the herpes virus OsHV-1 and *Vibrio* bacteria in the so called summer mortality events. However, Petton *et al*. (2015) suggests the mass-deaths of *Crassostrea gigas* caused by the herpes OsHV-1 and V*ibrio* infections depends on the oysters' susceptibility, with environmental factors possibly being involved. Pollution and other water born toxins could be considered as environmental factors, with oxidative stress often used as a biomarker in aquatic creatures (Hellou *et al.* 2012, Regoli and Principato 1995, Béguel *et al.* 2013 , Cossu *et al.* 1997). Although at the time of writing of this thesis, little work has been done measuring oxidative stress in oysters, a significant body of work exists when it comes to measuring oxidative stress in mussels as a biomarker for pollution (Hellou *et al.* 2012, Regoli and Principato 1995, Cossu *et al.* 1997). It is theorised that this should also be the case for other bivalves and *C. gigas*. As a theoretical starting point for this thesis, we decided to use samples taken from the field on the west coast of Sweden during the 2014 summer mortality event. By using the method of measuring for oxidative stress as an indicator of pollution or water born toxins, it may be possible to support the theory of Petton *et al*. (2015) that an environmental factor is involved in the mass deaths of *C. gigas* during summer mortality events.

4.2 Oxidative stress

All aerobic organisms and bacteria need O_2 for efficient production of energy by the use of O_2-dependent electron transport chains, however O_2 is also a mutagenic gas and toxic to most organisms if not properly managed. Aerobic organisms survive because they have antioxidant defences to protect against O_2 damage (Halliwell & Gutteridge, 1999). Oxidative stress is defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies, H. 1991). Pro-oxidants come in

the form of Reactive Oxygen Species (ROS) such as O_2^-, H_2O_2, $OH^\cdot$, and can be formed by external influences such as UV exposure, pollution and x-rays, but are also formed as part of the normal function of a cell within aerobic organisms. This is caused for example by leakage from the electron transport chain (Fig. 2) when oxygen molecules accept electrons resulting in free radicals (Halliwell & Gutteridge, 1999). To counteract ROS, antioxidants are present within the cell and come in two forms, molecular and enzymatic. In the case of this thesis, we are looking at the enzymatic antioxidants glutathione S-transferase (GST), glutathione reductase (GR), catalase, and superoxide dismutase (SOD).

SOD, catalase and GST are involved in the reaction to convert ROS molecules into H_2O, and GR is involved in converting the oxidized from of glutathione (GSSG) back into reduced glutathione (GSH) (Fig 4). By measuring these enzymatic antioxidants we are able to assess to what extent the *C. gigas* specimens were under oxidative stress when they were taken from the field.

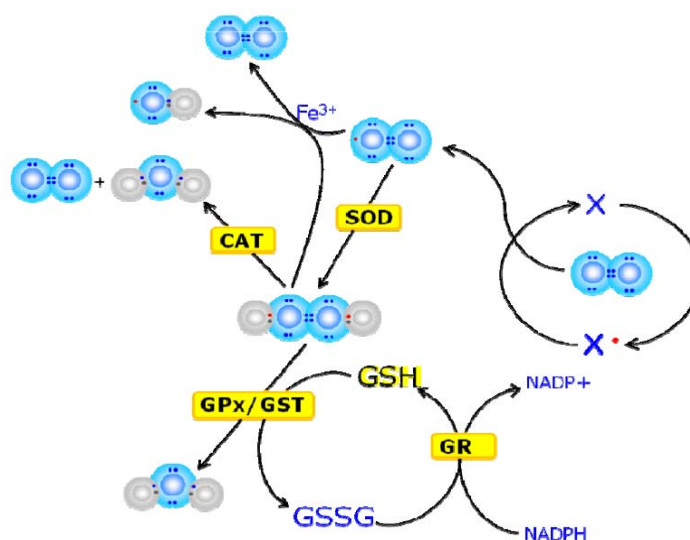


figure 4 Antioxidant activity withn a cell

4.3 Antioxidant enzymes

Antioxidant enzymes have been studied in fish and bivalves for some time with a focus on the link with chemical pollutants (Di Giulio *et al*. 1989); many bivalve studies having been with mussels (Livingstone *et al.* 1993, Solé *et al.*, 1995, Regoli and Principato, 1995). One reason there is a lot more data available on mussels, is that they are often used as sentinel organisms for monitoring the concentration of pollutants (Solé *et al.* 1995, Regoli and Principato 1995). There is very little previous work available looking at antioxidant

enzymes in the *Crassostrea* genus although Luna-Acosta *et al.* (2010) suggests *C. gigas* could also be a sentinel organism. When looking at oxidative stress in relation to the summer mortality events, no previous works has been found.

Aquatic studies conducting antioxidant measurements on bivalves exposed experimentally to chemicals or sampled from polluted areas, have focused on superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Di Giulio *et al*. 1989, Béguel *et al.* 2013), although GR and GST have also been measured (Regoli and Principato 1995, Béguel *et al.* 2013 , Cossu *et al.* 1997). Some studies show chemicals such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB's), polychlorinated dibenzofurans, dibenzodioxins produce an increase in measured activity for SOD and catalase (Solé *et al.* 1995a), but also decreases in other enzymes such as GPx (Livingstone *et al*. 1993). These enzymes have been detected in a number of bivalve species (Blum and Fridovich, 1984; Wenning and Di Giulio, 1988) including *C. gigas* (Béguel *et al.* 2013), and these increases in antioxidant activities have been observed after exposure to organic pollutants (Livingstone *et al*. 1990).

4.4 Triggers for oxidative stress

The main challenge with using oxidative stress as an indicator for the presence of pollution or external toxins, is that many triggers can induce elevated or reduced antioxidant activity. For example, superoxide dismutase (SOD) or catalase activity may be elevated owing to induction by the stressor or depressed as a toxic response to the stressor. In the case of exposure to mixed pollutants, the two processes might occur simultaneously with no net change in the observable activity (Di Giulio *et al*. 1989). Additionally, ecosystems are under pressure from a complex mixture of contaminants released in the environment due to various human activities and even if a link is established between oxidative stress in an aquatic organism and pollution, it may not be a single pollutant but a combination causing the oxidative stress effect (Stoliar and Lushchak, 2012)

A direct link has previously been established between low pH and carbon dioxide (CO_2). Even excluding factors such as lowering pH levels due to excess CO_2 in the atmosphere (climate change), excess CO_2 production due to respiration of the resident biota can exceed

the capacity of CO_2 sinks and in turn cause lower pH levels. A study on the eastern oyster *Crassostrea virginica* shows antioxidant proteins were significantly increased under high PCO_2 (partial pressure of carbon dioxide) conditions (Tomanek *et al.* 2011). Also seasonal variations in antioxidant defence activity have been measured previously (Viarengo *et al*. 1991,) although a recent study on *C. gigas* showed no seasonal variations for GPx and SOD (Luna-Acosta *et al.* 2010). In addition, there is evidence that viral infections can cause oxidative stress. For instance studies in fish showed excessive reactive oxygen (ROS) and nitrogen radical generation after viral haemorrhagic septicaemia virus (VHSV) infections (Novoa *et al.* 2010), although in a case of a study on turbot (*Scophthalmus maximus*) no excessive ROS production was observed (Novoa *et al.* 2010). A study on mice has also indicated an increased generation of ROS followed Herpes HSV-1 infection of neural cells (Kavouras *et al.* 2007). There may be synergistic effects, whereby the presence of a pathogen such as the Herpes OsHV-1 virus or V*ibrio* bacterium are combined with environmental factors causing oxidative stress.

5. Problem Definition

Antioxidants such as glutathione S-transferase (GST), glutathione reductase (GR), catalase, and superoxide dismutase (SOD) represent an important protective mechanism, and changes in their concentrations are often used as biomarkers of environmental stress (Hellou *et al.* 2012). Biomarkers are “any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status” (Walker *et al*. 2001) In this case we are measuring antioxidant enzymes to gage whether the individuals were under oxidative stress at the time they were collected as a indicator of an environmental factor in summer mortality events.

5.1 Hypothesis 1

When researching previous studies on the measurement of antioxidant enzymes in bivalves, no papers were found where the GR, GST and catalase assays were optimized for *C. gigas*. In order to produce the most reliable results, the assays should be pH optimized for *C. gigas* by assessing the suitability of standard pH levels for GR, and GST of pH 7.5 and pH 6.5 for catalase. This results in the first hypothesis for this thesis:

Hypothesis 1
Standard pH levels for measurement assays glutathione reductase (Cribb *et al.* 1989), glutathione S-transferase (Stephensen et al. 2002) and catalase (Aebi 1985) are not suitable for *C. gigas*.

5.2 Hypothesis 2

From the latest papers published on *C. gigas* summer mortality events, there is an indication that in addition to the herpes virus (OsHV-1 μ var) and bacterial populations of genus *Vibrio*, there may be another factor involved such as genetic traits or environmental factors (Petton *et al*. 2015). Unfortunately, Petton *et al.* 2015 does not define what is meant by environmental factors; are they abiotic, biotic or both. Using oxidative stress as a biomarker generally refers to abiotic stressors, although biotic stressors such as infections can cause oxidative stress. This being said, Petton *et al.* 2015 gives direction to this thesis and the work to measure antioxidant enzyme activity as an indicator of environmental factors in summer mortality events.

Hypothesis 2
Environmental factors, as measured by oxidative stress, are contributing to *C. gigas* summer mortality events.

6. Materials and Methods

In this thesis I measured several antioxidant enzymes (glutathione reductase (GR), glutathione S-transferase (GST), catalase, and superoxide dismutase (SOD)) on samples taken from the wild by a previous student Agnes Faxén. At the time of writing this thesis, little existing literary work had been found where these measurements have been used to assess oxidative stress in *Crassostera gigas.* This being the case, work for this thesis was approached with the view that the assays would need to be pH optimized for this species of bivalve, and with the uncertainty of whether the measuring would result in any data at all. All laboratory work was carried out at the Toxicology Laboratory of Göteborg University, Zoologihuset, Medicinaregatan 18A, 413 90 Göteborg.

The protocol developed for this work can be broken down into 5 parts:

- 1: Taking the first sample set for all sites and taking initial GR measurements as described by Cribb *et al.* 1989 and GST measurement as described by Habig *et al*. 1974 adapted to a microplate reader as described in Stephensen *et al*. 2002 at standard pH levels to assess suitability for *C. gigas* and whether there would be sufficient resulting data to analyse. Protein content determined as described by Lowry *et al*. (1951)
- 2: For selected sites, take a subset of samples from the second sample set and pH optimize GR, GST, and catalase as described below
- 3: Run final GR, GST, catalase measurements for selected sites on remaining samples from second sample set to provide data for analysis. Protein content determined as described by Lowry *et al*. (1951)
- 4: Superoxide dismutase activity measurements with a commercially available SOD Kit on third sample set for selected sites to provide data for analyses.
- 5: Statistics

Protein measurements were taken as GR, GST and Catalase results are expressed as enzyme activity per mg protein over time. It is not important the amount of tissue in a homogenised sample but the protein level. The enzyme activity results will fluctuate between the samples as the protein levels differ, however by knowing the protein level the results can be standardised.

6.1 Details of samples taken

During the summer mortality event of 2014, Agnes Faxén a student at Göteborg University collected *Crassostrea gigas* and *Ostrea edulis* samples (Tab. 1) from several sites on the west coast of Sweden (Fig. 5). These samples she later prepared as described above in section 6.1 in order for them to be measured for oxidative stress.

Table 1 samples taken during 2014 summer mortality event

Sampling site (Fig 5.0)	Species	Quantity	Size	Sample date	Condition
1. Lysekil	*C. gigas*	8	4-6cm	02/10/14	Sick, in very bad condition
2. Tjärnö channel	*C. gigas*	8	4-6cm	08/10/14	Obviously sick, very bad smell, necrosis
3. Lovén centre Laboritory	*C. gigas*	8	4-6cm	08/10/14	Healthy at freezing, had been kept in a constant flow of deep water
4. Koster Ostrea hatchery	*C. gigas*	20	1-2cm	02/10/14	Total mortality
5. Lovén centre Laboritory	*C. gigas*	20	1-2cm	08/10/14	Healthy at freezing, had been kept in a constant flow of deep water
6. Tjärnö bridge	*O. edulis*	20	-	14/10/14	No mortality (may have been affected by changes in environment during the week since collection of *C. Gigas* in Tjärnö)

The samples were collected from the regions around both Göteborg University's Sven Lovén Centre for marine science Tjärnö and Sven Lovén Centre for marine sciences Kristineberg (Fig 5).

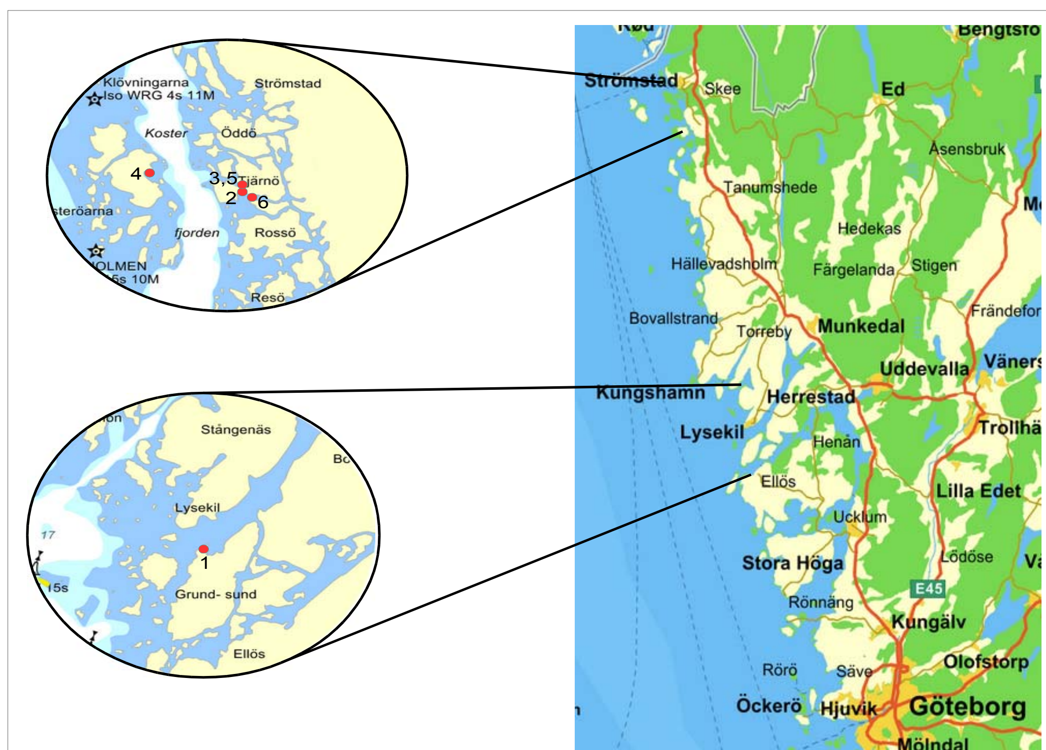


figure 5 Sample collection sites of C. gigas autumn 2014

6.2 Methods used in the collection/preparation of the samples

The samples used for the toxicological analysis of *C. gigas* in relation to the summer mortality event in 2014 were collected from wild populations affected by high mortalities by student Agnes Faxén of Göteborg University under supervision of researcher Åsa Strand, Göteborg University, Tjärnö, Sweden. Samples were taken in October 2014 from locations on the west coast of Sweden (Fig. 5), placed for 1 day in a fridge and then frozen to -80°C (except for 1-2cm control samples that were frozen to -20°C). The samples were then taken to the Toxicology Laboratory of Göteborg University, Göteborg and under supervision of professor Joachim Struve prepared for later analysis. Samples were prepared by removal of the gills and digestive gland from each oyster (except for 1-2cm samples, which were too small to separate the gills and digestive gland, so all tissue was removed and homogenised together), after which the separate gills and digestive gland samples were homogenised in 0.1 M Na^+/K^+ phosphate buffer (pH 7.4) including 0.15 M KCI. The resulting homogenate samples were centrifuged at 10 000 rpm for 20 minutes and returned to the -80°C freezer. All samples were kept on ice during this process. 7 seperate samples were taken at each site, with gills and digestive gland removed (except for the 1-2cm samples where the whole tissue was homogenised together due to the small size). Preperation of the sample tissue resulted in 3 replicates for each tissue type (gills, digestive gland or combined), which resulted in a total 3x14 samples from each site with 4-6cm oysters and 3x7 samples from sites with 1-2cm oysters.

After preparation of solutions and prior to running each set of antioxidant measurements (GR, GST, catalase, SOD), a dilution series was run on a subset of the samples to determine the correct dilution factor for that particular enzyme measurement. The results from the dilution series were used to dilute the remaining samples to the correct level for measuring.

6.3 Initial GR, GST, and Protein measurements

Glutathione reductase (GR) assay. All solutions were prepared as described by Cribb *et al.* (1989). Immediately prior to taking the GR measurements, samples from the first replicate set were taken from a -80°C freezer 20 at a time and while kept on ice, 20 µl of the samples were diluted 10x in homogenizing buffer with the original samples immediately returned to the -80°C freezer. The resulting diluted 20 samples were then prepared and measured as described by Cribb *et al.* (1989) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader and immediately returned to a -80°C freezer. This procedure was repeated until all samples had been measured for GR. The ratio of 10x diluted sample to reaction agents was 1:9.

Glutathione *S*-transferase (GST) assay. All solutions required were prepared as described by Habig *et al.* (1974) adapted to a microplate reader as described in Stephensen *et al*. (2002). Immediately prior to taking the GST measurements, the previously diluted samples from the GR assay (x10 dilution in homogenizing buffer) were removed from a -80°C freezer 20 at a time, and while kept on ice were prepared and measured as described by Habig *et al*. (1974) adapted to a microplate reader as described in Stephensen *et al*. (2002) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader. This procedure was repeated until all samples had been measured for GST. The ratio of 10x diluted sample to reaction agents was 1:20.

Protein measurements. Taking the same original samples from the first replicate set (defrosting 28 samples at a time), 25 µl was diluted 20x in MilliQ-water and the protein content determined as described in Lowry *et al*. (1951) and measured on the UV-Vis SpectraMax 190 Absorbance Microplate Reader. This procedure was repeated until the protein content had been determined for all samples

6.4 pH optimization of GR, GST, and catalase assay for *C. gigas*

pH optimization Glutathione reductase (GR) assay. Following the method described by Cribb *et al*. (1989) for GR measurements, 6 solutions sets were prepared to different pH levels 7, 7.2, 7.4, 7.6, 7.8, and 8, with pH measured on a INOLAND pH meter.

Immediately prior to taking the GR measurements, 6 randomly selected samples from the second replicate set were taken from the -80°C freezer with 20 µl of the samples diluted x10 in homogenizing buffer, kept on ice and the original samples immediately returned to -80°C freezer. These 6 diluted samples were then measured at each pH level following the method described by Cribb *et al*. (1989) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader. The pH results from this GR pH optimization were also used for determining the pH level for GST assay as the same homogenizing buffers are used for both GR and GST. The ratio of 10x diluted sample to reaction agents was 1:9.

pH optimisation catalase. The same 6 samples used in the pH optimisation for GR were also used for catalase. Following the assay described by Aebi (1985), solutions were prepared at a pH of 6.5 and pH 7.2. Each sample was measured for pH 6.5 and pH 7.2 on the UV-Vis SpectraMax 190 Absorbance Microplate Reader. pH 7.2 being the result from the GR pH optimization measurements and pH 6.5 the standard pH level described by Aebi, (1985) for the measurement of the catalase. The ratio of 20x diluted sample to reaction agents was 1:20.
After completing the first set of measurements for catalase, I was concerned that the result was influenced by the fact that the reagents were mixed and then the samples with different pH levels measured in serial (as per the assay). To reduce the influence on the results from running the measurements in serial, I remeasured catalase, this time measuring the samples with different pH levels in parallel and additionally mixing the reagents at the same time as taking the measurements.

6.5 Final GR, GST, catalase measurements

Results from the measurments on all samples confirmed enzyme activity could be measured in *C. gigas*, however a null result was obtained from the 1-2cm homoginised control samples. This may have been due to the samples initially only being stored at -20 and not -80. To focus the rest of the measurements in this Part 3 of the procedure, we decided not to re-measure the 1-2cm samples.

Glutathione reductase (GR) assay. All solutions were prepared as described by Cribb *et al*. (1989) to a pH of 7.2. Immediately prior to taking the GR measurements, the remaining

samples from the second replicate set were taken from a -80°C freezer 20 at a time and while kept on ice, 20 µl of the samples were diluted x10 in homogenizing buffer with the original samples immediately returned to -80°C freezer. The resulting diluted 20 samples were then prepared and measured as described by Cribb *et al*. (1989) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader and immediately returned to a -80°C freezer. This procedure was repeated until all samples had been measured for GR. The ratio of 10x diluted sample to reaction agents was 1:9.

Glutathione *S*-transferase (GST) assay. All solutions required were prepared as described by Habig *et al*. (1974) adapted to a microplate reader as described in Stephensen *et al*. (2002) to a pH of 7.2. Immediately prior to taking GST measurements, the previously diluted samples from the GR assay (10x dilution in homogenizing buffer) were removed 20 at a time, and while kept on ice were prepared then measured as described by Habig *et al*. (1974) adapted to a microplate reader as described in Stephensen *et al*. (2002) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader. This procedure was repeated until all samples had been measured for GST. The ratio of 10x diluted sample to reaction agents was 1:20.

Catalase. All solutions required were prepared as described by Aebi (1985) to a pH of 6.5. Immediately prior to taking the catalase measurements, the remaining samples from the second replicate set were taken from the -80°C freezer and diluted 1:20 with Potassium phosphate buffer at pH 6.5 then measured as described by Aebi (1985) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader. All samples were kept on ice while outside the -80°C freezer. The ratio of 20x diluted sample to reaction agents was 1:20.

Protein measurements. Taking the first 6 samples of the second replicate set, these samples were diluted in MilliQ-water to dilutions 10x,20x,30x,40x, and measured as described by Lowry *et al*. (1951) on a UV-Vis SpectraMax 190 Absorbance Microplate Reader to determine the correction dilution. Taking the remaining second replicate set of samples (defrosting 28 samples at a time), 25 µl was diluted 20x in MilliQ-water and the protein content determined as described by Lowry *et al*. (1951) and measured on the UV-Vis SpectraMax 190 Absorbance Microplate Reader. This procedure was repeated until the protein content had been determined for all samples. Samples were kept on ice throughout the process.

6.6 Superoxide dismutase (SOD) activity measurements

Superoxide dismutase activity based on xanthine oxidase and a colour reagent was determined using the third replicate set and a SOD Kit (Sigma-Aldrich), containing WST solution, enzyme solution, buffer solution, and dilution buffer. The first 6 samples (2x digestive gland, 2x gills, 1x control digestive gland, 1x control gills) were run at dilutions 10x,20x,30x to determine the correct dilution of 20x. Secondly, using a dilution of 20x, measurements were taken for the remaining samples using the SOD Kit and following the proscribed assay. By using the SOD Kit, Superoxide dismutase (SOD) activity was determined by measuring the reduction of cytochrome c by O_2 generated by the xanthine oxidase/hypoxanthine system (McCord and Fridovich, 1969), All measurements were taken on the UV-Vis SpectraMax 190 Absorbance Microplate Reader.

6.7 Statistics

Statistics were performed using PSPP 0.8.5 on data resulting from full measurements of GR, GST, Catalase and SOD; a statistical analysis on pH level measurements was not required. Testing for homogeneity of variance was done by using Levene's test ($p>0.05$) and statistical analysis of data by one-way ANOVA, Sheffe Post-hoc with significance ($p<0.05$). The data was visualized in graphs with means and ±SE (Standard Error) in Microsoft Excel.

7. Results

7.1 pH optimization of GR

To pH optimise GR (and in turn GST) for *Crassostrea gigas*, 6 randomly selected samples were taken and GR enzyme activity measured with solutions mixed at pH 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0. As can be seen in Fig. 6 there is a clear indication that the strongest result was measured with pH 7.2.

Samples	Details
T1	Gills, Lysekil
T2	Digestive Gland, Lysekil
T3	Gills, Tjärnö
T4	Digestive Gland, Tjärnö
T5	Gills, Control, Tjärnö
T6	Digestive Gland, Control, Tjärnö

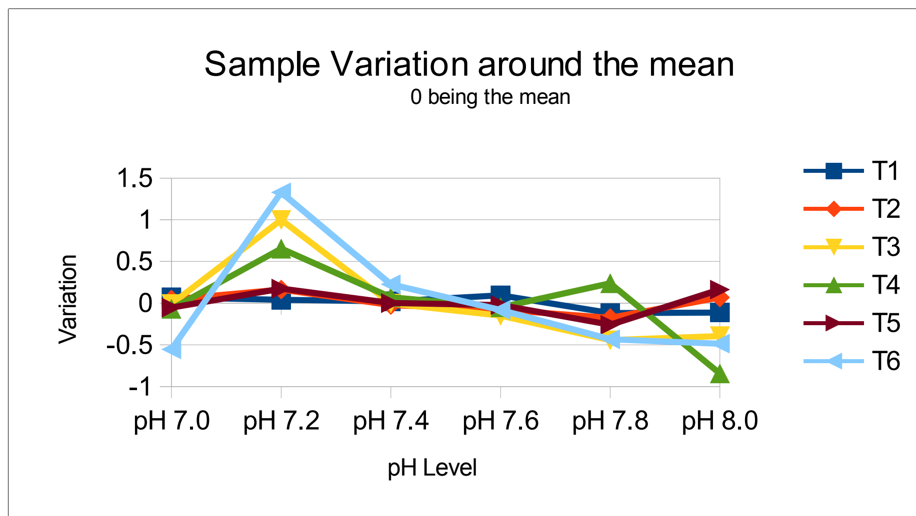


figure 6 GR measurement results with solutions at different pH levels for C. gigas

7.2 pH optimization of catalase

To focus the pH optimization of catalase I compared the standard assay pH level of 6.5 described by Aebi (1985) with the results of the GR optimization measurements (i.e. pH 7.2). Fig. 7 and 8 show the results of measuring the 6 randomly selected samples with solutions mixed at a pH of 6.5 and at a pH of 7.2. A negative value indicating a preference for pH 6.5 and a positive value a preference for pH 7.2. Even with 2 seperate measurment runs using the same samples the result was inconclusive, so pH 6.5 was used in all subsequent measurements taken in this thesis.

Samples	Details
T1	Gills, Lysekil
T2	Digestive Gland, Lysekil
T3	Gills, Tjärnö
T4	Digestive Gland, Tjärnö
T5	Gills, Control, Tjärnö
T6	Digestive Gland, Control, Tjärnö

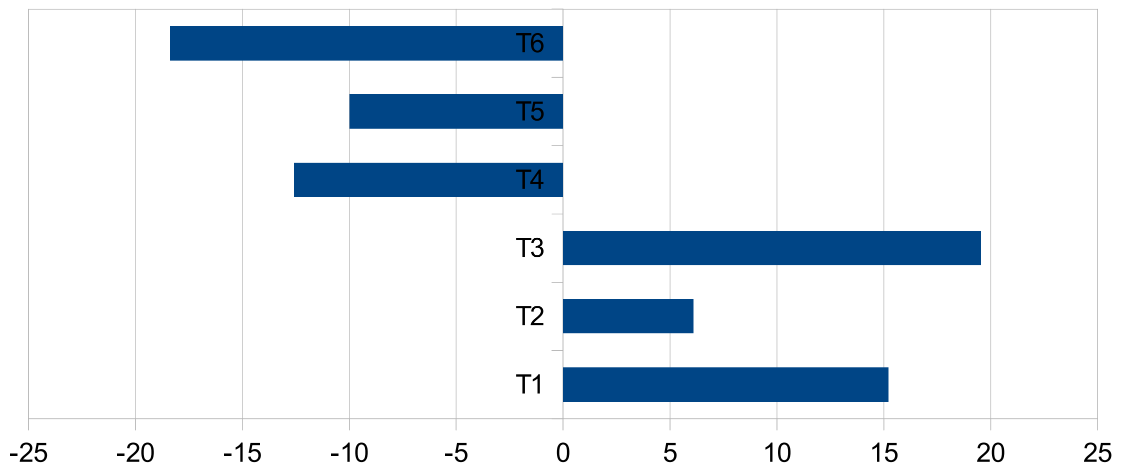


figure 7 Catalase measurement results with solutions at pH 6.5 & 7.2 for C. gigas first run

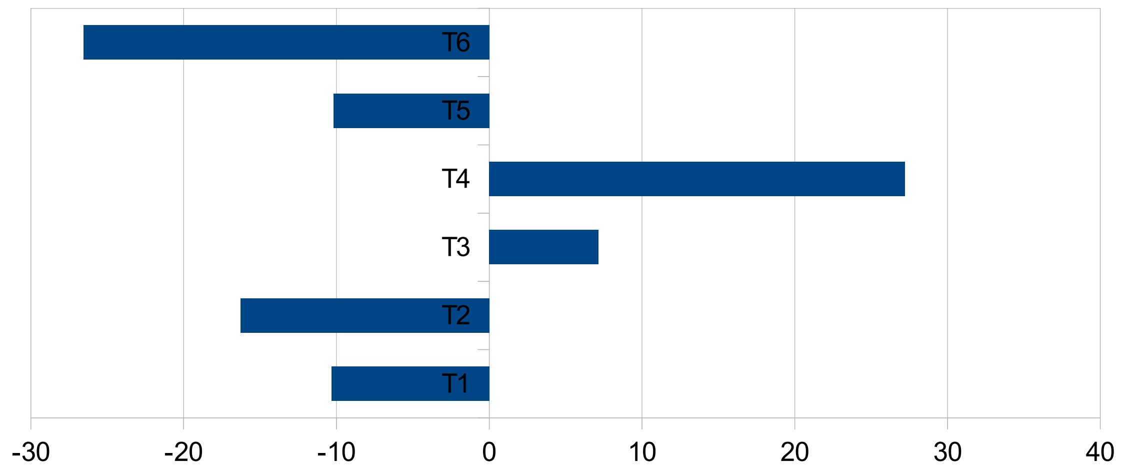


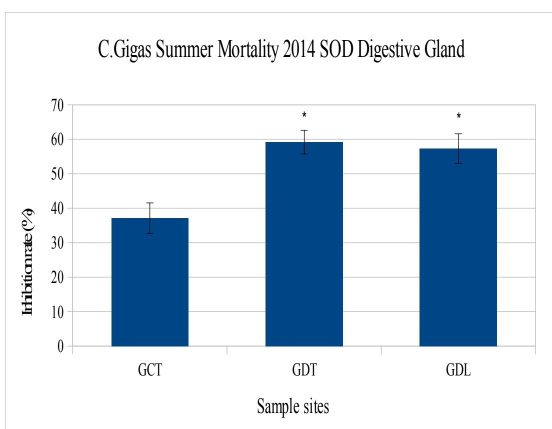
figure 8 Catalase measurement results with solutions at pH 6.5 & 7.2 for C. gigas second run

While the results for the GR measurements were clear, the catalase results were not. As can be seen by the 2 sets of catalase results, an influence can be seen by changing the measurements from serial to parallel. However, the overall result for catalase was still inconclusive.

7.3 Full Results Digestive Gland and Gills

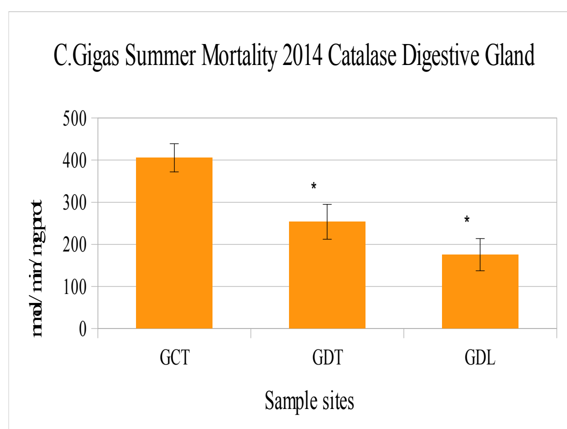
Results from the full antioxidant enzymes measurements (Fig 9 and Fig 10) varied depending on the sample type (Gills vs. Digestive gland) and on the enzyme measured. The results also varied depending on the site in which the samples had been taken. All graphs show the mean +SE of the 6 samples measured for each site and sample type (digestive gland vs. gills). Although some measurement results were not statistically different, Sheffe Post-hoc is seen as quite a conservative test and there is a general trend across the results that confirms that at the time the samples were taken, the oysters were under oxidative stress.

Measurement results from the digestive gland samples produced slightly clearer results than the gills samples.



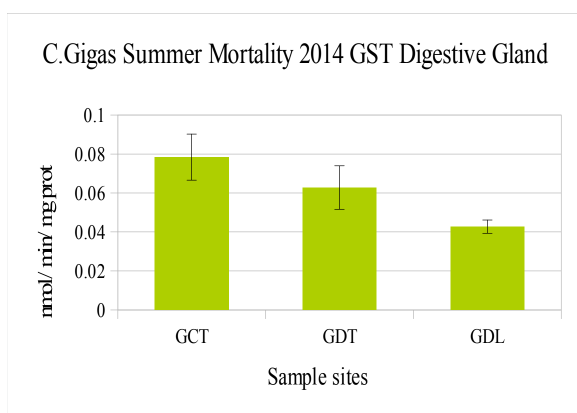
* Statistically significant vs Control

(GDL p=0.09, GDT p=0.05)



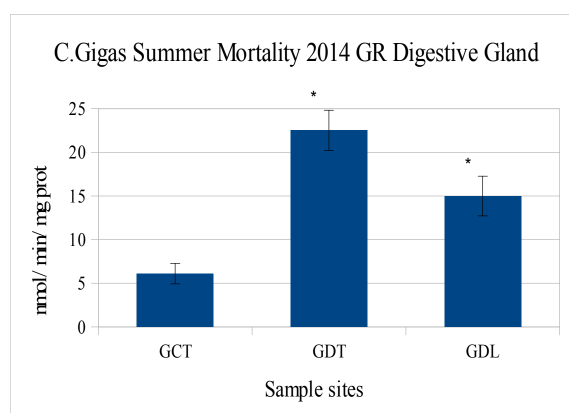
* Statistically significant vs Control

(GDL p=0.002, GDT p=0.035)



No statistically significant difference

(one-way ANOVA p=.053)



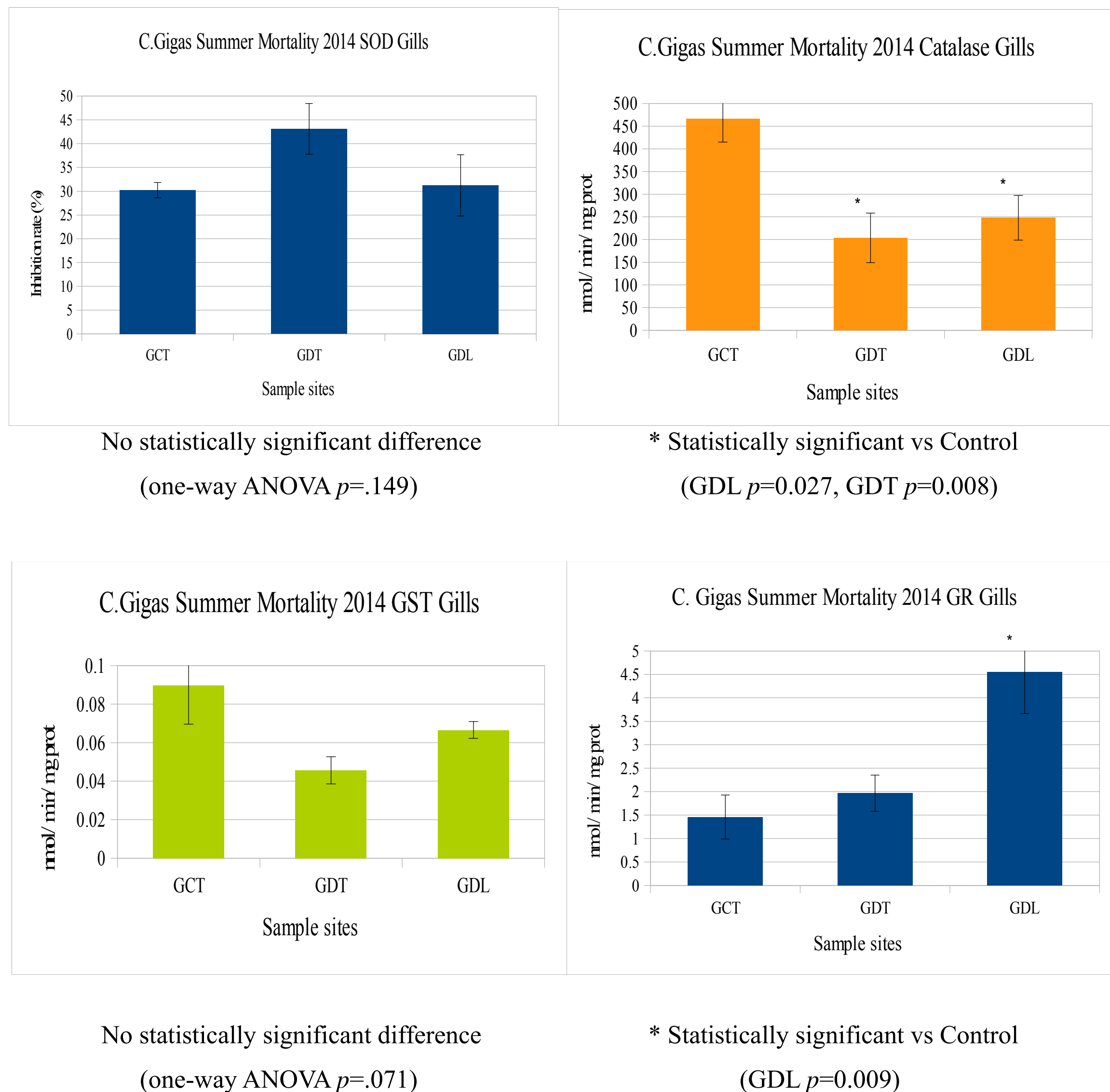
* Statistically significant vs Control

(GDL p=0.018, GDT p=0.000)

GCT= *C. gigas* Control Tjärnö, GDT=*C. gigas* Diseased Tjärnö, GDL= *C. gigas* Diseased Lysekil

figure 9 Digestive Gland GR,GST,Catalase and SOD full results for C. gigas

Measurement results from gills proved slightly less clear, however some interesting observations can be made. For example, the GR and GST results for the digestive gland samples showed higher measurements of enzyme activity from the Tjärnö site vs. Lysekil with the opposite seen from the gills samples. For SOD measurements taken from the digestive gland there is a clear, statistically significant result, however not for SOD measurements taken from gills.



No statistically significant difference (one-way ANOVA p=.149)

* Statistically significant vs Control (GDL p=0.027, GDT p=0.008)

No statistically significant difference (one-way ANOVA p=.071)

* Statistically significant vs Control (GDL p=0.009)

GCT= *C. gigas* Control Tjärnö, GDT=*C. gigas* Diseased Tjärnö, GDL= *C. gigas* Diseased Lysekil

figure 10 Gills GR,GST,Catalase and SOD full results for C. gigas

8. Discussion

Recent studies (Petton *et al*. 2015) recognized the possibility for additional factors involved in *Crassostera gigas* summer mortality events than the already extensively studied herpes OsHV-1 virus and *Vibrio* bacterium. The additional factors could be genetic or environmental. One method for assessing environmental factors affecting aquatic biota such as mussels, oysters and fish, is to measure antioxidant enzymes as an indicator for oxidative stress (Hellou *et al.* 2012, Regoli and Principato 1995, Béguel *et al.*, 2013 , Cossu *et al.*, 1997). In this thesis, I used this method to assess whether environmental factors in addition to OsHV-1 virus and *Vibrio* bacterium could explain the summer mortality events.

As few previous studies have been found using antioxidant enzymes measurements such as glutathione S-transferase (GST), glutathione reductase (GR), catalase, and superoxide dismutase (SOD) in *C. gigas*, the first hypothesis for this thesis was formed to assess whether previously established pH levels for GST, GR (pH 7.5) and catalase (pH 6.5) for these measurements would be suitable for this species. A clear result for GR was found at a pH of 7.2 thereby supporting the hypothesis that the standard pH level for GR and GST measurement assays is not suitable for *C. gigas*. However, in the case of catalase the results were inconclusive and no support could be made for the suggested hypothesis. This does not necessarily mean that for *C. gigas* the standard pH 6.5 is optimal for catalase measurements, just that no better pH level was found in this thesis. This work to assess pH level suitability for *C. gigas* enabled optimization of the GR and GST assays in order to complete measurements for the second hypothesis of this thesis.

The second hypothesis was formed to assess whether environmental factors are contributing to summer mortality events in addition to OsHV-1 and *Vibrio* (Petton *et al.* 2015). Using samples of sick oysters taken from the wild during the summer mortality event of 2014, antioxidant enzyme measurements were conducted for GR, GST, SOD and catalase. When analyzing the results, we can see that although not all the measurements on specific antioxidant enzymes showed a statistically significant difference between the affected sites vs. control, looking at the results as a whole there is clear evidence of oxidative stress. As measuring for oxidative stress can be used as a method to assess for

environmental factors affecting aquatic biota, this lends weight in support of the second hypothesis in this thesis.

When using oxidative stress as an indicator of an environmental factor in the summer mortality event, several angles need to be considered. The first being whether the Herpes OsHV-1 virus itself could be causing elevated antioxidant enzyme measurements. Previous studies in mice have shown the Herpes HSV-1 virus can induce ROS and oxidative stress, however it has also been shown that OsHV-1 virus is present in both oysters that survive a summer mortality event and those that do not. This may suggest that the OsHV-1 virus is not the cause of the oxidative stress results shown in this thesis.

Petton *et al*. 2015 suggested that some genetic strains of *C. gigas* could be more susceptible than others. This could be corroborated by a study on the OsHV-1 virus (Jouaux *et al.* 2013), which found that when oysters were introduced to an area where summer mortality occurs, they suffer a lower mortality rate than oysters already established in that site. It could be that the introduced oysters were a less susceptible strain. However it could also be that they have not been exposed to an environmental factor to the same extent that the oysters already existing at the site. Also from personal observations of summer mortality events on the west coast of Sweden during inventory field research in 2015, the common factor for all oysters found to still be alive related to their position; the few remaining live oysters that survived the mortality event were all concealed under stones/rocks or other oysters. If genetics was the contributing factor to their survival, I would have expected to see a larger variance in their locations within a site.

To suggest there is a direct link between pollution or other contaminant and oxidative stress as part of the summer mortality events is also difficult. Anthropogenic contaminants can induce oxidative stress in bivalves (Livingstone *et al.* 1993, Solé *et al.* 1995, Luna-Acosta *et al*. 2010), and mussels are used as sentinel species as indicators of pollution. However, the work carried out in this thesis can only indicate that the samples taken from the summer mortality event in 2014 were under oxidative stress. If pollution is a factor, it may not be a single pollutant but a cocktail that is causing the effect. In addition, if we make the assumption that pollutants could be the cause, this does not explain why summer mortality events only occur when there are periods of higher sunshine (UV), and warmer temperatures. These observations indicate that possible pollutants become more toxic with

warmer temperatures or increased UV, so-called synergistic effects of multiple stressors, and should be subject of future studies.

There are other abiotic environmental factors that could be affected by warmer temperatures, such as CO_2/pH levels. Tomanek *et al.* (2011) found that oxidative stress in oysters can be induced by high PCO_2 (partial pressure of carbon dioxide) levels. The warmer temperatures could also be inducing a naturally produced toxin that causes oxidative stress. Biotic factors such as Cyanobacteria which proliferate in warmer temperatures, produce several toxins, and have become more abundant due to increased nutrients from human activity (Smith 2003, Vahtera *et al.* 2007, Paerl and Paul 2012). Shelagh et al. 2009 suggests that a combination of high temperatures and high nutrients could be one possible cause of summer mortality, however is it this combination or a toxin from Cynobacteria that proliferates in these conditions that is the cause. Cynobacteria may also be a potential source of environmental stress in *C. gigas*. Another observation from this thesis is that the control samples were kept healthy with a constant flow of deep water. The intake for the deep water is in same region from where the sick *C. gigas* samples were taken but 35m below the surface. This would suggest that any environmental factor with negative effect on the survival of *C. gigas* is present in shallow water, which is their main habitat.

As is always the case when taking samples from the wild and not in a controlled environment, unknown elements may affect the results. Samples were taken from several sites for this study, and although different factors could be influencing the results from the different sites, there is a general trend of oxidative stress over the whole sample group. It was unfortunate that environmental measurements were not taken at the time the samples were collected, however the samples were taken and processed following accepted procedures and under supervision that should add weight to their reliability. Logically, as healthy *C. gigas* samples have been shown to also contain OsHV-1 in addition to sick *C. gigas* (Petton, 2015), it would seem unlikely that the oxidative stress is solely being caused by a pathogenic effect, however this could be fully discounted if a study would be conducted to measure both healthy and sick *C. gigas* for OsHV-1 and oxidative stress

When assessing the second hypotheses in relation to the results from the measurements of oxidative stress, previous studies, and field observations, there is support for the hypotheses that environmental factors are contributing to summer mortality events and more research should be conducted in this area.

Future research could focus on areas such as measuring environmental factors in and around *C. gigas* sites when summer mortality events occur, especially Cyanobacteria, CO_2, pH levels, and pollutants. Additionally, after assessing typical environmental conditions at *C. gigas* sites where summer mortalities occur, laboratory studies could be conducted whereby healthy oysters that contain the OsHV-1 virus and/or *Vibrio* are kept in similar environmental conditions and then have other factors introduced such as increased/reduced CO_2, temprature, sunlight (UV). Various toxins could also be introduced, such as common pollutants or common cyanobacteria toxins. By conducting studies in a controlled laboratory environment it may be possible to identify the combination of factors that are increasing *C. gigas's* susceptibility to OsHV-1 virus and/or *Vibrio* during summer mortality events.

Finally, testing several genetic strains for susceptibility to multiple environmental stressors in the lab would quantify the relative effects of genetic and environmental effects on summer mortality in populations of *C. gigas*.

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