The role of phytoplankton and environmental variables in Pacific oyster (*Crassostrea gigas*) aquaculture in British Columbia

by

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Abstract

Pacific oyster aquaculture in British Columbia faces serious challenges, such as high cadmium (Cd) concentrations, low growth and high mortalities during summer, and inability to directly gauge stress levels. The goal of this dissertation was to address these challenges by investigating the role of biological, physical and chemical oceanographic parameters in controlling them in various oyster farms in the Strait of Georgia. Three studies were undertaken. The first, from August 2004 to July 2005, investigated the role of phytoplankton in controlling Cd levels in the oysters in a Deep Bay farm. Phytoplankton mediated the transfer of dissolved Cd to the particulate phase, accounting for 90% of the summer reduction in dissolved Cd. This suggests that phytoplankton act as a sink for dissolved Cd, reducing the main source to the oysters. Two descriptive models for annual oyster Cd concentrations were developed based on environmental variables. The second study, from June to October of 2008, investigated how environmental factors, culture depth and seed size controlled oyster mortality and growth in four farms. Farms with less stratified, colder waters rich in diatoms fared better than those with highly stratified, higher temperature waters and persistent blooms of flagellates. Larger oyster seed presented low mortalities, while smaller seed were more susceptible to adverse conditions due to their ineffective particle processing capabilities. The best yield was obtained at a culture depth of 3 m, despite higher mortalities. A depth manipulation technique was investigated as a means to reduce summer mortalities without success. The third study, during the summer and fall of 2007 in Deep Bay, investigated a novel proteomic technique to detect and quantify heat-shock proteins (HSP) 70 and 90 in oysters to assess their stress levels. Mortalities were relatively low.
during that year (8.5% accumulated). The abundance of HSP 70 sequences was positively directly with non-harmful diatom biomass and negatively with high temperature and reproductive state. In contrast, the levels of HSP 90 were correlated negatively to the biomass of non-harmful diatoms, and positively to that of potentially-harmful algae, indicating that HSP 70s and 90s may have different triggers.
Preface

This thesis includes two published papers:


This publication forms Chapter 2 of this thesis. I had a minor role in the writing of the funding proposal for this project. I had a much larger role, together with Priyanka Lekhi, in the planning of the sampling procedures used. I was responsible for conducting monthly field collection of samples at one of the two study sites with Priyanka Lekhi. The other half of the sampling at this site was conducted by Nadene Ebell. During these sampling events we obtained oysters, filtered and non-filtered water, and size-fractionated suspended particulate matter samples, as well as measuring salinity and temperature. After the samples were brought back to the laboratory I analyzed them for dissolved nutrients and quantitative phytoplankton, while Priyanka Lekhi analyzed the filtered water and suspended particulate samples for cadmium. After obtaining the results of the sample analyses I performed most of the statistical analysis and wrote most of the research article. Drs. Pearce, Orians, and Maldonado helped shape and complete the publication. I was the corresponding author throughout the publication process and completed all the revisions requested by the reviewers and editor with the help of all the co-authors.

This publication forms Chapter 3 of this thesis. I had a large role in the identification of the research question and design of the experiments and field monitoring. I established and kept contact with the various participating organizations (University of British Columbia, Department of Fisheries and Oceans Canada, British Columbia Shellfish Growers Association, Mac’s Oysters Ltd., Taylor Shellfish Farms) with help from Dr. Pearce and David McCallum (British Columbia Shellfish Growers Association). I conducted half of the field sampling with the assistance of Lauren Moccia, while the other half of the sampling events was conducted by farm personnel (Simon Pickens, John Foster, and Gary Clark) I trained for this purpose. After the samples were brought back to the laboratory I analyzed them for dissolved nutrients, chlorophyll, and quantitative phytoplankton. After obtaining the results of the sample analyses I performed most of the statistical analysis and wrote most of the research article. Drs. Pearce and Maldonado helped shape and complete the publication. I was the corresponding author throughout the publication process and completed all the revisions requested by the reviewers and editor with the help of all the co-authors.

The information used to prepare Chapter 4 of this thesis was obtained during a field experiment in which I had a large role in the identification of the research question and design of the experiments and field monitoring along with Drs. Pearce, Maldonado,
Neil Ross (National Research Council), and Abayomi Alabi (Seed Science Ltd.). Dr. Pearce and David McCallum (British Columbia Shellfish Growers Association) coordinated the project between the various participating organizations and funding agencies. The field sampling was conducted in roughly equal parts by myself and John Blackburn (Fisheries and Oceans Canada) with minor assistance from Laurie Keddie (Fisheries and Oceans Canada). I analyzed the phytoplankton, dissolved nutrients, and chlorophyll samples and compiled all the data. I had a large role in the in silico part of the proteomic method developed to quantify stress proteins, selecting the fingerprinting peptide sequences. The oyster tissue sample analysis was performed by Dr. Juergen Kast and members of his laboratory Shujun Lin and Cordula Klockenbusch (Department of Chemistry, UBC). I conducted the statistical analysis and wrote most of the research article. Drs. Pearce, Maldonado, and Kast helped shape and complete the unsubmitted manuscript.

Ethical approval for lab experimentation with oysters was obtained from the Animal Care Committee (A07-0050).
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To my family.
Chapter 1 Introduction

1.1 Aquaculture of the Pacific Oyster

The Pacific oyster (*Crassostrea gigas* Thunberg, 1793) is the most widely-farmed and commercially important bivalve mollusc in the world (FAO, 2010), and it may be considered a model animal for studies of the physiological effects of environmental variables on estuarine molluscs. The total aquaculture production for this species reaches close to 4.5 million tonnes annually, with a total value of around USD 1.14 billion during 2009 (FAO, 2010). This mollusc has been a preferred species to cultivate because it grows rapidly, can be cultured in a variety of estuarine environments, and has high market value (Pauley et al., 1988).

In British Columbia (BC), the Pacific oyster has been cultured since 1912-13, when they were first introduced in the southern Strait of Georgia (Lavoie, 2005). The main farming areas are currently located in Baynes Sound, around Cortes Island, and in Okeover Inlet (Fig. 1.1) (BC Shellfish Growers Association, 2011). The production of this species in BC was around 2000 tonnes between 1950 and the early 1970s, but increased to more than 5400 tons by 2009 (FAO, 2010). Most of the production is mediated by small operations, with different degrees of mechanization in their productive processes and various culturing techniques. This, however, has started to change somewhat as large companies (*e.g.* Taylor Shellfish Farms) have recently begun multi-site operations in BC.
Figure 1-1 Location of main Pacific oyster culture areas in British Columbia (squares) and spat collection areas (arrows).

Oyster seed or spat (small oysters of approximately 5–25 mm in shell height), used for grow-out operations in BC, are collected in Pendrell Sound and Pipestem Inlet (Fig. 1.1) or bought from hatcheries (typically in the USA). The spat culture techniques used in BC vary from technologically-simple beach culture, where oyster spat is strewn on a cleared beach that is somewhat protected from predators with netting, to suspended methods, which reduce the need for predator protection and increase product yields (Quayle, 1988; Pauley et al., 1988). Suspended-culture methods also permit continuous seawater immersion of the oysters, providing them with constant potential food sources and more stable temperature conditions compared to beach cultures (Quayle, 1988; Hamdoun et al., 2003). Within the suspended techniques used in BC, the rope method is the simplest, with the small oysters being settled onto oyster shells or other substrates and then suspended by drop lines that are attached to either rafts or long lines until the oysters
reach market size. At the other end of the suspended-technique spectrum is tray culture, which includes a higher degree of handling and management of the oysters during grow-out. This tray method produces a better product than rope or beach culture, which results in a product that may be suitable for high-end and specialty markets.

Once the oysters are placed into culture trays, normally during the spring or in the fall, they are subjected to periodical size screening through shaking perforated metal trays. Other processes, such as tumbling in metal drums, may be used to eliminate new shell growth, thus obtaining a small and highly-cupped product for specialty markets. The oysters remain under culture from one to three years, depending on the local growth conditions and market size requirements. Harvesting is done throughout the year, as long as the oysters are not undergoing gametogenesis, which decreases the meat quality.

Several methods have been used for the selection of oyster culture areas, from graphical to computerized mathematical models (Brown and Hartwick, 1988a; Barillé et al., 1997; Pouvreau et al., 2006). In general, these methods determine the suitability of sites for grow-out operations based on various environmental variables, including temperature, salinity, and food availability (i.e. chlorophyll a) (Brown and Hartwick, 1988a; Pouvreau et al., 2006). Habitat suitability studies for suspended-tray culture of Pacific oysters in BC have established that oyster growth and mortality are largely determined by seawater temperature and food availability (Brown and Hartwick, 1988a; Roland and Brown, 1990).

Site selection is based on ideal conditions for grow-out operations with little information on the oyster seed’s environmental requirements. Small oysters (<24 mm shell height) are more susceptible to adverse conditions (e.g. prolonged periods of
harmful-algae blooms) than adults, as they have restricted ability to select particles (Cannuel and Beninger, 2007), relatively small energetic reserves, and high energetic demand due to somatic growth. As a result, adequate site selection is especially important for the placement of nursery operations in order to diminish mortalities and promote fast growth. In addition, existing site selection tools, although practical and useful, have limitations as they are based on linear growth of the oysters and are not dynamic [size increment at each time step (Gangnery et al., 2003)]. An additional challenge is estimating food availability based on chlorophyll concentration, which does not distinguish the favoured microalgae from detrital suspended solids and harmful algae.

Despite the rapid development of oyster aquaculture in BC in recent years, three major problems still impact this industry: closures of international markets due to high cadmium (Cd) levels in their product, high oyster mortality rates during summer, and the absence of adequate methods to measure sublethal oyster stress.

1.2 Cadmium in cultured Pacific oysters in British Columbia

Historically, Pacific oysters collected on the coast of BC can accumulate relatively high levels of Cd, up to 2 ppm (wet weight) or higher (Kruzynski, 2004). The high Cd concentrations in BC oysters are problematic for farmers as stringent international allowable limits for Cd in food have been set in their main export markets. In particular, Hong Kong has set the maximum allowable Cd concentration at 2 ppm (Hong Kong Centre for Food Safety). In other markets, the limits vary between 1 ppm (European Union) and 3.7 ppm (United States) (Kruzynski, 2004; United States Food and Drug Administration recommended guideline). These measures have been taken as the
consumption for extended periods of time of oysters with high Cd levels can lead to increased Cd concentration in the blood (Copes et al., 2008).

High levels of Cd in oysters do not seem to follow a geographical pattern in the northeast Pacific (Feng and Bendell, 2009; Ng et al., 2010). Possible point sources of Cd have been identified, such as fresh-water inputs, mines or mineral deposits, leaching of farm equipment, fertilizers, logging activities, and municipal waste-water disposal (Kruzynski, 2004). There is no clear correlation, however, between these factors and high Cd content in oysters. Indeed, the Cd isotopic signature of BC oysters indicates that the sources of seawater Cd are mainly natural, rather than anthropogenic (Shiel, 2010). In turn, the dissolved and particulate Cd pools in seawater are both possible sources of Cd to the oysters (Ray, 1984; Roesjadi and Robinson, 1994). This Cd may be internalized by the oysters through the seawater that comes in contact with their soft tissues (Borchard, 1983; Lekhi et al., 2008) and through food, mainly phytoplankton (Feng and Bendell, 2009; Ng et al., 2010).

The high concentrations of dissolved Cd (Cd\textsubscript{diss}) found in the north Pacific Ocean could be responsible for the higher concentrations of Cd seen in oysters from the Pacific coast of North America compared to those from the Atlantic coast (Kruzynski, 2004). Due to the deep ocean circulation, deep water formed in the north Atlantic travels south around Antarctica and ends up in the north Pacific Ocean. This water has accumulated Cd from sinking particles for ~1000 years, so the Cd\textsubscript{diss} concentrations at depth in the north Pacific Ocean are 3 to 5 times greater (values ranging from 0.08–0.10 ppb at depths greater than 1000 m) than the concentrations of the fairly “young” deep water of the north Atlantic Ocean (0.02–0.03 ppb at depths greater than 1000 m) (Bruland and Franks,
1983). This difference is also reflected in typical surface water Cd levels. Dissolved Cd values at ~7 m depth on a transect between the English Channel and the open northeast Atlantic Ocean had values up to 0.009 ppb (Kremling and Pohl, 1989), which is 3–10 times lower than values reported in BC at similar depths (Kruzynski, 2004). As a result, the Cd content of oysters cultured in the Atlantic Ocean is normally below the 1 ppm European Union limit, while that of those in the Pacific Ocean ranges between 1.5 and 3 ppm (Kruzynski, 2004). In extraordinary cases, the Cd levels of some Pacific oysters have been up to 7 ppm (Glynn et al., 2004; Lekhi et al., 2008).

The Cd$_{\text{diss}}$ brought to the Pacific Ocean by these deep currents reaches the surface through upwelling (Lares and Orians, 1997). Once close to the surface, some of the Cd$_{\text{diss}}$ is taken up by phytoplankton and other organisms, thus reducing its concentration. Local physical processes (such as coastal upwelling, entrainment, and water mixing due to wind) replenish the Cd$_{\text{diss}}$ in the surface with cold/saline waters below the pycnocline, which are also rich in Cd$_{\text{diss}}$ (Lekhi et al., 2008). These physical processes can mediate heightened Cd levels in shellfish (Lares and Orians, 1997; Lekhi et al., 2008).

Particulate Cd (Cd$_{\text{part}}$) can be found in both inorganic and organic forms. The latter is thought to be a possible source of Cd to oysters (Ettajani et al., 2001) as the oysters have a feeding preference and are able to select for organic particles rich in phytoplankton. Phytoplankton abundance and species composition follows an annual cycle, which is determined by various environmental factors. These changes in phytoplankton composition and biomass may influence Cd accumulation in oysters throughout the year, as Cd is known to accumulate differently in various algal groups. For example, when grown with high Cd concentrations, diatoms are able to maintain low
cellular Cd levels, while naked chlorophycean flagellates accumulate Cd readily (Payne and Price, 1999). Although Cd was not historically known to have any biological role in phytoplankton, its distribution profile in the open ocean follows that of nutrients such as phosphate (Bruland et al., 1978; Abe, 2004). Recently, Cd has been shown to have a nutrient role in phytoplankton by substituting for zinc in the metalloenzyme carbonic anhydrase (Price and Morel, 1990; Lane et al., 2005; Dixon et al., 2006). As Cd$_\text{diss}$ is thought to enter the algal cells through non-specific transporters, microalgae use resilient intracellular organic ligands, such as phytochelatins, to detoxify the metal (Lee and Morel, 1995; Payne and Price, 1999).

The relative importance of various Cd pathways into shellfish has been widely debated. Uptake of dissolved Cd has been identified as the main pathway in some locations (Lekhi et al., 2008), but high Cd concentrations in shellfish stomachs suggest that trophic transfer from ingested particles might also be important (Bendell and Feng, 2009). Recent studies, both field- (Lekhi et al., 2008; Bendell and Feng, 2009; Ng et al., 2010) and lab-based (Strady et al., 2010), have been able to shed some light into this debate. The results of these studies point towards a large role for Cd$_\text{diss}$, as it is rapidly taken up through the gills and other tissues that are exposed to seawater. Cadmium in phytoplankton has a smaller impact on oyster Cd (Cd$_\text{oys}$) concentrations and its accumulation rate in oysters is much slower than from Cd$_\text{diss}$, although retention is greater (Ettajani et al., 2001, Lekhi et al., 2008; Strady et al., 2010). The accumulation of Cd by shellfish and the relative importance of these two main pathways can be strongly modified by environmental variables (Bendell and Feng, 2009; Ng et al., 2010). Once the
Cd is absorbed through either pathway it is then distributed throughout the oyster’s organs and tissues (Strady et al., 2010).

Cadmium, as well as other heavy metals, exists in two main forms in oysters: as a labile and easily released form and as resilient intracellular complexes (Boisson et al., 2003). The labile form is normally found as small inorganic particles in the diverticula of the hepatopancreas, while the resilient form is present intracellularly and in the hemolymph (Roesijadi and Robinson, 1994). Cytosolic Cd is stored in various chemical forms – in inorganic compounds, ionic form, and bound to proteins. The main metal-binding protein has been identified as a 10-kDa metallothionein (Engel, 1999) in American oysters (*Crassostrea virginica*), a resilient organic compound used by some organisms to detoxify and store toxicants in an innocuous chemical form.

Practical ways of reducing Cd levels in oyster products are not commercially feasible. For example, short-term depuration has been tried, but is not very efficient as only a small percentage (10–20%) of Cd is stored in labile compounds with a short half-life (2–10 days) (Boison et al., 2003). In contrast, most of the Cd is stored in resilient forms, which have a very long half-life (+200 days) and are difficult to depurate (Boison et al., 2003). The production of processed products (smoked, soaked in brine, frozen) was thought to provide an alternative solution, but failed to do so (Eccles, 2000). Thus, it seems that the only option for farmers to avoid high Cd in oysters is to manage the culturing and harvesting of their product. For this purpose, the following studies would be beneficial: 1) investigating the environmental variables that control the intake of Cd by BC oysters, 2) determining the geographical and temporal variations of Cd_{oys} levels in
field oysters, and 3) designing mathematical models to predict Cd levels in cultured oysters throughout the year.

My research was designed to provide answers to these three research questions. A year-long field monitoring study was conducted to elucidate the role of environmental variables in modulating Cd\textsubscript{diss} and Cd\textsubscript{part} in seawater, and how, in turn, the resulting Cd\textsubscript{diss} and Cd\textsubscript{part} affected Cd concentrations in oysters in their second year under culture conditions. Among the environmental variables investigated, special focus was given to phytoplankton biomass and community composition, as well as oysters’ growth and Cd content. Our results were used to create two mathematical models, one that explains the maximum variability of Cd\textsubscript{oys}, while the other uses variables easily measured under field conditions (Cassis \textit{et al.}, 2011a).

1.3 Summer mortalities of cultured Pacific oysters in British Columbia

Reports of high summer mortality rates have been common since the Pacific oyster was first introduced to western North America (Cardwell \textit{et al.}, 1979; Burge \textit{et al.}, 2007), although this is a worldwide problem with cultured shellfish (Pauley \textit{et al.}, 1988; Burge \textit{et al.}, 2007; Samain and McCombie, 2007; FAO, 2010). Some of these events have reached massive proportions causing the death of 50–90\% of the oyster farm stock. These losses affect juvenile as well as adult oysters, deepening the economic problems that they cause.

Some of the factors that could be involved in these oyster mortalities are: 1) abrupt changes in temperature or high temperatures, 2) abrupt changes in salinity or low salinity, 3) harmful-algal blooms (HAB), 4) energetic misbalance due to reproductive
effort, 5) pollutants, 6) farming practices involving rough handling and shaking of the oysters, 7) anoxia due to upwelling of oxygen-depleted deep water, and 8) opportunistic bacterial or viral diseases (Galtsoff, 1964; Gainey and Shumway, 1988; Pauley et al., 1988; Burge et al., 2007; Samain and McCombie, 2007; Li et al., 2009). However, salinity changes, diseases, and pollutants normally have a limited geographical distribution and, rather than cause mortalities themselves, they accentuate the strain experienced by the oysters due to other stressful factors (Li et al., 2009). The main suspected causes of high summer mortality rates of shellfish are high temperature, high energy expenditure during the reproductive cycle, and HABs (Werner and Hinton, 1999; Burge et al., 2007; Samain and McCombie, 2007). The exact role of these factors (both individually and in combination) in stressing the oysters, leading ultimately to their death, however, is not fully understood. A combination of several of these stressors is thought to be the most common cause of summer massive mortalities of cultured oysters, as high temperatures and energy expenditure during the reproductive effort can render the oysters unable to cope with lesser sources of stress, such as starvation and bacterial infections (Werner and Hinton, 1999, Li et al., 2009).

Practical ways to reduce summer oyster mortality rates under culture conditions have been sought, but have generally not been successful due to the amount of labour required and the complications they would add to farming procedures. The mortality problem has been addressed through management improvements (i.e. change in timing of introduction to culture or harvest) or techniques focused on certain growth stages of the oysters, such as the use of intensive nursery systems before small oysters are introduced to tray culture (Ralonde, 1998). A second way to reduce mortality rates is through site
selection for conditions better suited for oyster culture based on the environmental factors described above. Site selection has been a powerful tool in the optimization of oyster culture throughout the world (Brown and Hartwick, 1988a; Gangnery et al., 2003; Pouvreau et al., 2006; Barnes et al., 2007). These mortality reduction measures require a thorough knowledge of the interactions between the environment and cultured oysters, as well as the environment prevalent in the prospective culture area. For this purpose, research needs to be focused on: 1) investigating the effect of environmental variables on growth and survival of cultured oysters throughout a geographical range, and 2) testing new methods to reduce the effect of harmful levels of environmental variables.

My research was designed to provide useful information to improve the management of cultured oysters, including a technique to reduce the oyster’s exposure to detrimental high temperatures and harmful algal blooms. This was achieved by monitoring four farm areas for oyster growth and mortality rate during the first year under culture, as well as for various environmental variables, including detailed phytoplankton abundance and composition. A depth manipulation technique was also tested for its possible effect in reducing oyster mortalities triggered by high temperatures and HABS near the surface of the water column.

1.4 Responses of Pacific oysters to the main stressors identified

1.4.1 Temperature

The main factor implicated in summer shellfish mortalities throughout the world is high temperature, but normally this occurs in combination with other physiological and environmental stressors (Cardwell et al., 1979; Samain and McCombie, 2007), making it
difficult to assess the relative contributions of each factor in oyster mortality. Pacific oysters present an allometric physiological response to temperature (Bougrier et al., 1995). Clearance rate increases at a rate proportional to the temperature, reaching a maximum at 19°C and decreasing sharply with higher temperatures (Bougrier et al., 1995). Oxygen demand, however, showed a continuous increase with temperature throughout the range tested by Bougrier et al. (1995) (5-32°C). The different behaviour of clearance rate and oxygen demand with increasing temperature could generate an energetic gap during warm (>19°C) periods, decreasing the food input while increasing the energy usage (Bougrier et al., 1995). This could contribute to high stress levels and mortalities of oysters during the summer. The surface temperature in the Strait of Georgia rarely exceeds 20°C during the summer months, although exceptionally warm spells can drive the water temperature several degrees above this mark in enclosed bays (Thomson, 1981; Davenne and Masson, 2001; Cassis et al., 2011b).

Temperatures above 20°C, or fast changes in temperature (varying by 6 to 8°C in 24 h) may cause cellular damage in oysters thus inducing the expression of molecular chaperones that capture and repair or dispose of these damaged proteins (Hofmann and Somero, 1995). The large pool of molecular chaperones produced by stressed oysters can, in turn, protect the shellfish from other temperature stresses for up to two weeks after the initial shock (Clegg et al., 1998; Hamdoun et al., 2003). The effects of high temperatures on oysters can also include an increase in the energetic demand, as the damaged proteins have to be re-natured or replaced and the high concentrations of HSPs necessary to counteract the damage have to be produced and maintained (Hofmann and Somero, 1995). Although a temperature of 20°C is not high enough to kill cultured Pacific oysters
[lethal temperature 40–43°C (Clegg et al., 1998; Hamdoun et al., 2003)], the combination of stressors – such as HABs, reproduction, and mechanical disturbance caused by rough handling – could become unbearable to the oysters, resulting in high mortality rates.

1.4.2 Reproduction

The reproductive effort normally observed during the summer, when high quality food may be scarce, may produce an energy deficiency in the oysters and a reduced capacity to deal with other environmental stressors (such as high temperature and HABs), ultimately leading to their death (Cho and Jeong, 2005; Li et al., 2009). The production of gametes by Pacific oysters is an energy demanding process that may precede other biological processes in energy allocation (Cardwell et al., 1979; Li et al., 2009). Though oyster spawning is sporadic and occurs in several irregular bursts, it is most common in spring and summer throughout the world (Pauley et al., 1988; Samain and McCombie, 2007).

1.4.3 Harmful algae

Traditionally, the effects of harmful algae on BC oysters have been related to toxin production, which prevents the sale of the oysters until they have been depurated of the toxins. Harmful-algae blooms (HABs) have also been shown to contribute to mortalities of cultured shellfish directly through toxin production and hypoxia, or indirectly through feeding efficiency reduction (Gainey and Shumway, 1988; Landsberg, 2002; Cassis and Taylor, 2006). Oyster spat can suffer a reduction in filtration rate and an increase in the amount of pseudo-feces produced by the strong rejection reactions elicited
by the toxic *Alexandrium* and *Protoceratium*, limiting their food capture capabilities during times of fast growth (Gainey and Shumway, 1988; Wildish *et al*., 1998; Lassus *et al*., 1999; Cassis and Taylor, 2006).

The most prominent toxic harmful algae in BC belong to the genera *Alexandrium, Protoceratium, Pseudo-nitzschia, Dinophysis*, and *Heterosigma*, which produce paralytic shellfish poison (PSP), yessotoxin, amnesiac shellfish poison (ASP), diarrheic shellfish poison (DSP), and a yet unknown toxin, respectively (Lansberg, 2002; Fryxell and Hasle, 2003; Taylor *et al*., 2003; Twiner *et al*., 2005). The phytoplankton toxins can enter the oysters via direct consumption of the harmful algae, or by the algal release of the toxins into the seawater and subsequent internalization of the toxins by the oysters as they filter the water (Lassus *et al*., 1999; Landsberg, 2002; Pate *et al*., 2005). So far, only a few studies have linked physiological stress in oysters with HABs. The toxigenic diatom *Pseudo-nitzschia*, which produces the neurotoxin domoic acid, was shown to produce immune stress in Pacific oysters by reducing the number and phagocytic activity of their hemocytes in a similar manner as the antifouling agent tributyltin (Jones *et al*., 1995a, b). Flow cytometry was used by Hégaret and Wikfors (2005) to trace the immune response of eastern oysters (*Crassostrea virginica*). When exposed to *Prorocentrum minimum*, the oysters presented an increase in hemocyte number and in phagocytosis that were both dependent upon the duration of the exposure. A related species, *P. rhathymum*, damages the digestive system of Pacific oysters and is believed to be a contributor to massive mortalities of oysters elsewhere (Pearce *et al*., 2005). In contrast, however, members of the *Alexandrium* genus were found to have no effect on the eastern oyster immune system (Hégaret *et al*., 2005, 2007).
Annual blooms of the raphidophite *Heterosigma akashiwo* in the inlets surrounding the Strait of Georgia (Taylor and Haigh, 1993) can cause mortalities of a wide variety of marine organisms (Landsberg, 2002; Keppler *et al.*, 2005). The uncharacterized toxin produced by this alga causes lysosomal destabilization in the hepatopancreas of oysters and, when ingested in high quantities, it may produce long-term, negative, sublethal effects in cultured oysters (Keppler *et al.*, 2005). Another toxic raphidophite *Chattonella globosa* has been recently detected in the Strait of Georgia. This species is considered potentially dangerous to BC finfish and shellfish aquaculture, as previously shown in Japan and California (Barraza *et al.*, 2004).

In addition to toxigenic effects, small oysters are also more susceptible to starvation during HABs as they are forced to close their valves at a time of accelerated growth and very little energetic reserves (Gangnery *et al.*, 2003). In contrast, HABs (*e.g.* *H. akashiwo*) are less likely to harm larger oysters with reasonable energy reserves, unless they are preparing for reproduction and spending large amounts of energy in gametogenesis (Li *et al.*, 2009).

A number of non-toxic algae may also pose a danger to oyster aquaculture in BC. Some algal species (*e.g.* *Chaetoceros* and *Rhizosolenia*) have long projections or spines which make it impossible for oysters to ingest them (Cassis and Taylor, 2006), whereas others (*e.g.* *Cylindrotheca closterium*, *Euglena viridis*, *Trichodesmium erythraeum*, and *Chlorella* sp.) can clog the gills of shellfish due to their exceptionally high biomass during blooms (Landsberg, 2002). The former species can cause irritation and a “spitting” behaviour in cultured oysters while the latter can smother the oysters and decrease their nutritional state, leading to mortalities (Galtsoff, 1964; Baldwin and Newell, 1995; Negri
et al., 2004). The non-toxic dinoflagellates *Ceratium fusus* and *Akashiwo sanguinea* form part of the normal phytoplankton species succession of the Strait of Georgia (Taylor et al., 1994). Both have been blamed, in part, for oyster summer mortalities in Puget Sound, possibly damaging the oysters through the bacteria associated with these dinoflagellates or reducing the filtration rate because of their large size and shape (Cardwell, 1978; Landsberg, 2002).

Algal blooms can also produce physiological stress in oysters through a reduction in the seawater dissolved oxygen (David et al., 2005). As phytoplankton respire during the night and bacteria use oxygen while decomposing the large amounts of algal biomass, some high-biomass algal blooms can cause hypoxia, and even anoxia (Landsberg, 2002). The nutritional status of the oysters can also be a determining factor in the stress they experience during algal blooms. Shellfish can reach a low physiological condition and a compromised immunological response due to starvation and malnutrition. In contrast, oysters fed large quantities of high-quality food, such as small flagellates, can have a strong immune response and resistance to stressful conditions (Wikfors et al., 2000; Hégaret et al., 2004).

1.5 Quantifying stress in Pacific oysters

Oysters are exposed to a highly variable environment during culture, with large and sometimes abrupt changes in variables such as temperature, salinity, and food availability. These changes in the environment may produce physiological stress in the oysters, damaging proteins and membranes in the cells and necessitating the existence of defence and repair mechanisms (Hofmann and Somero, 1995). Oysters do not exhibit
external signs of stress, however, and growers are unable to identify stressed animals until they develop into mortalities. Due to this, various biochemical methods are used to measure stress under controlled conditions and in the field (Clegg et al., 1998; Lacoste et al., 2002).

Several methods to detect and quantify physiological stress in shellfish have emerged during the last decade. Among them, those based on proteins produced to counteract the damaging effects of stressors [i.e. heat-shock proteins (HSPs), methallothioneins, and ubiquitins (Clegg et al., 1998; Lewis et al., 1999; Cruz-Rodriguez et al., 2000; Boutet et al., 2003b; Sokolova, 2004)] are of special interest due to the wealth of recent information and relative simplicity of the quantification methods. Drawbacks, such as low sensitivity in the lower range of the stimulus and low specificity have stimulated interest and research into the use of multiple biomarkers for assessing the stress level of bivalves, including the use of several families of HSPs to obtain a characteristic profile of the stress response (Hofmann and Somero, 1995; Downs et al., 2002).

Heat-shock proteins are part of the stress and immune response found in all organisms which help them cope with environmentally stressful situations. These proteins are ubiquitous and highly conserved throughout evolution, being detected in all organisms from bacteria to humans (Werner, 2000). Under normal conditions they have an important role in protein-protein interactions, helping stabilize unfolded proteins, preparing proteins for disposal, preventing unwanted protein aggregations, transporting proteins through cell membranes, and assisting in the establishment of proper protein conformation (Hamdoun et al., 2003; Padmani and Usha Rani, 2009). Under
physiologically stressful conditions the expression of this group of proteins is upregulated. A wide variety of stressors can elicit an HSP response (Lewis et al., 1999) including infection, hypoxia, hot and cold shocks, and exposure to toxins and heavy metals (Clegg et al., 1998; Cruz-Rodriguez et al., 2000; Downs et al., 2002; Boutet et al., 2003b; Sokolova, 2004).

The major, most highly-conserved, and best studied of the HSPs are the 70 kDa family (Hamdoun et al., 2003; Encomio et al., 2007). This group is comprised of important molecular chaperones whose over expression can produce an acquired tolerance to environmental stresses for a limited time (Clegg et al., 1998). The HSP 70 family is composed of environmentally-inducible (HSP) and constitutively-expressed (HSC) forms. The former are normally found in the cells in low concentrations, but are produced in large amounts after a sublethal stress, while the latter form part of the normal cellular protein pool (Boutet et al., 2003a). The expression of HSP 70 may be curtailed by exposure to heavy metals (Ivanina et al., 2009) and anoxia (Ueda et al., 2009).

Other HSP families that perform important physiological roles as molecular chaperones are HSP 60 and 90. These proteins are also upregulated after a sublethal stress, but have different responses to various stressors (Snyder et al., 2001). HSP 60 is produced in large quantities after heat-shock (Rispoli et al., 2001), but also after an increase in oxidative stress in response to various toxins and high concentrations of metals (Smith and Treblay, 1999; Malagoli et al., 2004; Moraga et al., 2005; Franco et al., 2006). HSP 90 is expressed in response to sublethal heat-shock and bacterial infections and helps regulate apoptosis and metamorphosis in larval animals, as well as having a strong role in controlling gametogenesis and reproduction (Bishop and
This family of stress proteins also helps in the folding and repair of receptor proteins (Jackson et al., 2004; Chakraborty et al., 2008). The main role of HSP 90 under conditions of stress seems to be to enhance the reactivation rate of stress-damaged proteins, rather than to protect proteins from inactivation (Csermely et al., 1998). Other families of HSPs exist, such as the less studied HSP 27 and 23 (Meistertzheim et al., 2007; Lang, 2008).

HSPs have been widely used to study the effects of stressors due to their fast expression and ease of detection, as well as due to their response to a wide range of stressors. These proteins are well suited to study short-term physiological effects of stressors and the damage they can produce in an organism, ultimately leading to its death, as well as the development of tolerance to stressors resulting from sublethal shocks. Several drawbacks exist to the use of HSPs as stress indicators: 1) the threshold for the induction of a response can be dependent upon the recent environmental history of the animal (Buckley et al., 2001), 2) they are difficult to link to higher biological order and longer term effects, such as growth or reproductive output (Lewis et al., 1999), and 3) they respond to multiple stress agents, thus the effect of individual stressors is difficult to tease apart in field studies (Werner and Hinton, 1999; Rossi et al., 2006). The seasonality of HSP expression has been discussed much, but still remains poorly defined (Farcy et al., 2007). As current protein-based methods for the quantification of HSP have issues in terms of high resolution and reproducibility, this has stimulated interest and research into the use of multiple biomarkers and novel technologies for assessing the stress protein level of bivalves.
This study was focused on: 1) using a new proteomic method to detect and quantify two stress biomarkers (HSP 70 and 90) in oysters under field conditions, 2) determining the oysters’ stress levels throughout the summer during their second year under culture conditions, and 3) studying the effects of multiple environmental variables and phytoplankton on the levels of stress indicators and their relationship with oyster mortalities. The research identified proteomic fingerprinting as a feasible technique to identify and quantify HSP 70 and 90 in Pacific oysters. The levels of HSP 70 and 90 and their variations were described in oysters throughout the summer at Deep Bay. Potential environmental stressors were studied in relation to levels of HSP 70, 90, and oyster mortalities. Special attention was given to the role of various phytoplankton taxonomic subgroups in stressing the oysters.

1.6 Objectives

In order to reduce the impact of the current challenges faced by the oyster aquaculture industry in BC today, such as high Cd levels and large-scale summer mortalities, oyster farmers require the ability to monitor the environment and predict its effect on shellfish stocks. To facilitate this, it is necessary to understand the roles of various environmental factors (biological, physical and chemical) on oyster growth, stress, and survival. With this goal in mind, my research was focused on determining: 1) the role of phytoplankton in modulating Cd levels in cultured Pacific oysters, 2) the effect of multiple environmental variables on growth and mortality of cultured Pacific oysters, and 3) stress levels in cultured Pacific oysters and the environmental variables that affect them.
The first general objective had the following specific sub-objectives: 1) investigating the role of phytoplankton in modulating Cd concentrations in the environment and in the oysters and 2) designing mathematical models to predict Cd levels in cultured oysters throughout the year.

The second general objective had the following specific sub-objectives: 1) investigating the effect of environmental variables and culture depth on growth and mortality of cultured oysters throughout the Strait of Georgia and 2) testing a new technique (depth manipulation) to obtain the maximum oyster yield by reducing exposure to harmful algae and high temperature that are most prominent close to the water surface.

The third general objective had the following specific sub-objectives: 1) using a new proteomic method to fingerprint, detect, and quantify two stress biomarkers (HSP 70 and 90) in oysters under field conditions, 2) determining the oyster stress levels present throughout the summer during their second year under culture conditions, and 3) studying the effects of environmental variables and phytoplankton on the levels of stress indicators and their relationship with oyster mortalities.

Overall, this PhD had the broad objective of providing management tools to oyster farmers to help optimize oyster growth and survival rates and reduce oyster Cd levels by helping them understand the complex relationship between their stock, the environment, and the algae, which constitute their most important food source. This information could serve as the basis for better husbandry practices and site selection, as well as for further research on the role of phytoplankton composition in oyster production.
Chapter 2 The role of phytoplankton in the modulation of dissolved and oyster cadmium concentrations in Deep Bay, British Columbia, Canada

2.1 Introduction

Cadmium (Cd) is a heavy metal that can produce deleterious effects on human health when ingested in high concentrations. The consumption of oysters with high Cd levels for extended periods of time can lead to increased Cd concentration in the blood (Copes et al., 2008). In recent years, Cd has been a concern of Pacific oyster (Crassostrea gigas) growers in British Columbia (BC), Canada due to the fact that shipments of local product have been above the 2 ppm wet weight (w.w.) limit established by some export markets (Kruzynski, 2004).

Cadmium has two main pathways of entry into oysters and other aquatic organisms (Ray, 1984; Roesijadi and Robinson, 1994) directly through the seawater that comes in contact with their soft tissues (Borchard, 1983; Dixon et al., 2006; Lekhi et al., 2008) and through food, mainly phytoplankton (Ettajani et al., 2001). Cadmium is rapidly accumulated from the dissolved form through exposed tissues such as gills (Roesijadi and Robinson, 1994; Ettajani et al., 2001). Phytoplankton can accumulate trace metals (including Cd) through absorption for use in various cellular functions (Lane et al., 2005; Dixon et al., 2006). The absorption of dissolved Cd (Cd\textsubscript{diss}) by phytoplankton in the photic layer leads to a nutrient-type depth profile of this metal in the ocean, where Cd\textsubscript{diss} is found at lower concentrations closer to the surface (Bruland et al., 1978; Abe, 2004). Microalgae can, in turn, be a potential source of Cd to shellfish.
The concentrations of metals in planktonic particulate matter and the particular microalgal species present have an important impact on the assimilation efficiency (AE) of metals by oysters (Ettajani et al., 2001). The AE of oysters feeding on particulate matter depends on the quality and digestibility of the particles, as oysters may reject detritus and inorganic particles in favor of more nutritious microalgae (Borchard, 1983; Ettajani et al., 2001; Ng et al., 2005). Although the Cd contained in phytoplankton is accessible to oysters during digestion (Amiard et al., 2007), it is mostly contained in tightly-bound depuration proteins such as phytochelatins and metallothioneins that are not easily assimilated (Lee and Morel, 1995; Payne and Price, 1999).

Deep Bay is part of Baynes Sound, one of the main areas used for the cultivation of Pacific oysters in BC. Several oyster growing companies are based on the west side of the bay, while a small fishing and recreational harbor operates on the more protected east part of the basin. Vancouver Island University has recently developed a shellfish research and education facility in Deep Bay, highlighting the need to establish baseline conditions prior to the further development of human activities in the area. Deep Bay also has importance from a conservation standpoint as it is regularly visited by migrating birds and is used by herring to spawn (Haegle, 1978). Lekhi et al. (2008) previously observed a strong seasonal variation in oyster (Cd_{oys}), dissolved (Cd_{diss}), and particulate (Cd_{part}) Cd concentrations in Deep Bay, with lower Cd_{oys} levels during summer and higher ones in winter. At their study site, Cd_{oys} was strongly positively correlated with Cd_{diss} but strongly negatively correlated with Cd_{part} (>20 µm), leading to the conclusion that the latter was not a significant source of Cd in the oysters at this site (Lekhi et al., 2008). The seasonality of Cd_{diss}, and thus that of Cd_{oys}, was hypothesized to be controlled by two
main factors, $Cd_{diss}$ drawdown by phytoplankton during the summer and $Cd_{diss}$ replenishment during the winter by oceanographic processes (Lekhi et al., 2008).

Particulate Cd ($>20 \mu m$), although cited by other authors as a possible source of Cd in oysters (Borchard, 1983; Hardy et al., 1984; Amiard et al., 2007), had a negative correlation with $Cd_{oys}$ concentrations in Deep Bay, indicating that phytoplankton were not a source of Cd in oysters at this site, but rather a sink. The high salinities and low temperatures observed in the winter and fall in the bay were correlated with high $Cd_{diss}$ concentrations and were indicative of increased entrainment, disruption of the thermocline, upwelling, and other oceanographic processes that bring water from below the pycnocline towards the surface. These processes replenish $Cd_{diss}$ and nutrients in surface waters (Lares and Orians, 1997; Lekhi et al., 2008). The correlations found by Lekhi et al. (2008) indicated that phytoplankton may play an important role in indirectly modulating $Cd_{oys}$ concentrations via uptake of $Cd_{diss}$. The role of different taxonomic and ecological components of the phytoplankton community in modulating Cd concentrations in oysters is, however, unknown.

Predicting $Cd_{oys}$ levels through the measurement of environmental variables can be challenging. Several authors have proposed mathematical models describing accumulation of Cd in various food chains based on laboratory and field observations (Hardy et al., 1984; Blackmore and Wang, 2004; Baines and Fisher, 2008). These models are complex and not easily applied by shellfish growers, mostly due to the use of variables that are difficult to measure routinely by farm staff. In order to prevent market rejection and loss of product, BC oyster growers need management tools to predict Cd concentration in their product before shellfish are harvested. The main objective of the
The present study was to complement our previous work (Lekhi et al., 2008) through further analysis of the role of various taxonomic groups within the phytoplankton community in the modulation of Cd concentrations in Deep Bay. A secondary objective was to examine the available chemical, physical, and biological environmental data to explore whether simple descriptive models could be developed that reliably explain the observed trends and variations in Cd<sub>oys</sub> concentration (using Deep Bay as a case study). The information on Cd<sub>oys</sub>, Cd<sub>diss</sub>, Cd<sub>part</sub>, oyster meat weight, temperature, and salinity used by Lekhi et al. (2008) were statistically analyzed in conjunction with unpublished detailed phytoplankton and dissolved nutrient data to examine more closely the role of various groups of phytoplankton in modulating Cd<sub>diss</sub> and Cd<sub>oys</sub> and to develop predictive models that could be used by farm staff.

2.2 Materials and methods

2.2.1 Site description

The experimental site (49°27′67″N, 124°43′45″W) was situated in Deep Bay, a protected meso-tidal estuary located on the south-western part of Baynes Sound on the east coast of Vancouver Island (Fig. 2.1). The maximum depth of this bay is 48 m, although the depth of the study site is closer to 25 m. This bay is exposed to estuarine conditions of varying temperature and salinity (7–19 °C and 23–29, respectively, Lekhi et al., 2008). The main freshwater input in this area is Cook Creek, which has a mean annual discharge of 1261 L s<sup>-1</sup> (Braybrook et al., 1995). The freshwater discharge from this source varies seasonally from 18 to 2270 L s<sup>-1</sup> (August and December, respectively). At this particular site, Pacific oysters are cultured at 2–5 m depth (within surface waters).
using suspended-tray culture methods. This culture depth range is common throughout BC, although it may vary slightly among sites and companies. The bay contains three oyster culture tenures that hold a total of approximately $3 \times 10^6$ oysters at any given moment throughout the year.

![Figure 2-1 Location of study site at Deep Bay, Vancouver Island, British Columbia, Canada.](image)

**Figure 2-1** Location of study site at Deep Bay, Vancouver Island, British Columbia, Canada.

2.2.2 Sampling and analyses

Filtered seawater, unfiltered seawater, particulate matter, oysters, Secchi depth,
temperature, and salinity were sampled/measured at 5 m depth (oysters at 2–5 m depth) biweekly during the spring and summer and monthly during the winter between August 2004 and July 2005. The filtered (0.4 µm) seawater samples, collected for Cd$_{\text{diss}}$ determination via a peristaltic pump, were acidified (pH 6), concentrated using Chelex 100 ion exchange resin, and then eluted with 2 N HNO$_3$ prior to analysis by graphite furnace atomic absorption spectrometry (GFAAS), as detailed in Lekhi et al. (2008). Part of this filtered seawater was used for determination of nitrate (nitrate + nitrite), phosphate, and silicic acid by means of an Autoanalyzer 3 continuous flow autoanalyzer (Bran + Luebbe, Norderstedt, Germany). The filters used to capture the size-fractionated (0.4, 3, and 20 µm pore size) suspended particulate matter were dissolved using NH$_4$OH and then digested in a 4:4:1 mixture of HNO$_3$, HCl, and HF. Particulate Cd (nM) was determined in the product of the digested filters using GFAAS (Lekhi et al., 2008). Cadmium concentrations (µg g$^{-1}$ w.w.) in five randomly-selected oysters at each sample time were determined in a private laboratory (Norwest Labs, Vancouver) following protocols set by the Canadian Food Inspection Agency (CFIA). The oysters were blotted and weighed to obtain meat wet weight data prior to the chemical analysis. The unfiltered seawater samples were obtained using a metal-free Niskin bottle. Subsamples of the unfiltered water were kept in 250-ml amber glass jars, preserved with Lugol's iodine solution, and stored in the dark until further analysis for microalgae. Aliquots (10–25 ml) of unfiltered seawater were placed in settling chambers and analyzed quantitatively for phytoplankton by the Utermöhl method, with modifications described in Hasle (1978), under a Zeiss Axiovert 10 inverted microscope (Carl Zeiss, Oberkochen, Germany). Carbon biomass per cell of the microalgae was calculated using estimates per species
produced by Haigh et al. (1992), which were based on equations from Strathmann (1967) and Montagnes and Franklin (2001). Secchi depth measurements were performed with a standard Secchi disk, while seawater temperature was measured using a ClineFinder® digital probe (Catalina Technologies, Tucson, Arizona). A manual refractometer (STX-3, VeeGee Scientific, Washington, USA) was used to measure salinity in the filtered seawater samples.

2.2.3 Statistical analysis

The data were tested for normality using the Kolmogorov–Smirnov test. The datasets that failed this test (i.e. harmful algae biomass and Cd\textsubscript{par}) were normalized with a natural-logarithm transformation before further statistical analysis. Homoscedacity of the datasets was assessed using Bartlett's test. The data were analyzed using Pearson product–moment correlations ($\alpha = 0.05$), correlating various chemical, physical, and biological variables with Cd\textsubscript{oys} and Cd\textsubscript{diss}. Several time lags were tested, but the best correlations were always obtained with co-occurring data. The main variables correlated with Cd\textsubscript{oys} were then further analyzed using multiple linear regressions, producing two descriptive models. The models were selected by step-wise regression to obtain the highest level of correlation with the original data and lowest mean square error. All the models constructed were then tested against random sub-groups of the data used. Sensitivity tests were run to determine the effect of the different variables on the models. The statistical analyses were performed with XLStat for Windows (Addinsoft SARL, Paris, France).
2.2.4 Mass-balance calculations

The area of Deep Bay was calculated from navigational charts using ImageJ image processing and analysis software (National Institutes of Health, Bethesda, Maryland, USA). Based on our average summer Secchi depth, the 1% sea-surface irradiance was calculated to be at a depth of 15 m. Thus, the volume of the photic zone for the whole bay was calculated assuming a 15-m deep photic layer. The total amount of Cd\textsubscript{diss} lost from the photic zone of Deep Bay during the summer was determined using the difference in the Cd\textsubscript{diss} concentrations between April 4, 2005 and July 17, 2005 and then multiplying this by the volume of the photic zone in Deep Bay. This was calculated assuming that the water in the photic zone was homogeneous throughout the bay and that salinity-driven stratification, common throughout the Strait of Georgia during the summer (Masson and Peña, 2009), limits the exchange of Cd\textsubscript{diss} and nutrients between the photic zone and deeper water. The uptake by phytoplankton was calculated using the reduction in nitrate observed between April 4, 2005 and July 17, 2005. The extended Redfield ratio (Ho \textit{et al}., 2003) was then used to calculate the Cd\textsubscript{diss} equivalent to the amount of nitrate lost from the photic zone.

The Cd burden per oyster (\(\mu g\) oyster\(^{-1}\)) was calculated by multiplying the Cd concentration in the oysters (\(\mu g\) g\(^{-1}\) w.w.) by the oyster meat wet weight (g). The Cd\textsubscript{diss} that oysters acquired during the summer was estimated by multiplying the change observed in the Cd burden of oysters (\(\mu g\) oyster\(^{-1}\)) between April 4, 2005 and July 17, 2005 by an estimate of the number of the oysters being grown in the bay. This was based on the assumption that \(3 \times 10^6\) oysters were being grown in the bay at any given time and that Cd\textsubscript{diss} is the main source of Cd in the oysters (Lekhi \textit{et al}., 2008).
2.3 Results

2.3.1 Dissolved nutrients

2.3.1.1 Annual cycle

The nutrients at 5-m depth showed a typical annual trend for temperate estuarine surface waters, being depleted during the spring and summer and replenished during winter (Harrison and Yin, 1998) (Fig. 2.2). This trend was interrupted by several spikes in the nutrients' surface concentrations during spring and summer. All the nutrients reached maximum concentrations during winter; silicic acid at 52.75 µM, nitrate at 27.31 µM, and phosphate at 2.50 µM. The lowest concentrations of silicic acid, nitrate, and phosphate were detected during August 2004 (0.54, 0.42, and 4.48 µM, respectively). The salinity and Cd\textsubscript{diss} attained their highest values during the winter and early spring (29 and 0.81 nM, respectively) (Fig. 2.3B), while the Cd\textsubscript{part} was higher during the fall of 2004, the early spring of 2005, and late summer of 2005, reaching peaks of 0.044, 0.035, and 0.050 µM, respectively (Fig. 2.3C).

2.3.1.2 Summer drawdown

The largest reduction in dissolved nutrients was observed between April 4, 2005 and July 17, 2005 (Fig. 2.2). During this period, nitrate was reduced by more than 97%, with phosphate and silicic acid at 64 and 50%, respectively (Table 2.1). As well, the Cd\textsubscript{diss} concentration was reduced by 37% over the same period.
Figure 2-2. Concentrations of phosphate (µM), nitrate (µM), and silicic acid (µM) at 5-m depth in Deep Bay from August 2004 to July 2005 (n=1).

2.3.2 Phytoplankton

2.3.2.1 General description and annual cycle

A typical annual cycle of phytoplankton production in cold estuarine waters was observed at this site. Diatoms normally dominated the phytoplankton carbon-based biomass, while dinoflagellates and other species constituted typically less than 10% of the overall biomass (Fig. 2.4A). Late summer and fall of 2004 were dominated by a bloom of *Rhizosolenia setigera*, a diatom, which persisted until November when it mixed with other fall-bloom diatom species. Low abundances of small diatoms, flagellates, and ciliates were noted during the winter months. The spring bloom of 2005 was characterized by various diatom species and was followed by a bloom of *R. setigera*.
during July and August. A bloom of the diatom *Chaetoceros socialis* was observed at the end of July, 2005. Due to the small size of the cells, however, this bloom did not produce a proportional peak in biomass. The phytoplankton biomass averaged $190.5 \pm 49.9 \, \mu g C L^{-1}$ ($n = 22$) throughout the year of sampling with the highest value ($1003.7 \, \mu g C L^{-1}$) being measured during the bloom of *R. setigera* in August 2004 (Fig. 2.4A).

### Table 2-1

Concentrations on April 4 and July 17, 2005 and summer drawdown of dissolved cadmium (from Lekhi *et al.*, 2008), phosphate, silicic acid, and nitrate in Deep Bay. Drawdown of dissolved phosphate, silicic acid, and nitrate relative to April 4, 2005 and normalized to cadmium. Cadmium-normalized extended Redfield ratio (Ho *et al.*, 2003) is shown for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Cadmium (nM)</th>
<th>Phosphate (µM)</th>
<th>Silicic acid (µM)</th>
<th>Nitrate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apr-05</td>
<td>0.81</td>
<td>2.48</td>
<td>40.13</td>
<td>21.11</td>
</tr>
<tr>
<td>Jul-05</td>
<td>0.51</td>
<td>0.90</td>
<td>20.01</td>
<td>0.55</td>
</tr>
<tr>
<td>Summer drawdown</td>
<td>0.30</td>
<td>1.58</td>
<td>20.12</td>
<td>20.56</td>
</tr>
<tr>
<td>% Drawdown relative to April</td>
<td>37.04</td>
<td>63.71</td>
<td>50.14</td>
<td>97.39</td>
</tr>
<tr>
<td>C\text{d}-normalized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep Bay</td>
<td>1</td>
<td>5.27</td>
<td>67.07</td>
<td>68.53</td>
</tr>
<tr>
<td>Ho <em>et al.</em> (2003)</td>
<td>1</td>
<td>4.76</td>
<td>71.43</td>
<td>76.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Cadmium (nM)</th>
<th>Nitrate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apr-05</td>
<td>0.33</td>
<td>8.51</td>
</tr>
<tr>
<td>Jul-05</td>
<td>0.57</td>
<td>0.61</td>
</tr>
<tr>
<td>Summer drawdown</td>
<td>0.19</td>
<td>13.01</td>
</tr>
<tr>
<td>Ho <em>et al.</em> (2003)</td>
<td>0.21</td>
<td>16.01</td>
</tr>
</tbody>
</table>
Figure 2-3 (A) Cadmium content (µg Cd oyster$^{-1}$) of oysters in Deep Bay from August 2004 to July 2005. Error bars represent standard deviations and n=5. (B) Salinity (n=1) and dissolved Cd (nM) (n=2). (C) Particulate Cd per size fraction and total particulates (µM) (n=1) (re-drawn from Lekhi et al., 2008).
Figure 2-4 (A) Biomass (µg C L⁻¹) of diatoms, dinoflagellates, and other phytoplankton groups in Deep Bay from August 2004 to July 2005. (B) Percentage of total algal biomass (%) of toxic and potentially-harmful algae in Deep Bay from August 2004 to July 2005.

2.3.2.2 Harmful algae

Several species of harmful algae were noted during the sampling period (Fig. 2.4B). These included several toxic species such as the domoic acid-producing diatoms
*Pseudo-nitzschia* spp., (causative agent of amnesiac shellfish poison) and the dinoflagellate *Alexandrium catenella* (responsible for paralytic shellfish poison) (Taylor and Harrison, 2002). Toxic species were present in relatively low numbers and constituted a maximum of 10% of the total biomass when present. Other algal species were identified as potentially-harmful (but not toxic) to cultured marine organisms (*e.g.* *R. setigera, Chaetoceros convolutus, Heterosigma akashiwo, Dictyocha speculum*). The abundance of these potentially-harmful phytoplankton species was low during the winter and spring (0–7% of total biomass) and higher from July to November (up to 96% of total biomass) (Fig. 2.4B). The most abundant and prevalent potentially-harmful species was *R. setigera*, which may prevent the oysters from feeding properly due to its sharp spines and large size (Cassis and Taylor, 2006). Quick and repeated clapping of the valves was observed in the oysters whenever this species was present in high densities.

### 2.3.3 Summer mass balance

#### 2.3.3.1 Oyster cadmium burden

Oyster Cd burden (µg Cd oyster⁻¹) increased throughout the study period (Fig. 2.3A). The average burden at the start of the sampling period was 25.7 ± 1.7 µg oyster⁻¹ (n = 5), reaching 65.9 ± 3.1 µg oyster⁻¹ (n = 5) by the end of the study. The average Cd burden between April 4, 2005 and July 17, 2005 increased from 61.9 ± 3.9 to 63.1 ± 2.1 µg oyster⁻¹, an average increase of 1.2 ± 0.6 µg oyster⁻¹ (n = 5). Cd burden was only correlated with total phytoplankton biomass (Table 2.2).
Table 2-2 Pearson correlations between dissolved nutrients (µM) and biomass of various phytoplankton components (µg C L\(^{-1}\)) and cadmium concentration in oysters [µg g\(^{-1}\) wet weight (w.w.)], cadmium burden (µg Cd oyster\(^{-1}\)), dissolved cadmium (nM), and particulate cadmium (nM). Potentially-harmful algae include the species *Rhizosolenia setigera*, *Chaetoceros convolutus*, *Heterosigma akashiwo*, and *Dictyocha speculum*. P-values are given in parentheses. Values in **bold** are significant at P<0.05. Oyster, dissolved, and particulate Cd information obtained from Lekhi *et al.*, 2008.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Oyster Cd (µg g(^{-1}) w.w.)</th>
<th>Cd burden (µg Cd oyster(^{-1}))</th>
<th>Dissolved Cd (nM)</th>
<th>Particulate Cd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4–3.0</td>
</tr>
<tr>
<td>Phosphate (µM)</td>
<td>0.764</td>
<td>0.337</td>
<td>0.672</td>
<td>-0.300</td>
</tr>
<tr>
<td></td>
<td>(-0.0001)</td>
<td>(0.146)</td>
<td>(0.001)</td>
<td>(0.199)</td>
</tr>
<tr>
<td>Silicic acid (µM)</td>
<td>0.565</td>
<td>0.097</td>
<td>0.564</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>(0.010)</td>
<td>(0.683)</td>
<td>(0.010)</td>
<td>(0.012)</td>
</tr>
<tr>
<td>Nitrate (µM)</td>
<td>0.006</td>
<td>0.079</td>
<td>0.530</td>
<td>-0.264</td>
</tr>
<tr>
<td></td>
<td>(0.005)</td>
<td>(0.741)</td>
<td>(0.016)</td>
<td>(0.261)</td>
</tr>
<tr>
<td>Phytoplankton biomass (µg C L(^{-1}))</td>
<td>-0.466</td>
<td>-0.491</td>
<td>-0.183</td>
<td>-0.048</td>
</tr>
<tr>
<td></td>
<td>(0.039)</td>
<td>(0.028)</td>
<td>(0.440)</td>
<td>(0.841)</td>
</tr>
<tr>
<td>Potentially-harmful algae biomass (ln µg C L(^{-1}))</td>
<td>-0.620</td>
<td>-0.370</td>
<td>-0.183</td>
<td>-0.132</td>
</tr>
<tr>
<td></td>
<td>(0.004)</td>
<td>(0.108)</td>
<td>(0.033)</td>
<td>(0.580)</td>
</tr>
<tr>
<td>Toxic algae biomass (ln µg C L(^{-1}))</td>
<td>-0.478</td>
<td>-0.006</td>
<td>-0.442</td>
<td>0.323</td>
</tr>
<tr>
<td></td>
<td>(0.033)</td>
<td>(0.982)</td>
<td>(0.051)</td>
<td>(0.164)</td>
</tr>
<tr>
<td>Diatom biomass (µg C L(^{-1}))</td>
<td>-0.452</td>
<td>-0.287</td>
<td>-0.184</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>(0.045)</td>
<td>(0.220)</td>
<td>(0.436)</td>
<td>(0.686)</td>
</tr>
<tr>
<td>Dinoflagellate biomass (µg C L(^{-1}))</td>
<td>-0.106</td>
<td>0.247</td>
<td>-0.117</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>(0.657)</td>
<td>(0.293)</td>
<td>(0.623)</td>
<td>(0.820)</td>
</tr>
<tr>
<td>Others biomass (µg C L(^{-1}))</td>
<td>-0.414</td>
<td>-0.600</td>
<td>-0.237</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>(0.069)</td>
<td>(0.800)</td>
<td>(0.314)</td>
<td>(0.710)</td>
</tr>
</tbody>
</table>
Table 2-3 Calculated dissolved cadmium removal by phytoplankton, cultured oysters, and other processes from the photic zone in Deep Bay between April 4 and July 17, 2005 based on an approximate volume of the photic zone (L), approximate number of cultivated oysters in the bay, the observed and estimated dissolved Cd drawdown (nM), extended Redfield ratio (Ho et al., 2003), and the variation in the Cd burden of oysters.

<table>
<thead>
<tr>
<th></th>
<th>Difference between April and July</th>
<th>Volume of photic layer (L) or number of oysters grown in Deep Bay (inds)</th>
<th>Calculated drawdown of Cd (moles)</th>
<th>% of dissolved Cd drawdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolved Cd Phytolankton</td>
<td>$0.3 \pm 0.009$ (nM)</td>
<td>$1.89 \times 10^{10}$</td>
<td>$5.67 \pm 0.017$</td>
<td>100 $\pm$ 0.3</td>
</tr>
<tr>
<td></td>
<td>$0.269$ (nM Cd equivalent from nitrate uptake)</td>
<td>$1.89 \times 10^{10}$</td>
<td>5.08</td>
<td>89.67</td>
</tr>
<tr>
<td>Oysters Other processes</td>
<td>$0.011 \pm 5.5 \times 10^{-4}$ (µmoles oyster$^{-1}$)</td>
<td>$3 \times 10^8$</td>
<td>$3.3 \times 10^{-5} \pm 1.65 \times 10^{-6}$</td>
<td>$5.8 \times 10^{-4} \pm 2.9 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10.33$</td>
<td></td>
</tr>
</tbody>
</table>
2.3.3.2 Mass balance of cadmium in Deep Bay

The calculations indicated that approximately 90% of the \( \text{Cd}_{\text{diss}} \) drawdown observed during the summer was due to uptake by phytoplankton, while the oysters cultured in the bay accounted for less than 0.001% (Table 2.3). The remaining 10% of the \( \text{Cd}_{\text{diss}} \) drawdown could be lost due to uptake by other organisms (e.g. mesozooplankton) and other forms of sedimentation (e.g. fecal pellets and biogeochemical processes).

2.3.4 Correlations between nutrients/phytoplankton and oyster, dissolved, and particulate cadmium

The Pearson correlation analyses indicated that dissolved phosphate, silicic acid, and nitrate all had strong correlations with all forms of Cd measured, being positively correlated with \( \text{Cd}_{\text{oys}} \) and \( \text{Cd}_{\text{diss}} \) and negatively correlated with \( \text{Cd}_{\text{part}} \) (>20 µm). Among all the nutrients measured, phosphate had the highest correlations with the different forms of Cd, while nitrate and silicic acid shared similar levels of correlation with \( \text{Cd}_{\text{oys}} \) and \( \text{Cd}_{\text{diss}} \) (Table 2.2). The various components of the phytoplankton had negative correlations with \( \text{Cd}_{\text{oys}} \) and positive ones with \( \text{Cd}_{\text{part}} \), but these correlations only reached significant levels for total phytoplankton biomass, potentially-harmful algae biomass, and diatom biomass (Table 2.2). Potentially-harmful algae biomass had the most significant correlation with \( \text{Cd}_{\text{oys}} \) and \( \text{Cd}_{\text{part}} \) and was the only phytoplankton group to reach a significant correlation with \( \text{Cd}_{\text{diss}} \).
2.3.5 Models

Two linear descriptive models for Cd\textsubscript{oys} concentration (µg g\textsuperscript{-1} w.w.) were created using multiple linear regressions with variables identified during the Pearson correlation analyses (Fig. 2.5). It should be noted that these models were produced for the specific environmental conditions at 5-m depth present at Deep Bay during two years only (2004 and 2005) and that there is a large spatial variability in Cd\textsubscript{oys} and the environmental variables that affect them (Bendell and Feng, 2009; Ng \textit{et al.}, 2010).

\textbf{Figure 2-5} Observed and predicted cadmium concentrations [µg g\textsuperscript{-1} wet weight (w.w.)] in cultured oysters in Deep Bay from August 2004 to July 2005. Error bars represent standard deviations and n=5.
Table 2-4 Number of observations, degrees of freedom (df), coefficient of determination ($R^2$), adjusted coefficient of determination (Adjusted $R^2$), mean square error (MSE), root mean square of the error (RMSE), and mean absolute percentage error (MAPE) as goodness of fit measures for two models describing Cd levels in oysters in Deep Bay from August 2004 to July 2005.

<table>
<thead>
<tr>
<th>Model</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>df</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.870</td>
<td>0.806</td>
</tr>
<tr>
<td>Adjusted $R^2$</td>
<td>0.794</td>
<td>0.761</td>
</tr>
<tr>
<td>MSE</td>
<td>0.056</td>
<td>0.080</td>
</tr>
<tr>
<td>RMSE</td>
<td>0.237</td>
<td>0.283</td>
</tr>
<tr>
<td>MAPE</td>
<td>6.530</td>
<td>8.394</td>
</tr>
</tbody>
</table>

The first model ($R^2 = 0.870$) was developed to explain as much of the variability in the $Cd_{oys}$ ($\mu g \cdot g^{-1} \cdot w.w.$) as possible using any measured variables from the study, including those that might not be so easily measured by farm personnel (e.g. phytoplankton biomass).

$$Cd_{oys} (\mu g \cdot g^{-1} \cdot w.w.) = 0.92 + 0.00378 \text{ oyster age (days)} - 0.074 \text{ temperature (°C)} + 0.973 \text{Cd}_{diss} (nM) - 0.0569 \text{ total phytoplankton biomass (µg C L}^{-1}) + 0.0866 \ln \text{ biomass of toxic algae (µgC L}^{-1}) - 0.118 \ln \text{ Cd}_{part} (nM \text{ in particles >20 µm}) - 0.0368 \text{ oyster meat wet weight (g)}.$$  

The second model ($R^2 = 0.806$) was built with environmental variables that could be easily measured in the field by farm personnel.

$$Cd_{oys} (\mu g \cdot g^{-1} \cdot w.w.) = 0.362 + 0.003836 \text{ oyster age (days)} - 6.942 \text{ temperature (°C)} + 0.908 \text{Cd}_{diss} (nM) - 0.0518 \text{ total phytoplankton biomass (µg C L}^{-1}) + 0.0646 \ln \text{ biomass of toxic algae (µgC L}^{-1}) - 0.0968 \ln \text{ Cd}_{part} (nM \text{ in particles >20 µm}) - 0.0228 \text{ oyster meat wet weight (g)}.$$
\[ (°C) + 0.052 \text{ salinity} - 0.03597 \text{ oyster meat wet weight (g)}. \]

The measurements of the goodness of fit for each model are listed in Table 2.4. The sensitivity analysis indicated that both models were heavily affected by the age of the oysters. The first model was secondarily affected by temperature, while the second model was more sensitive to changes in salinity (data not shown).

2.4 Discussion

2.4.1 Dissolved nutrients and phytoplankton

The environment in Deep Bay is typical of semi-enclosed, temperate estuarine areas in the Strait of Georgia with seasonal stratification and a diatom-dominated phytoplankton community (Haigh et al., 1992; Masson and Peña, 2009). The spring bloom in Deep Bay was fuelled by high concentrations of dissolved nutrients brought up to the photic zone during late fall/winter as a result of vertical mixing. These nutrients were gradually depleted over the summer months (as in Margalef, 1978; Harrison and Yin, 1998). Occasionally, in the summer, the dissolved nutrients in the surface waters were partially replenished by wind events or coastal upwelling (Richie and Pawlowicz, 2006; Lekhi et al., 2008). The waters below the pycnocline normally present high and relatively stable concentrations of dissolved nutrients (Masson and Peña, 2009) and \( \text{Cd}_{\text{diss}} \) (Abe, 2004).

The relatively strong positive correlations between the three nutrients and \( \text{Cd}_{\text{diss}} \) suggest that the same sources and sinks affect their concentrations in the photic zone. As discussed by Lekhi et al. (2008) the main source of \( \text{Cd}_{\text{diss}} \) in the photic zone in Deep Bay is sub- surface water that originates in the north Pacific Ocean, which is naturally high in
this metal compared to other areas (Bruland et al., 1978; Paulson et al., 1991). This sub-surface water, enriched in Cd\textsubscript{diss}, is characterized by high salinity and low temperature, being carried towards the surface by strong vertical mixing and upwelling during the winter. Another possible source of Cd\textsubscript{diss} could be a combination of terrestrial and anthropogenic sources via freshwater inputs to the Strait of Georgia. For example, the influx of freshwater has been described as a possible contributor to the variation of nutrient and Cd\textsubscript{diss} concentrations in the Gironde Estuary in France (Dabrin et al., 2009). This potential source probably has a very limited contribution at this particular site, however, as the Cd\textsubscript{diss} levels were associated with high salinity (Lekhi et al., 2008). Although the mouth of the local creek is located close to the oyster rafts, its effect on Cd\textsubscript{diss} was probably negligible. This stream has its maximum flow during winter (Braybrook et al., 1995) when the salinity at the site was at its highest. Further evidence for a lack of effect from local freshwater sources was observed between the 6\textsuperscript{th} and 17\textsuperscript{th} of July, 2005 when the salinity changed from 27 to 25. This change was associated with a large reduction in nitrate (4.66 to 0.55 µM), phosphate (1.43 to 0.90 µM), and Cd\textsubscript{diss} (0.53 to 0.51 nM). The plankton community also experienced a large change between these two dates, as a bloom of the weakly silicified diatom Chaetoceros socialis was observed on April 17\textsuperscript{th}. This points towards a stronger biological role in the modulation of the dissolved elements during the summer.

Both Cd\textsubscript{diss} and dissolved macronutrient concentrations in the photic zone were reduced by phytoplankton uptake during the summer (Table 2.1; Lekhi et al., 2008). The drawdown of Cd\textsubscript{diss} and dissolved phosphate during the summer (0.30 nM, 1.58 µM, respectively) was close to the ideal Cd:P Redfield ratio (0.19 and 0.21 mmol mol\textsuperscript{-1}, for
indicating that phytoplankton was the main sink of Cd$_{\text{diss}}$ in the bay. During the winter months, the water column is well mixed and the light-limited phytoplankton are scarce, thus macronutrient and Cd$_{\text{diss}}$ levels remained high throughout this period. Phosphate was better correlated with Cd$_{\text{diss}}$ than any of the other measured nutrients. This may be due to the fact that phosphate is used by all phytoplankton groups, whereas silicic acid is mainly used by diatoms and nitrogen can exist in seawater in several bioavailable forms (nitrate, nitrite, and ammonium) (Harrison and Yin, 1998).

2.4.2 Modulation of dissolved and particulate cadmium by phytoplankton

The fast removal of Cd$_{\text{diss}}$ in Deep Bay during the summer, which closely matches the extended Redfield ratio proposed by Ho et al. (2003), suggests that the reduction of Cd$_{\text{diss}}$ in the photic zone was largely mediated by phytoplankton. According to our calculations, phytoplankton were the largest sink for Cd$_{\text{diss}}$, accounting for ~ 90% of the summer Cd$_{\text{diss}}$ drawdown in the top 15 m of the water column (37% of the winter concentrations). Thus, phytoplankton Cd uptake significantly reduced the concentration of Cd$_{\text{diss}}$ available to the cultured oysters (which are normally grown in the top 8 m of the water column) during these warmer months. This hypothesis is supported by the high phosphate and low titanium content of the suspended particles in Deep Bay which suggests that most suspended particles in the bay were organically derived (Lekhi et al., 2008). Thus, it is feasible to compare the measured Cd:P ratios of the suspended particles to the calculated Cd:P ratios from the summer drawdowns of dissolved Cd and phosphate. Indeed, the average Cd:P ratios in the suspended particulate matter (>20µm) in Deep Bay
during the summer varied between 0.12 and 0.14 mmol mol$^{-1}$, as indicated by Lekhi et al. (2008), while the summer drawdown of dissolved Cd and P was similar at 0.19 mmol mol$^{-1}$. Furthermore, these Cd:P ratios were close to the extended Redfield ratio (0.21 mmol mol$^{-1}$; Ho et al., 2003), suggesting that the drawdown of Cd$_{diss}$ was determined by phytoplankton growth, especially the potentially-harmful diatoms. Other organisms may also participate in the reduction of Cd$_{diss}$ and reduction of the phytoplankton standing stock, such as mesozooplankton, which can show high assimilation efficiencies for trace metals (Twining and Fisher, 2004).

2.4.3 Effect of harmful algae blooms

Potentially-harmful algae [belonging to the Bacillariophyceae (e.g. *Rhizosolenia setigera* and *Chaetoceros convolutus*), the Raphidophyceae (*Heterosigma akashiwo*), and the Dictyochophyceae (*Dictyocha speculum*)], dominated the phytoplankton community in Deep Bay throughout the summer and was the only phytoplankton group significantly negatively correlated to Cd$_{diss}$. This group also exhibited the best correlation with Cd$_{part}$ of all the different phytoplankton groups analyzed, further supporting the role of potentially-harmful algae in mediating the transfer of Cd$_{diss}$ to Cd$_{part}$.

The Pearson correlation coefficient is better suited for the comparison of processes that co-vary with time (i.e. those that have an immediate and proportional response), rather than for the analysis of processes that have a cumulative effect across time. Because of this, the slow removal of Cd$_{diss}$ by phytoplankton did not produce a significant correlation between Cd$_{diss}$ and total phytoplankton biomass nor diatom biomass (Table 2.2, Fig. 2.6A). In contrast, the relatively fast removal of Cd$_{diss}$ by short-
lived, potentially-harmful algal blooms produced a significant correlation (Table 2.2, Fig. 2.6B). A similar effect of harmful algae on $\text{Cd}_{\text{diss}}$ concentration was observed by Garcia-Hernandez et al. (2005).

### 2.4.4 Modulation of oyster cadmium concentrations

As indicated by Lekhi et al. (2008), the annual cycle of $\text{Cd}_{\text{oys}}$ in Deep Bay was highly dependent on $\text{Cd}_{\text{diss}}$. A link between the replenishment of $\text{Cd}_{\text{diss}}$ by upwelling waters and higher Cd concentrations in shellfish has been previously observed in nearby waters (Lares and Orians, 1997; Apeti et al., 2009). Ample evidence indicates a fast acquisition and retention of Cd from the dissolved phase by oysters (Kerfoot and Jacobs, 1976; Borchard, 1983; Ettajani et al., 2001). Once the Cd is in the oyster's tissues, some of it stays in solution in the cytosol (10–20%), forming part of the easily removable fraction that has a relatively short biological half-life (2–10 days) (Boisson et al., 2003). This fraction is probably the most affected by short-term variations in $\text{Cd}_{\text{diss}}$. Most of the Cd absorbed by the oyster, however, is stored in more resilient forms such as metallothioneins and other detoxification organic ligands, which have a half-life of upwards of 200 days (Boisson et al., 2003). These resilient forms of Cd storage are probably affected more by the diluting effect of rapid growth periods, experienced by the oysters when faced with high abundance of nutritive particles, than by fluctuations in $\text{Cd}_{\text{diss}}$.

The fast uptake of $\text{Cd}_{\text{diss}}$ by phytoplankton during the summer effectively transfers the oyster-accessible Cd into/onto particles, which are then eaten by various filter-feeders (including oysters) or sink to the bottom of the bay. We believe that fast sedimentation to
the benthos of a large proportion of the summer phytoplankton—as previously shown in other fjords (up to 50% of the diatom biomass; Skjoldal and Wassmann, 1986; Sancetta, 1989)—may reduce the availability of Cd\textsubscript{part} to the oysters. This may prevent the oysters from acquiring much of the Cd in phytoplankton and other particulate matter. This flux of phytoplankton and other particles from the surface waters to the seafloor can be accelerated further by the production of fecal and pseudo-fecal pellets by grazers or transport by vertically migrating zooplankton (Thompson et al., 2008). The sediment samples analyzed by Lekhi et al. (2008) from Deep Bay had a Cd:P ratio of 0.32 (mmol mol\textsuperscript{-1}), being highly enriched with organic matter in comparison to crustal-derived sediments which have a Cd:P ratio of about 0.14 (mmol mol\textsuperscript{-1}). This indicates that the flux of organic material from the photic zone is strong and rapid in Deep Bay. Diagenesis probably only had a limited effect on the biological particles at this site (Lekhi et al., 2008), as cells with intact chloroplasts have been observed in sediment traps under similar conditions (Sancetta, 1989). In addition, potentially-harmful algal blooms, such as those of the large and spiny Rhizosolenia setigera, reduce the overall feeding capacity of oysters (Cognie et al., 2003; Cassis and Taylor, 2006) and increase the sedimentation of particles out of the photic zone (Kremling et al., 1978; Garcia-Hernandez et al., 2005).
Figure 2-6 Main correlations (Pearson correlation coefficient and P values) between the different components of the system including dissolved Cd concentration (nM), oyster Cd concentration (µg g⁻¹ wet weight), phytoplankton biomass (µg C L⁻¹), harmful algal biomass (µg C L⁻¹), and oyster age (days) during (A) normal phytoplankton conditions and (B) harmful-algal bloom conditions. Statistically significant values (i.e. P<0.05) are in bold. Correlations for dissolved, oyster, and particulate Cd were obtained from Lekhi et al. (2008).
The increased phytoplankton biomass during the summer is accompanied by larger abundances of zooplankton, which can also act as a sink for trace metals (Twining and Fisher, 2004). All of these processes may act to reduce Cd\textsubscript{diss} available to the oysters, increase the flux of Cd-containing organic matter from the photic zone, and decrease the absorption of Cd from the ingested particles. This scenario, where periods of high phytoplankton biomass result in low Cd\textsubscript{diss} and low Cd\textsubscript{oys}, contrasts with periods of extremely low phytoplankton biomass (as seen under winter conditions) which result in higher Cd\textsubscript{oys} as the elevated Cd\textsubscript{diss} concentrations remain unchecked by the phytoplankton.

In addition, oysters may be only partially assimilating the phytoplankton Cd in the gut and/or eliminating it with the feces (Galtsoff, 1964; Boisson \textit{et al.}, 2003). Indeed, the retention of trace metals from food has been determined to be relatively low, although highly variable (9–25\% according to Ettajani \textit{et al.}, 2001). In contrast with our findings, Christie and Bendell (2009) suggested that Cd\textsubscript{part} is the main source of Cd for Pacific oysters in BC. In their study, they examined Cd levels in oyster gut tissues versus non-gut tissues. They also applied stable isotope analyses to determine the origin of particulate matter ingested by the oysters. They found that 40\% of the variation in non-gut tissue Cd could be explained by gut tissue Cd and concluded that Cd\textsubscript{part} must be the main source of Cd\textsubscript{oys}. Furthermore, these authors used the isotopic signature of N and C (not Cd) in gut tissues of oysters as these stable isotopes can be used to distinguish between terrigenous and oceanic signatures. The general pattern they observed between ocean-exposed and inlet-grown oysters was as expected, as the isotopic signature of the predominant local food would dominate that of the gut. This is not direct evidence, however, of Cd\textsubscript{part} being
the main source of Cd\textsubscript{oys}. Our data suggest that phytoplankton may be a source for Cd\textsubscript{oys}, but the general effect of high abundances of phytoplankton lowering Cd\textsubscript{diss} masks this uptake. If phytoplankton, or any component of the suspended particulate matter, were an important source of Cd to oysters, higher Cd\textsubscript{oys} concentrations would be observed during spring and summer, rather than during fall and winter. Ultimately, however, both Cd\textsubscript{part} and Cd\textsubscript{diss} may play a role in Cd\textsubscript{oys} and the relative importance of the two sources will be dependent on a variety of biotic and abiotic factors under field settings. Recent efforts to quantify the importance of each pathway under laboratory conditions, using multiple stable Cd isotopes, have indicated that the main input was from Cd\textsubscript{diss} (Strady \textit{et al.}, 2011). The dissolved metal was shown to be rapidly taken up through the gills and other exposed tissues, while the uptake of Cd\textsubscript{part} was slower, being detectable only after two weeks of exposure and with a trophic efficiency of only 1\% (Strady \textit{et al.}, 2011). Once the Cd was absorbed through either pathway it was then distributed throughout the oyster's organs and tissues (Strady \textit{et al.}, 2011).

Other factors may also influence Cd levels in the oysters. A strong reduction in Cd content was observed at the end of April, 2005. This reduction was probably caused by a large reduction in the oysters' meat weight (from 22.7 to 15.7 g) due to farming techniques, such as tumbling and selection by size, which impacted their Cd content but not the Cd\textsubscript{oys} concentrations.

2.4.5 Models

The models accurately described the major factors affecting Cd concentration in oysters of Deep Bay. The first model explained 87\% of the Cd\textsubscript{oys} variability with a
combination of environmental factors, phytoplankton groups, and oyster variables. The environmental factors included were $\text{Cd}_{\text{diss}}$ as the main source of variability and temperature as a seasonality indicator. Phytoplankton was represented, as a negative term, by the total biomass and the $\text{Cd}_{\text{part}} > 20 \mu\text{m}$ (which was highly correlated to diatom biomass). In contrast, toxic algae, most of which were dinoflagellates, were included as a positive term in the model. The oyster variables involved in this model, age and meat weight, show how growth can have seemingly contrary effects on $\text{Cd}_{\text{sys}}$, depending on the time scale used. The appearance of age in the model indicated that accrual of Cd in oysters over long periods of time was important, as described by Rasmussen et al. (2007). The appearance of meat weight in the model, however, showed that fast changes in the meat weight during brief growth periods may serve to temporarily dilute the Cd already accumulated, thus reducing the overall Cd concentrations (Lekhi et al., 2008).

The second model, which explained 81% of the variability, was designed to be useful to oyster growers in Deep Bay as a way to obtain a quick estimate of the Cd concentration in their product, based on variables that are easy to measure under farm conditions. The environmental variables included in this model (temperature and salinity) can act as seasonality indicators and as proxies for $\text{Cd}_{\text{diss}}$, as they had strong correlations with this form of Cd. Low values of temperature and high values of salinity are normally indicative of upwelling, which brings high $\text{Cd}_{\text{diss}}$ waters to the surface (Lares and Orians, 1997), while low salinity and high temperatures could indicate a higher influx of fresh water. The Cd content of the freshwater influx in Deep Bay was not measured, although it seems to be lower than that of the more saline subsurface waters, as high salinity was strongly correlated to $\text{Cd}_{\text{diss}}$ (Lekhi et al., 2008). The oyster’s age and meat weight
represent the constant accrual of Cd over time (age) as well as the short bursts of growth which temporarily dilute the Cd already accumulated (meat weight). The oyster's meat weight might also be used as a partial proxy for phytoplankton biomass within this model, as growth bursts were only observed when abundant non-harmful phytoplankton were present in the water column. This model also was useful to illustrate the key parameters that the growers should monitor and use in their management to obtain the lowest Cd concentrations possible. This could be done by reducing the age of the oysters (i.e. selling smaller oysters for specialty Cd-sensitive markets) or by avoiding harvesting in times when high salinity and low temperatures are common (i.e. winter). The models presented are applicable only to the Deep Bay area as the relative concentrations of Cd$_{\text{diss}}$ and Cd$_{\text{part}}$ (and their importance to Cd accumulation in oysters), vary immensely among different regions of the northeast Pacific Ocean (Bendell and Feng, 2009; Ng $et$ $al.$, 2010).

2.5 Conclusions

The results of this study and Lekhi $et$ $al.$ (2008) point towards a modulation of Cd$_{\text{diss}}$, and ultimately of Cd$_{\text{oys}}$ concentrations, by phytoplankton and various oceanographic processes. Phytoplankton uptake accounted for most of the Cd$_{\text{diss}}$ loss during the summer, while upwelling, increased entrainment, and water mixing replenished it to the surface waters during the fall and winter. Although phytoplankton are believed to be a significant source of Cd in shellfish in some areas, such was not the case in Deep Bay. Accumulation of Cd$_{\text{diss}}$ by phytoplankton reduced the availability to oysters. Sedimentation of up to half of the phytoplankton biomass out of the photic zone
(see Sancetta, 1989) may create a sink of Cd, effectively taking it out of the reach of cultured oysters in the upper water column. The reduction of $C_{diss}$ by phytoplankton can be a lengthy process as 37% of its winter concentration was lost during the course of three and a half months of the spring and summer. Alternatively, it can be a relatively fast process; such was the case with algal blooms that created short-lived, but strong, pulses of $C_{diss}$ reduction and burial (as well as limiting the acquisition of $C_{part}$ by oysters).

Two descriptive models, developed from the environmental and oyster data, explained 81–87% of the variability observed in $C_{oys}$ throughout the year. One of these models was specifically designed for the oyster growers of Deep Bay, being based on simple oyster and environmental variables such as oyster age, oyster meat weight, seawater temperature, and salinity.
Chapter 3 Effects of the environment and culture depth on growth and mortality in juvenile Pacific oysters in the Strait of Georgia, British Columbia

3.1 Introduction

Globally, the Pacific oyster *Crassostrea gigas* Thunberg is the most common bivalve in aquaculture, owing to its handling ease, fast growth, euryhaline/eurythermal tolerance, and the variety of culture techniques available (FAO, 2010). In British Columbia (BC), Canada, the Pacific oyster is the largest cultured crop in terms of tonnage, the main areas of production being in the Strait of Georgia (Fig. 3.1) in Baynes Sound and Okeover Inlet. Nursery systems are often used for both wild-collected and hatchery-produced seed before grow-out. The oysters are then grown using a variety of methods, but generally suspended in the water column (Quayle, 1988).

Pacific oysters grow rapidly during their first year and more slowly thereafter (Gangnery et al., 2003). Their growth rate is dependent on seawater temperature (reaching a metabolic optimum at 19°C; Bougrier et al., 1995), food availability, and food quality (Brown and Hartwick, 1988a,b; Hyun et al., 2001; King et al., 2006). These variables can, in turn, be linked to culture depth (Ngo et al., 2006), water-column stratification, and nutrient abundance (Harrison and Yin 1998).
Figure 3-1 Study site locations in the Strait of Georgia, British Columbia, Canada: Metcalf Bay (MB), Sykes Island (SI), Thor’s Cove (TC), and Trevenen Inlet (TI).

Oysters are suspensivores that prefer seston rich in small diatoms and flagellates and poor in harmful algae and detritus (Baldwin and Newell, 1995; Chu et al., 2002). Oysters use their gills for particle selection, complemented by clapping of valves and other rejection reactions (Wildish et al., 1998; Cassis and Taylor 2006). Pacific oysters develop particle-processing capabilities as they grow, reaching the adult level of selectivity at approximately 2.4 cm shell length (Cannuel and Beninger, 2007). This
selectivity may have implications for their feeding capacity and the effects of blooms of harmful and noxious algae on growth and mortality (Cannuel and Beninger, 2007).

Mortalities of Pacific oysters during the summer months have been documented throughout the world and can affect between 10 and 50 % of the juveniles (Samain and McCombie, 2007), with extreme cases involving > 90 % mortality (Pauley et al., 1988; FAO, 2010; Burge et al., 2007). Mortality rates are typically higher in smaller oysters than in larger ones and this may be associated with the physiological stress of fast growth (García-Esquivel et al., 2000). Larger oysters, however, can also suffer mortalities during the summer, mostly due to physiological stress and exertion during the reproductive period (Moal et al., 2007). Extreme mortality rates have been observed during periods of high seawater temperature, low salinity close to the surface, or phytoplankton blooms (Shumway et al., 1990; Landsberg, 2002). Temperatures above 19°C can stress oysters and increase their metabolism during periods of low food availability, possibly causing an energetic deficit (Moal et al., 2007). Harmful algal blooms (HABs) can cause shellfish mortalities by means of oxygen depletion and/or toxin production (Shumway et al., 1990; Landsberg, 2002). HABs can also reduce shellfish growth rates (Alexander et al., 2006) and filtration efficiencies (Gainey and Shumway, 1988; Cassis and Taylor, 2006), as well as damaging their digestive systems (Keppler et al., 2005; Galimany et al., 2008). Oysters may succumb to opportunistic viral and bacterial infections during or after periods of heightened environmental and/or physiological stress (Friedman et al., 1991; Burge et al., 2007).

Growth and mortality of oysters have been investigated in large-scale (Brown and Hartwick, 1988a,b; Samain and McCombie, 2007) and local (Toro et al., 1999; García-
Esquivel et al., 2000; King et al., 2006) studies. Salinity was the determining factor for oyster growth in the Strait of Georgia (Brown and Hartwick, 1988a), while phytoplankton composition had a significant effect on oysters grown in Wales, UK (King et al., 2006). A number of studies (e.g. Sumner, 1981; Gagnaire et al., 2006; King et al., 2006) have indicated that oysters typically grow faster closer to the surface, where food supply is abundant, although others have reported lower growth for oysters held close to the surface (Toro et al., 1999).

Salinity in the Strait of Georgia is typically around 32, but can reach values of 15 and lower in the vicinity of local sources of freshwater, producing a strong density-driven stratification in the top 10 to 15 m of the water column (Thomson, 1981). During the summer, the surface seawater temperature generally ranges between 15 and 24°C (Thomson, 1981; Masson and Cummins, 2007), but summer temperature peaks of 25°C and greater can be observed close to the surface in some areas (Thomson, 1981; this study). The water below the pycnocline generally maintains winter values of 6 to 8°C (Masson and Cummins, 2007). These temperature and salinity values are in the ranges considered acceptable for Pacific oysters (Pauley et al., 1988). Nonetheless, large oyster mortality events have been correlated with periods of extreme values and/or strong fluctuations of temperature (Cardwell et al., 1979; Pauley et al., 1988). The phytoplankton community in the Strait of Georgia follows typical annual cycles and successions of temperate estuaries: spring and fall blooms of diatoms and dominance of flagellates in summer. The main species present are generally determined by the availability of nutrients and the water-column structure (Harrison and Yin, 1998).
Due to intense summer stratification, high temperature spikes and HABs in the Strait of Georgia normally occur in the upper 10 m of the water column (Taylor and Harrison, 2002), causing oyster mortalities and reducing oyster growth (Brown and Hartwick, 1988a,b). The objectives of the current study were to: (1) assess the effects of various environmental variables on oyster growth and mortality at 3 m, the shallowest depth typically used by oyster growers in the Strait of Georgia; (2) determine the optimum depth for oyster culture (3, 10, or 15 m); and (3) establish if the manipulation of culture depth could be used to reduce exposure of oysters to damaging environmental conditions (e.g. high temperatures, large temperature fluctuations, HABs) and thus improve oyster growth and survival. Temperature, previously identified as one of the main variables involved in summer mortalities (Brown and Hartwick, 1988a,c; Burge et al., 2007), was selected as the trigger for depth manipulation in our experiment. A possible drawback of this approach is that moving oysters to deeper waters could result in lower food intake and reduced growth, since the abundance of beneficial phytoplankton is also highest near the water surface. We had 2 main hypotheses: (1) oyster growth rate would be highest at the shallowest depth tested (3 m) where temperatures and food levels would typically be greatest, and (2) oyster mortality rate would decrease with increasing depth (due to reduced spikes in high temperatures and concentration of harmful algae). The results of this research may allow oyster growers to reduce oyster mortalities and to optimize the distribution of their stock based on environmental monitoring.
3.2 Materials and methods

The depth-manipulation experiment was conducted from June to October, 2008, at 4 commercial oyster farms within 3 of the main oyster-producing areas in the Strait of Georgia: Metcalf Bay (MB) in Baynes Sound; Sykes Island (SI) in Jervis Inlet; and Thor’s Cove (TC) and Trevenen Inlet (TI) in Okeover Inlet (Fig. 3.1). At each of these sites, 27 oyster culture trays (L × W × H: 56.25 × 56.25 × 21.25 cm) were stocked with hatchery-produced, diploid seed oysters. As no BC-wide industry standard exists, seed sizes and stocking densities particular to the companies that operate these commercial aquaculture leases were used for the experiment. Each individual tray at TC and TI was seeded with 1000 juvenile oysters (mean ± SE shell height: 27.6 ± 4.3 mm; n = 10), while each tray at MB and SI was stocked with ~2500 juvenile oysters (shell height: 5.3 ± 0.4 mm; n = 10). Shell height was the longest distance from the umbo to the ventral margin of the shell. The seeded trays were distributed randomly on 2 rafts (MB, TC, and TI) or 2 sets of long lines (SI) at each site, again depending on company protocol. During the study, the experimental oysters were managed in the same manner as the other oysters at each site, which included thinning to avoid density-related growth problems. This was normally done by placing half of the oysters of the original tray into a new one, which was kept along with the old tray in the same tray stack. During sampling, both trays were counted and the data averaged across trays.

All treatments were conducted in triplicate, with 3 random trays being assigned to each treatment; 3 sets of triplicate trays were kept at fixed depths (3, 10, and 15 m) throughout the experiment, while the rest of the trays were divided into 3 sets of 6 trays each and assigned a trigger temperature (14, 16, or 18°C) (Fig. 3.2). The 6 trays assigned
to each temperature trigger were divided into 2 groups, one to be lowered to 10 m and the other to 15 m. The temperature-triggered trays were kept at 3 m depth until the seawater temperature at 3 m reached their temperature trigger. Once each particular temperature trigger was reached, the trays of oysters were lowered to their predetermined depths (10 and 15 m). These lowered trays were then brought back to 3 m depth when the seawater temperature at 3 m dropped below the temperature trigger. The depths chosen represented the upper depth normally used by oyster farmers (3 m), the depth of the pycnocline, which was also the lower depth typically used by the growers (10 m), and the deeper and colder water layer below the pycnocline (15 m).

Every 2 wk, the experimental and control trays were lifted out of the water and the shell heights of 5 randomly chosen oysters from each tray were measured with digital callipers. Oyster volume was estimated by measuring the water volume displaced by a random sample of oysters from each replicate tray. The sample sizes used during this experiment (> 60 oysters per tray at MB and SI and > 20 oysters per tray at TC and TI) are similar to other oyster growth studies (Toro et al., 1999; King et al., 2006) and were the highest number possible under the commercial farm conditions. Instantaneous growth and mortality rates were calculated using the difference between one sample and the next, divided by the number of days between measurements. Oyster mortality was only determined at MB and SI during the volume measurements, as the sample size used for volume estimation at TC and TI was too small to accurately gauge mortalities.
Figure 3-2 Experimental design for depth manipulation trial. Control trays (n = 3) were kept at 3, 10, and 15 m depth throughout the study, whereas the temperature-triggered trays (n = 3 per trigger temperature and target depth) were held at 3 m and dropped to either 10 or 15 m when the seawater at 3 m reached the trigger temperature.

Temperature was monitored daily at 3 m with a Clinefinder digital probe (Catalina Technologies). Temperature at 3, 10, and 15 m depth was recorded at each site every 3 h for the duration of the experiment by duplicate automatic data loggers (Tidbit V.2, Onset). Water samples were obtained every 2 wk at 3, 10, and 15 m for salinity measurement (STX-3 refractometer, Vee Gee Scientific) and quantitative evaluation of phytoplankton abundance. Secchi depth measurements and vertical tows of a plankton net (20 µm mesh) from 15 m depth were also conducted every 2 wk at each site. Seawater
density was calculated using UNESCO’s Equation of State for Seawater (Gill, 1982). The stratification intensity of the water column was defined as the difference in density between 3 and 15 m.

Water mounts were analyzed quantitatively for micro-phytoplankton and small zooplankton by the Utermöhl method with modifications described in Hasle (1978). Gently homogenized water samples were placed in 5, 10, or 25 ml settling chambers for 24 to 48 h. The detailed composition of these samples was obtained under an Axiovert 10 inverted microscope (Carl Zeiss). Once the phytoplankton were counted and identified, the bio-volumes were estimated using previously determined bio-volumes of local species (Haigh et al., 1992). These estimates were then used to calculate phytoplankton carbon biomass using equations from Strathmann (1967) and Montagnes and Franklin (2001). The phytoplankton counts were converted to carbon biomass to avoid problems associated with quantitative phytoplankton estimates based solely on cell counts. Usually phytoplankton cell counts, without estimates of carbon or bio-volume, overestimate the importance of small species that are present in large numbers and underestimate large species at low abundances. Chlorophyll samples were taken monthly from 3, 10, and 15 m depth. Seawater samples (250 ml) were GF/F filtered (0.7 µm), and the filters were frozen at −20°C until analysis by chlorophyll extraction in 90 % acetone and fluorescence measurement in a 10AU fluorometer (Turner Designs). We conducted 2- and 3-way ANOVAs on oyster volume and mortality per pair of sites that shared the same initial seed size (i.e. MB and SI, and TC and TI). Tukey’s multiple comparison post hoc tests were used to determine significant (p < 0.05) differences among 3 or more treatment means. Prior to the ANOVAs, the data sets were tested for normality using the
Kolmogorov-Smirnov test and for homoscedasticity using Bartlett’s test. All data sets were normally distributed and homogeneous. Two-tailed Dunnett’s tests were used to compare the 6 experimental treatments against the 10 m control within sites (comparisons with 15 m controls were also tried, with similar results). Correlations between environmental variables and instantaneous oyster mortality and growth were examined using Pearson correlation coefficients with $\alpha < 0.05$. The statistical analyses were performed with XLStat for Windows (Addinsoft). The data analysis was tried with several different time lags, but the best results and most significant correlations were obtained with data of the same time period (i.e. no lag).

3.3 Results

3.3.1 Environmental variables

Seawater temperature varied between 26.2°C during August at SI at 3 m, and 6.0°C in October at TC at 15 m. The average temperature was highest at SI (mean ± SE: 16.3 ± 2.2°C) and lowest at MB (14.9 ± 2.2°C), whereas TC (15.2 ± 1.8°C) and TI (15.3 ± 2.0°C) were intermediate and shared a very similar temperature regime. Several short periods of increased temperature throughout the water column were detected at all sites, with the longest periods occurring during July and August (Fig. 3.3). Seawater temperature rapidly decreased in September and October. In terms of the percentage of days on which the temperature at 3 m depth was above the trigger temperature, oysters in the 14°C group remained dropped to lower depths for the longest time at SI and for the shortest time at MB (Table 3.1). The oysters in the 16 and 18°C treatments spent the least amount of time at lower depths at TC and the most at SI (Table 3.1).
Salinity varied from 19 at SI during early July, to 30 in October at MB. Low values of salinity, ~20 to 24, were observed close to the surface at most sites during June and July (Fig. 3.4). Higher salinities were common at greater depth and throughout the water column from August onwards. Among all sites, SI presented the lowest values of salinity and for the longest period. Salinity-driven stratification was high at all sites during June and July, and especially strong at SI, which continued with high to moderate stratification until the fall (Fig. 3.4).

**Figure 3-3** Daily average (n = 8) seawater temperature (°C) at each study site (see Fig. 3.1 for abbreviations).
**Figure 3-4** Salinity at each study site, sampled every 2 wk (n = 1) (see Fig. 3.1 for abbreviations).

**Table 3-1** Percentage of days with water temperatures at 3 m depth above the trigger temperatures at each study site (see Fig. 3.1 for abbreviations).

<table>
<thead>
<tr>
<th>Site</th>
<th>Trigger temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>MB</td>
<td>64.5</td>
</tr>
<tr>
<td>SI</td>
<td>86.9</td>
</tr>
<tr>
<td>TC</td>
<td>79.7</td>
</tr>
<tr>
<td>TI</td>
<td>73.2</td>
</tr>
</tbody>
</table>
Table 3-2 Monthly (n = 1) chlorophyll concentrations (mg m\(^{-3}\)) (see Fig. 3.1 for abbreviations).

<table>
<thead>
<tr>
<th>Month</th>
<th>Depth (m)</th>
<th>MB</th>
<th>SI</th>
<th>TC</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>3</td>
<td>1.82</td>
<td>5.29</td>
<td>4.25</td>
<td>15.92</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.26</td>
<td>6.40</td>
<td>0.92</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.78</td>
<td>1.78</td>
<td>0.44</td>
<td>1.02</td>
</tr>
<tr>
<td>July</td>
<td>3</td>
<td>3.95</td>
<td>1.82</td>
<td>3.14</td>
<td>6.48</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.56</td>
<td>2.29</td>
<td>0.61</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.65</td>
<td>0.67</td>
<td>0.34</td>
<td>1.05</td>
</tr>
<tr>
<td>August</td>
<td>3</td>
<td>3.12</td>
<td>13.08</td>
<td>4.28</td>
<td>13.30</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.31</td>
<td>12.72</td>
<td>0.71</td>
<td>9.77</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.42</td>
<td>1.79</td>
<td>0.41</td>
<td>4.20</td>
</tr>
<tr>
<td>September</td>
<td>3</td>
<td>5.20</td>
<td>7.25</td>
<td>3.22</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.28</td>
<td>3.74</td>
<td>0.75</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.65</td>
<td>0.94</td>
<td>0.71</td>
<td>2.01</td>
</tr>
<tr>
<td>October</td>
<td>3</td>
<td>3.45</td>
<td>0.73</td>
<td>0.84</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.20</td>
<td>0.76</td>
<td>1.16</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.55</td>
<td>1.32</td>
<td>0.48</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Chlorophyll concentrations ranged from 15.92 mg m\(^{-3}\) (3 m in June at TI) to 0.34 mg m\(^{-3}\) (15 m in July at TC). A 3-way ANOVA indicated that Site \((F = 5.41, \text{df} = 3,24, p = 0.005)\), Depth \((F = 14.63, \text{df} = 2,24, p < 0.0001)\), and Date \((F = 7.04, \text{df} = 4,24, p = 0.001)\) all had significant effects on chlorophyll concentration. The TC site had the lowest chlorophyll values, while TI and SI had the highest chlorophyll peaks during June and August (Table 3.2). The chlorophyll values observed at MB were intermediate. During the summer months, the chlorophyll concentrations were normally higher at 3 and 10 m depth than at 15 m at all sites, with a large variation among months. The average difference between values at 3 and 15 m was lowest at MB with 2.7 times more chlorophyll at the surface than at 15 m depth. TC had the largest average depth difference in chlorophyll with around 7.1 times higher values at the surface than at 15 m, while SI and TI averaged 4.2 and 5.3 times more chlorophyll, respectively, at the shallowest depth than at 15 m.
The total phytoplankton biomass varied widely throughout the experimental period and among sites, generally being concentrated towards the surface with much lower values at depth (Fig. 3.5). This pattern was broken several times by blooms of different species during the summer and by the fall bloom of diatoms. The fall bloom was barely noticeable in the biomass profiles at most sites, but it was easily recognizable due to a sudden increase in the population of *Skeletonema costatum* (Greville) Cleve. The phytoplankton community at MB had a high abundance of mainly diatoms (mean of 95 % of total carbon biomass across all sample dates) at all depths and months except for August. TI and TC had similar average proportions for various phytoplankton types with dinoflagellates comprising about 45 % of the biomass, while equal proportions of diatoms and other phytoplankton species accounted for the remaining 55 %. SI was the site with the lowest overall diatom abundance (6 %), with dinoflagellates and other phytoplankton species dominating. HABs, which were detected at all sites, were divided into 2 groups: (1) species potentially-harmful to shellfish (potentially-harmful algal blooms or pHABs) due to spiny projections, associated harmful bacteria, and/or the production of irritants or other harmful chemicals (*i.e.* *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara and M. Chihara), *Dictyocha speculum* Ehrenberg, *Ceratium fusus* (Ehrenberg) Dujardin, *Proteroceratium reticulatum* (Claparède et Lachmann) Bütschli, and *Rhizosolenia setigera* Brightwell), and (2) species that potentially cause toxicity in shellfish (toxic HABs or tHABs) (*i.e.* *Alexandrium* spp. Halim, *Dinophysis* spp. Ehrenberg, and *Pseudo-nitzschia* spp. H. Peragallo in H. and M. Peragallo). Most HABs were produced by pHABs, with tHABs only appearing in medium to low abundances and mostly during short periods of time.Potentially-harmful algae comprised 16 to 34 % of the total phytoplankton biomass.
per site across all sample dates, with the highest proportion occurring at SI and TI (34 % at both sites) and the lowest occurring at TC and MB (16 and 20 %, respectively). Throughout the sites studied, pHABs were normally more abundant towards the surface, with some blooms affecting the whole water column (Fig. 3.6). The intensity of the blooms was highest at SI, where up to 93 % of the total phytoplankton biomass was made up of pHABs during 2 large blooms in June and August (Fig. 3.6). TC and TI had a small bloom in June and a large one during August and September. Potentially-harmful algae were scarce at MB where only 2 short blooms were observed (Fig. 3.6), reaching a maximum of 40 % of the total phytoplankton biomass. Although pHABs were present at all sites, SI and TI were the most affected by blooms of *Heterosigma akashiwo*, *Dictyocha speculum*, and *Protoceratium reticulatum* during June, and *Ceratium fusus* and *P. reticulatum* during August and September. *Rhizosolenia setigera* was only present at MB in low abundances during October.

Normally, tHABs reached only marginal levels of biomass, being scarce in SI while constantly present at low levels in MB. At the latter site, *Pseudo-nitzschia* spp. were common with sporadic appearances by *Alexandrium* spp. and *Dinophysis* spp. TC and TI had the highest relative abundance of toxic algae of all sites, as *Alexandrium* spp. were observed throughout July and August while *Pseudo-nitzschia* spp. were detected during the fall bloom.
Figure 3-5 Total phytoplankton biomass (mgC L\(^{-1}\)) at each study site, sampled every 2 wk (n = 1) (see Fig. 3.1 for abbreviations).
Figure 3-6 Percentage of potentially-harmful algae of the total phytoplankton biomass (%) at each study site, sampled every 2 wk (n = 1) (see Fig. 3.1 for abbreviations).

3.3.2 Depth-manipulation experiment

At all sites and in all treatments, Oyster volume and Shell height were closely correlated in an allometric relationship described by the formula: Volume = (0.0004 × Shell height) 2.7234 ($R^2 = 0.9757$, $p > 0.0001$). Given that the initial oyster seed size was substantially different between the 2 companies participating in this study (i.e. shell
height of 5.3 ± 0.04 mm at MB and SI, and 27.6 ± 4.3 mm at TC and TI), the results of the experiment are divided by sites that shared the same initial seed size (i.e. MB and SI, and TC and TI).

3.3.3 Correlations of instantaneous growth and mortality with environmental variables

Instantaneous growth rate of the oysters at 3 m depth at the various sites was positively correlated only with different components of the phytoplankton community: diatoms (% of total phytoplankton biomass) at SI, dinoflagellates (% of total phytoplankton biomass) at MB, dinoflagellates (biomass) at TC, and pHABs (biomass) at TI (Table 3.3). Growth rate was negatively correlated with Date at TC. Instantaneous oyster growth was not significantly correlated with Temperature, Salinity, Secchi depth, or Chlorophyll concentration at any of the 4 sites (Table 3.3).

Instantaneous oyster mortality rate at SI was strongly positively correlated with Temperature, Diatom biomass, pHAB biomass, and Chlorophyll concentration (Table 3.4). The much lower mortality rate registered at MB was positively correlated with Secchi depth and negatively correlated with Temperature (Table 3.4). Instantaneous mortality rates at TC and TI were very low and random, not being associated with any specific environmental variable (data not shown). The cumulative mortality rates observed at TC and TI were 3 and 7 %, respectively.
Table 3-3 Pearson correlations between oyster instantaneous growth (% d\(^{-1}\)) and selected variables at 3 m at each study site (see Fig. 1 for abbreviations). p-values are in brackets; **bold**: p < 0.05. pHAB = potentially-harmful algal bloom.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MB</th>
<th>SI</th>
<th>TC</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>-0.364 (0.376)</td>
<td>-0.110 (0.795)</td>
<td><strong>-0.700 (0.040)</strong></td>
<td>-0.657 (0.071)</td>
</tr>
<tr>
<td>Daily average temperature (°C)</td>
<td>0.003 (0.995)</td>
<td>-0.061 (0.849)</td>
<td>-0.297 (0.475)</td>
<td>-0.172 (0.683)</td>
</tr>
<tr>
<td>Diatom biomass (mg C L(^{-1}))</td>
<td>-0.241 (0.565)</td>
<td>0.413 (0.393)</td>
<td>-0.547 (0.200)</td>
<td>-0.147 (0.729)</td>
</tr>
<tr>
<td>Dinoflagellate biomass (mg C L(^{-1}))</td>
<td>0.136 (0.642)</td>
<td>-0.059 (0.817)</td>
<td><strong>0.797 (0.032)</strong></td>
<td>0.446 (0.268)</td>
</tr>
<tr>
<td>pHAB biomass (mg C L(^{-1}))</td>
<td>-0.189 (0.219)</td>
<td>-0.369 (0.369)</td>
<td>-0.275 (0.530)</td>
<td><strong>0.478 (0.004)</strong></td>
</tr>
<tr>
<td>% diatoms of total biomass</td>
<td>-0.606 (0.110)</td>
<td><strong>0.740 (0.033)</strong></td>
<td>-0.543 (0.207)</td>
<td>-0.335 (0.417)</td>
</tr>
<tr>
<td>% dinoflagellates of total biomass</td>
<td>0.836 (0.010)</td>
<td>0.097 (0.819)</td>
<td>0.684 (0.090)</td>
<td>0.119 (0.779)</td>
</tr>
<tr>
<td>% pHAB of total biomass</td>
<td>-0.267 (0.523)</td>
<td>-0.329 (0.427)</td>
<td>-0.359 (0.429)</td>
<td>0.302 (0.468)</td>
</tr>
<tr>
<td>Chlorophyll concentration (mg m(^{-2}))</td>
<td>-0.735 (0.263)</td>
<td>0.441 (0.274)</td>
<td>0.651 (0.349)</td>
<td>0.810 (0.071)</td>
</tr>
<tr>
<td>Secchi depth (m)</td>
<td>-0.267 (0.523)</td>
<td>-0.183 (0.661)</td>
<td>-0.773 (0.125)</td>
<td>-0.673 (0.219)</td>
</tr>
<tr>
<td>Salinity</td>
<td>-0.520 (0.187)</td>
<td>-0.251 (0.548)</td>
<td>-0.345 (0.360)</td>
<td>-0.606 (0.270)</td>
</tr>
</tbody>
</table>

3.3.4 Final oyster volume at MB and SI

A 2-way ANOVA on the 3, 10, and 15 m controls indicated that the final volume of control oysters at the fixed depths was significantly affected by both Site and Depth, with no significant interaction between the 2 factors (Table 3.5). Oysters were significantly larger at MB than at SI and significantly larger when held at 3 m than when held at 10 or 15 m, with no significant difference between oyster size at 10 and 15 m (Fig. 3.7A).

A 3-way ANOVA on the temperature-triggered treatments indicated that the final volume of experimental oysters at MB and SI was only affected by Site with no other significant main effects or interactions (Table 3.6). The depth-manipulated oysters at MB were significantly larger at the end of the experiment than those at SI (Fig. 3.7A). No significant differences (using Dunnett’s 2-tailed tests) were observed between the 10 or
15 m temperature-triggered treatment groups and the respective 10 m control groups at either site (Fig. 3.7A).

3.3.5 Final oyster volume at TC and TI

A 2-way ANOVA on the 3, 10, and 15 m controls indicated that only Depth significantly affected the final volume of control oysters at these 2 sites, with no significant effect of Site or the Site × Depth interaction (Table 3.5). Oysters held at 3 m were significantly larger than those held at 10 or 15 m, but there was no significant difference in oyster volume between 10 and 15 m depth (Fig. 3.7B).

A 3-way ANOVA on the temperature-triggered treatments indicated that the final volume of experimental oysters at TC and TI was affected by Site and Test depth with no other significant main effects or interactions (Table 3.6). The depth-manipulated oysters at TC were significantly larger at the end of the experiment than those at TI, and oysters that were dropped to 10 m were significantly larger than those moved to 15 m (Fig. 3.7B). Comparisons between the temperature-triggered treatments and the 10 m control using Dunnett’s 2-tailed test indicated no significant differences at either site (Fig. 3.7B). Oysters in the 15 m TC control group had the lowest final volume of all treatments.
Figure 3-7 Final oyster volume (ml, mean ± SE, n = 3) at (A) Sykes Island (SI) and Metcalf Bay (MB), and (B) Thor’s Cove (TC) and Trevenen Inlet (TI) for controls and for each treatment combination (14, 16, or 18°C temperature trigger and 10 or 15 m depth). Note scale difference between graphs. Different letters above bars indicate significantly different (Tukey’s, p < 0.05) depths among sites.
Table 3-4 Pearson correlations between oyster instantaneous mortality (% d\(^{-1}\)) and selected variables at 3 m at Metcalf Bay (MB) and Sykes Island (SI). p-values are in brackets; **bold**: p < 0.05. pHAB = potentially-harmful algal bloom.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MB</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>0.632 (0.093)</td>
<td>-0.451 (0.262)</td>
</tr>
<tr>
<td>Daily average temperature (°C)</td>
<td><strong>-0.700 (0.040)</strong></td>
<td><strong>0.735 (0.038)</strong></td>
</tr>
<tr>
<td>Diatom biomass (mg C l(^{-1}))</td>
<td>0.054 (0.898)</td>
<td><strong>0.924 (0.001)</strong></td>
</tr>
<tr>
<td>Dinoflagellate biomass (mg C l(^{-1}))</td>
<td>-0.165 (0.696)</td>
<td>-0.242 (0.564)</td>
</tr>
<tr>
<td>pHAB biomass (mg C l(^{-1}))</td>
<td>0.076 (0.857)</td>
<td><strong>0.707 (0.050)</strong></td>
</tr>
<tr>
<td>% diatoms of total biomass</td>
<td>0.399 (0.327)</td>
<td>0.645 (0.084)</td>
</tr>
<tr>
<td>% dinoflagellates of total biomass</td>
<td>-0.358 (0.385)</td>
<td>-0.598 (0.117)</td>
</tr>
<tr>
<td>% pHAB of total biomass</td>
<td>-0.148 (0.727)</td>
<td>-0.284 (0.495)</td>
</tr>
<tr>
<td>Chlorophyll concentration (mg m(^{-3}))</td>
<td>-0.020 (0.980)</td>
<td><strong>0.771 (0.025)</strong></td>
</tr>
<tr>
<td>Secchi depth (m)</td>
<td><strong>0.906 (0.002)</strong></td>
<td>-0.396 (0.331)</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.628 (0.096)</td>
<td>-0.316 (0.446)</td>
</tr>
</tbody>
</table>

Table 3-5 Two-way ANOVAs on final oyster volume (ml) and cumulative oyster mortality (%) in the 3, 10, and 15 m fixed depth controls at study sites (see Fig. 3.1 for abbreviations) that share the same initial oyster size; **bold**: p < 0.05; n = 3.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oyster volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB and SI</td>
<td>1</td>
<td>420.494</td>
<td>57.304</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Site</td>
<td>2</td>
<td>466.551</td>
<td>63.580</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Depth</td>
<td>2</td>
<td>5.692</td>
<td>0.776</td>
<td>0.482</td>
</tr>
<tr>
<td>Site x Depth</td>
<td>12</td>
<td>7.338</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TC and TI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td>1</td>
<td>18.801</td>
<td>0.336</td>
<td>0.573</td>
</tr>
<tr>
<td>Depth</td>
<td>2</td>
<td>1216.208</td>
<td>21.737</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site x Depth</td>
<td>2</td>
<td>114.540</td>
<td>2.047</td>
<td>0.172</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>55.950</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oyster mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB and SI</td>
<td>1</td>
<td>22865.793</td>
<td>503.918</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Site</td>
<td>2</td>
<td>286.292</td>
<td>6.309</td>
<td>0.013</td>
</tr>
<tr>
<td>Depth</td>
<td>2</td>
<td>141.397</td>
<td>3.115</td>
<td>0.081</td>
</tr>
<tr>
<td>Site x Depth</td>
<td>12</td>
<td>45.376</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.6 Oyster mortality at MB and SI

A 2-way ANOVA on the cumulative mortality of the fixed-depth controls at 3, 10, and 15 m at MB and SI indicated that Site and Depth significantly affected oyster mortality, whereas the interaction between the factors was not significant (Table 3.5). SI had significantly higher cumulative oyster mortality than MB (Fig. 3.8). Despite the significant Depth effect in the ANOVA, a Tukey’s test on data combined across the 2 sites did not indicate any significant pair-wise comparisons among depths. The Site × Depth interaction term was non-significant (p = 0.081), but it may be more appropriate to examine the effect of Depth within sites instead. At SI there was significantly higher cumulative mortality at 3 m than at 10 or 15 m, while there were no significant pair-wise comparisons among depths at MB (Fig. 3.8).

A 3-way ANOVA on the temperature-triggered treatments indicated that cumulative oyster mortality at MB and SI was affected by Site and Depth with no other significant main effects or interactions (Table 3.6). The experimental oysters experienced higher mortalities at SI than at MB and had higher cumulative mortality when dropped to 10 m as opposed to 15 m (Fig. 3.8).
**Table 3-6** Three-way ANOVAs on final oyster volume (ml) and cumulative oyster mortality (%) at sites (see Fig. 1 for abbreviations) that share same initial oyster size. TT: temperature trigger; **bold**: $p < 0.05$; $n = 3$.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>MS</th>
<th>$F$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oyster volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB and SI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td>1</td>
<td>1314.616</td>
<td>191.947</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TT</td>
<td>2</td>
<td>23.046</td>
<td>3.365</td>
<td>0.052</td>
</tr>
<tr>
<td>Depth</td>
<td>1</td>
<td>1.786</td>
<td>0.261</td>
<td>0.614</td>
</tr>
<tr>
<td>Site × TT</td>
<td>2</td>
<td>16.632</td>
<td>2.428</td>
<td>0.110</td>
</tr>
<tr>
<td>Site × Depth</td>
<td>1</td>
<td>0.041</td>
<td>0.006</td>
<td>0.939</td>
</tr>
<tr>
<td>TT × Depth</td>
<td>2</td>
<td>5.862</td>
<td>0.856</td>
<td>0.438</td>
</tr>
<tr>
<td>Site × TT × Depth</td>
<td>2</td>
<td>4.593</td>
<td>0.671</td>
<td>0.521</td>
</tr>
<tr>
<td>Error</td>
<td>23</td>
<td>6.849</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TC and TI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td>1</td>
<td>121.474</td>
<td>4.882</td>
<td>0.037</td>
</tr>
<tr>
<td>TT</td>
<td>2</td>
<td>71.236</td>
<td>2.863</td>
<td>0.077</td>
</tr>
<tr>
<td>Depth</td>
<td>1</td>
<td>513.571</td>
<td>20.638</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Site × TT</td>
<td>2</td>
<td>11.768</td>
<td>0.473</td>
<td>0.629</td>
</tr>
<tr>
<td>Site × Depth</td>
<td>1</td>
<td>30.836</td>
<td>1.239</td>
<td>0.277</td>
</tr>
<tr>
<td>TT × Depth</td>
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<td></td>
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<td>MB and SI</td>
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3.4 Discussion

3.4.1 Temperature, salinity, and chlorophyll

Seawater temperature and salinity at the 4 sites had typical profiles for estuaries around the Strait of Georgia during the summer and fall (Thomson, 1981). The temperature at most of the sites reached a maximum close to 19°C, which is the metabolic optimum for Pacific oysters (Bougrier et al., 1995). The exception was SI, which presented slightly higher temperatures during the summer. The sites presented a gradient of salinity regimes with MB on one end, with relatively high values throughout the water column due to strong tidal currents, TI and TC in an intermediate position, and SI with the lowest values and the largest difference between surface and deeper waters.
The salinities measured during the present study were always at levels that are not likely to jeopardize oyster physiology (Bernard, 1983), and salinity did not have significant correlations with instantaneous oyster growth or mortality at any of the study sites. Chlorophyll levels were within the values expected for inlets around the Strait of Georgia during the summer (Haigh et al., 1992; Masson and Peña, 2009).

3.4.2 Phytoplankton and harmful algae

The sites monitored during this study cover many of the marine environments present around the Strait of Georgia (Thomson, 1981), being similar in oceanographic conditions, phytoplankton composition and species succession to those studied by Haigh et al. (1992): a diatom-dominated narrow channel with strong currents mediating seawater mixing (MB), a flagellate-dominated and strongly stratified bay with important freshwater input (SI), and 2 intermediate (mid-channel) sites with an equal representation of the major taxonomic phytoplankton groups (TC and TI). The phytoplankton species succession also followed the normal general pattern for fjords located around the Strait of Georgia — a bloom of flagellates at the end of spring, followed by the onset of summer species and the fall diatom bloom (Harrison and Yin, 1998). Temperature and salinity determined the composition of the phytoplankton community.

Two bloom periods of pHABs were identified at the 4 study sites during the summer: Heterosigma akashiwo and Dictyocha speculum during June and July, and Ceratium fusus and other dinoflagellates mainly during August, although with large differences in the intensity, persistence, and species composition among the sites. Strong and enduring summer stratification was associated with blooms at SI (Taylor et al., 1994;
Taylor and Harrison, 2002). TC and TI had a small *H. akashiwo* bloom at the end of spring and a larger, mixed dinoflagellate bloom during late summer. Potentially-harmful algae at MB were limited to transient periods of low abundance. A high abundance of pHABs can lead to lower oyster growth rates and survival (Gainey and Shumway, 1988; Alexander *et al.*, 2006; King *et al.*, 2006). During the present study, pHABs and oysters interacted differently at the 4 sites: pHABs were positively correlated with instantaneous oyster mortalities at SI and with instantaneous growth rate at TI, suggesting that larger oysters (used at TC and TI) were not as susceptible to the blooms’ harmful effects.

*Heterosigma akashiwo* was the most common and abundant potentially-harmful algal species noted during this study, with the duration and intensity of its blooms being the most evident differences among sites in the phytoplankton community composition. This raphidophyte damages the digestive system in shellfish (Keppler *et al.*, 2005), and pHAB biomass was indeed positively correlated with SI oyster mortality. Diatoms were also highly correlated with instantaneous oyster mortality at SI, although they only represented < 6 % of the total biomass at this site. The highest abundance of diatoms (22 % of total biomass) occurred during the peak and decline of a bloom of *H. akashiwo*, thus, this correlation could be a coincidence. An increase in oyster mortalities was registered during the fall at MB after a bloom of the potentially-harmful diatom *Chaetoceros socialis* (Lauder). This and other bloom-forming diatoms may cause clogging of the gills in shellfish (Landsberg, 2002).

Toxic dinoflagellates and diatoms were more common when the water column was well mixed, such as at MB throughout the sample period, and towards the end of summer at TC and TI. These algae normally cause shellfish harvest closures, due to
paralytic shellfish poison, in areas of the Strait of Georgia in August, as previously observed at TC and TI (Taylor and Harrison, 2002).

3.4.3 Depth-manipulation experiment

Fixed oyster-culture depth significantly affected the final volume and cumulative mortalities of oysters: individuals held at 3 m depth had larger final volumes than those in the 10 and 15 m depth control groups at all 4 sites, and oysters experienced significantly higher cumulative mortalities at 3 m than at 10 and 15 m at SI.

In addition, the test depth to which the temperature-triggered treatments were dropped had a significant effect on final oyster volume at TC and TI and on cumulative mortalities at MB and SI. Those oysters that were dropped deeper had lower volumes and cumulative mortalities, but did not differ significantly from the fixed 10 and 15 m controls. These results agree with other studies in which higher growth and mortality rates were registered closer to the surface (Dégremont et al., 2005; Gagnaire et al., 2006; Ngo et al., 2006). For instance, Pacific oysters grown in Gosung Bay (Korea) at 0 to 2 m had a higher growth rate, gonadosomatic index, and fecundity than those held at a depth of 3 to 5 m, most likely due to higher temperatures and/or an increased food supply (Ngo et al., 2006).

3.4.4 Growth

Several studies have investigated the growth and mortality of Pacific oysters across geographically and oceanographically distinct areas (e.g. Brown and Hartwick 1988a,b; García-Esquivel et al., 2000, King et al., 2006). These studies and our results
indicate that oysters grow better and have lower mortality rates at sites with higher diatom relative abundances, weaker haline and thermal stratification, and smaller temperature variations. The final oyster volumes in the current study were strongly site specific, confirming results from previous studies (e.g. Brown and Hartwick, 1988a). Despite identical initial oyster seed size, large final differences were found between MB and SI; oysters at MB grew to a final size ~20 times larger than at SI in most treatments (the exception was the 3 m control treatment where SI oysters attained 65 % of the volume achieved by MB oysters). However, major differences in final oyster volume were not evident between TC and TI.

Phytoplankton is the main driving force for oyster growth (Ren and Schiel, 2008), and phytoplankton quality and quantity are strongly correlated with both oyster growth rates (Toro et al., 1999) and survival (Hyun et al., 2001; King et al., 2006). Similarly, the main variables significantly correlated with instantaneous oyster growth rate in the present study were components of the phytoplankton community. Diatoms are the preferred food for oysters (Dupuy et al., 2000, Marshall et al., 2010) and were positively correlated with oyster instantaneous growth at SI despite being scarce at this site. In contrast, dinoflagellates were correlated with instantaneous oyster growth at MB and TC, although the phytoplankton at MB was dominated by diatoms. Dinoflagellates have a higher content of carbon and protein per unit volume than diatoms, which could make them trophically preferable for oysters in a diatom-rich environment (Menden-Deuer and Lessard, 2000). The lack of significant correlations of chlorophyll concentration and Secchi depth with instantaneous oyster growth rates indicates that the phytoplankton
composition, rather than the total amount of phytoplankton biomass in the water, is the main factor in fuelling oyster growth.

Current speed is an important factor for oyster performance, mainly due to its effects on food acquisition (Lenihan, 1999). All sites were subjected to tidal and wind-driven currents and, although no direct measurements of flow speed were made at the sites, no large differences in flow were observed during sampling. Our data did not differ significantly between protected (TI) and exposed (TC) sites, corroborating the results of a previous study which used nearby locations in Desolation Sound (Wiley and Zahradnik, 1981).

The initial size of the oysters in the present study was also an important factor for their growth rate, as oysters grow rapidly during their first year and more slowly thereafter (Sumner, 1981). Our results reflect this growth pattern, as the larger seed oysters (at TC and TI) reached ~8 times their initial size while the smaller seed (at MB and SI) grew up to 200 times their starting volume, similar to results obtained by Gangnery et al. (2003).

### 3.4.5 Mortality

Large differences in cumulative oyster mortality were found between MB and SI as oysters at the latter site had 3 to 4 times lower survival than those at the former site. Cumulative oyster mortality observed at MB during the present study was similar or lower than the 25 to 30% reported as being normal for Pacific oysters during their first year in culture (García-Esquivel et al., 2000; Burge et al., 2007). Instantaneous oyster mortality rates varied at MB throughout the summer, similar to results reported by
Soletchnik et al. (2006), but increased towards the fall, as was also observed by King et al. (2006). The increase we recorded in the fall was possibly linked to a bloom of *Chaetoceros socialis*. Conversely, SI had high oyster mortality rates at the start of the study, as was also seen in 7 mm long oysters by Dégremond et al. (2005), levelling off later in the summer. Soletchnik et al. (2006) also described a peak in summer mortalities during June, which was associated with physiological stress due to accelerated growth. At SI, instantaneous oyster mortality was positively correlated with temperature, pHAB biomass, diatom biomass, and chlorophyll concentration, while at MB it was negatively correlated with temperature and positively correlated with water transparency. Although seawater temperatures at these sites rarely reached levels that could cause stress in the oysters, high temperature periods have been identified as one of the main factors in summer mortality events (Cardwell et al., 1979; Brown and Hartwick, 1988a,b; Burge et al., 2007). In the present study, the oyster mortality rate at SI was higher during the warmer, more stratified periods and near the water surface. At MB, the correlation with temperature was negative, as the highest mortality rates occurred during the fall.

Pacific oysters acquire adult capabilities for particle processing and selectivity (being able to reject large, spiny and some toxic algal species; Cassis and Taylor, 2006) at a shell length of ~2.4 cm (Cannuel and Beninger, 2007). The oysters at TC and TI were at or above this critical size at the start of our study, whereas the seed at MB and SI only reached it during late July. The reduced particle selectivity of smaller oysters and the high abundance of harmful algae at SI could be major causes of the large oyster mortality rate at this site. In contrast, oyster growth was fast and mortality rates were negligible during the early summer at TC and TI despite abundant pHABs. Chlorophyll concentration was
also positively correlated with instantaneous oyster mortality at SI, probably because abundant pHABs produced a similar increase in the total chlorophyll concentration. Transparency (Secchi depth) was correlated with instantaneous mortality at MB throughout the study period. Nonetheless, the elevated mortalities observed during the fall might have been partially caused by a bloom of *Chaetoceros socialis*, which could clog the gills of shellfish due to the large size of their colonies. High temperature and low salinity were the factors that best separated high (MB) and low (Si, TC, TI) mortality sites (Fig. 3.9). Salinity was not significantly correlated with instantaneous mortality at either MB or SI; nevertheless, it may have acted as the initiator in a cascade of events that led to increased oyster mortalities at SI. High freshwater input during the spring reduced the surface salinity, inducing stratification in the water column. This surface water was then heated by the sun, and the strong stratification probably prevented heat transfer to deeper waters. These high-temperature, low-salinity, and strongly stratified waters have been described as ideal environments for halotolerant motile algae but inadequate for diatoms (Bearon *et al.*, 2006). SI was characterized by large blooms dominated by *Heterosigma akashiwo* during June and *Ceratium fusus* during August, while diatoms accounted for only 6% of the total phytoplankton biomass. The oysters at this site were then faced with periods of high temperatures (up to 26.7°C), HABs, and lack of nutritive particles overlapping in quick succession. Stress, starvation, and malnutrition caused by pHABs during the summer could result in oysters with a reduced immune response (Galimany *et al.*, 2008). This could then lead to a higher susceptibility to infection by parasites and opportunistic diseases (Friedman *et al.*, 1991; Chu *et al.*, 2002; Burge *et al.*, 2007) and thus increased mortalities in the stressed oysters. The particularly extended
period of low salinity and strong stratification that was conducive to large blooms of *H. akashiwo* and *C. fusus* seen at SI was not observed at any other site, except for a short period at TC and TI. The low stratification and strong tidal mixing prevalent at MB were favorable to diatoms. TC and TI presented intermediate values of salinity and temperature, although closer to MB than SI (Fig. 3.9); in addition, their phytoplankton was an average of the SI and MB extremes.

![Figure 3-9 Seawater temperature (°C) and salinity at each study site, measured every 2 wk](image)

**Figure 3-9** Seawater temperature (°C) and salinity at each study site, measured every 2 wk (see Fig. 3.1 for abbreviations).
3.5 Conclusions

MB had the best oyster-growing conditions, having a well-mixed water column, high salinity, low temperature, and dominance by preferred diatoms. Undesirable conditions at the SI site — strong and enduring stratification, a long period of low salinity, high temperature, and pHABs which resulted in large mortality rates and reduced growth — should be avoided for the culture of small oyster juveniles. Site selection is critical for culture of seed oysters throughout the Strait of Georgia.

Significantly higher growth and mortality rates were seen in oysters held closer to the surface (3 m) than in those cultured deeper (10 or 15 m). The 3 m fixed culture depth was seen as the optimum, due to the larger final oyster volumes obtained, despite the higher mortality rates registered at this depth. Depth manipulation, based on temperature as the trigger for oyster movement, failed to produce significantly better growth or survival than the fixed controls at similar depths. Depth manipulation of oysters remains as a potential management option to reduce summer mortalities, but needs to be researched further as the present study showed that temperature was inadequate as a sole trigger for significantly reducing oyster mortality rate. Potential trigger variables for further study would include various phytoplankton taxonomic groups. Oyster culture sites should be studied in terms of temperature and salinity regimes as well as phytoplankton composition and species succession, to optimize the balance between oyster growth and mortality.
Chapter 4 Differential proteomic analysis of heat-shock proteins (HSP 70 and 90) to study environmental summer stress of Pacific oysters (*Crassostrea gigas*) in Deep Bay, British Columbia, Canada

4.1 Introduction

The Pacific oyster (*Crassostrea gigas*, Thunberg) is the most widely farmed and commercially-important bivalve mollusc in aquaculture in the world (FAO, 2010). It is an ideal species for culture as it has broad salinity and temperature tolerances allowing cultivation in a wide variety of coastal and estuarine environments, is easily manipulated in culture, and grows well under a variety of different culturing techniques (FAO, 2010). *Crassostrea gigas* was first introduced to the province of British Columbia (BC) from Japan in the 1912-13 (Lavoie, 2005) and has been cultured in consistently increasing quantities since then. Baynes Sound, located on the east coast of Vancouver Island in the Strait of Georgia (Fig. 2.1), is one of the main culturing areas in the province.

Oysters are suspension feeders that prefer seston rich in small diatoms and flagellates and poor in harmful algae and detritus, selecting it in their gills (Baldwin and Newell, 1995; Brown and Hartwick, 1998a; Chu *et al.*, 2002; Ward *et al.*, 2003). Various organism-level feeding responses exist against harmful particles such as closing of valves (*i.e.* stopping feeding), clapping of valves, and other rejection reactions (Wildish *et al.*, 1998; Ward *et al.*, 2003; Cassis and Taylor, 2006).
Oyster mortalities during the second year under culture conditions typically range from 3 to 40% in suspended tray operations (Bodoy, 1986; Burge et al., 2007). Extreme cases of mortalities sometimes occur and can affect greater than 50% of the cultured oysters (e.g. Pauley et al., 1988; Burge et al., 2007). The seasonality of these mortality events is highly site-specific (Soletchnik et al., 2007; Cassis et al., 2011b), but large die-offs occur mainly in the summer. At some locations, however, oyster mortalities may peak in late spring (Bodoy, 1986) or early fall (King et al., 2006).

Summer oyster mortalities in BC and the Pacific northwest of the USA have been observed in conjunction with periods of high seawater temperature, low salinity, and phytoplankton blooms of various toxic and noxious species (Cassis et al., 2011b). Temperatures above 19°C has been identified as one of the main environmental stressors (Moal et al., 2007), although the upper lethal thermal limit of Pacific oysters is substantially higher [i.e. 43–44°C (Clegg et al., 1998)] than the temperatures normally observed in the Strait of Georgia. Increasing temperatures can increase the metabolism of oysters (Bougrier et al., 1995), potentially causing an energetic deficit during periods of low food availability (Moal et al., 2007).

Diatoms represent a good source of food for oysters (Marshall et al., 2010) and their low abundance could be a defining factor in oysters’ growth and mortality (Cassis et al., 2011b). Harmful and toxic algal blooms may increase shellfish mortality rates by means of oxygen depletion and toxin production (Landsberg, 2002). Some of these harmful or toxic phytoplankton blooms may also cause sublethal effects such as reduced growth (Alexander et al., 2006), damage to the oyster’s digestive system (Keppler et al., 2005; Galimany et al., 2008), and reduced filtration efficiency (Cassis and Taylor, 2006).
Reproduction is one of the most important physiological processes in the life cycle of any bivalve (Enríquez-Díaz et al., 2009). In Pacific oysters, gametogenesis is initiated by active development of the gonads during late winter and spring. Spawning occurs during the summer, when the gametes reach maturity, and resorption of the unspent gonad occurs during the fall (Enríquez-Díaz et al., 2009). During part of the spring and summer, gametogenesis may take precedence in energy allocation over other important metabolic processes (Werner and Hinton, 1999). This may deplete the glycogen and fatty-acid reserves that the oysters rely on to combat, adapt to, and overcome stress, thus potentially reducing their immunocompetence (Li et al., 2009). This compromised immune response may leave the oysters more susceptible to opportunistic diseases (Li et al., 2009).

Heat-shock proteins (HSP) are highly homologous and well-conserved molecular chaperones in living cells. The main roles of HSP 70 and 90 are folding of nascent proteins, re-folding of mis-folded proteins, and degradation of severely damaged proteins (Hamdoun et al., 2003). They are normally present at all times (constitutive forms), but there are also isoforms that are expressed in response to environmental, physical, and chemical stresses (inducible forms). Both the constitutive (HSC 72 and 77) and inducible (HSP 69) forms of the HSP 70 family help stabilize the hydrophobic protein domains that are exposed to aqueous cellular environments during synthesis and translocation (Hamdoun et al., 2003). The inducible form of HSP 70 (HSP 69) contributes, in time of physiological stress, to the tolerance of otherwise lethal conditions (Clegg et al., 1998; Hamdoun et al., 2003). The stressors known to activate the expression of HSP 70 in shellfish include temperature extremes (Clegg et al., 1998), hypoxia (David et al., 2005),
heavy metals, hydrocarbons and other contaminants (Boutet et al., 2004). The expression of HSP 70 is curtailed, however, by simultaneous exposure to heavy metals and temperature, or very low salinity and temperature (Werner, 2004), indicating a possible limitation in the response to multiple stresses (Ivanina et al., 2009).

The 90 kDa heat-shock protein family are important molecular chaperones in the production of a wide variety of signal transduction proteins and has a role in regulating gene expression, cellular functions, and apoptosis (Zhao and Houry, 2005). The variables that can induce expression of HSP 90 include heat and cold shock, heavy metal exposure (Choi et al., 2008), and cellular growth factors expressed during oogenesis and muscle development (Csermely et al., 1998).

Physiological stress in oysters may be measured by the expression of HSPs, of which the 70 and 90 kDa families are commonly used as biomarkers (Hamdoun et al., 2003; Rossi et al., 2006). These proteins have received considerable attention as a way to gauge stress in shellfish, mostly under well controlled laboratory conditions (Clegg et al., 1998; Hamdoun et al., 2003; Li et al., 2009), although some recent strides have been made in the study of these proteins in field settings (Farcy et al., 2007; Luna-González et al., 2008). Despite this, drawing direct correlations between the concentrations of HSPs in oysters and specific environmental variables has been hampered by the effects of preconditioning (Hamdoun et al., 2003), chronic stress (Farcy et al., 2007), and the expression of these proteins in response to multiple stressors (Werner and Hinton, 1999; Rossi et al., 2006).

The objectives of the present study were: 1) to determine, using proteomic methods, the abundance of induced heat-shock proteins (HSP 70 and 90) in Pacific
oysters under suspended culture conditions throughout the summer, and 2) to explore possible links between induced HSP 70 and 90 concentrations and oyster instantaneous mortalities, phytoplankton abundance/composition, and various environmental variables.

4.2 Methods

Monitoring of oyster parameters and environmental variables was conducted at a commercial, suspended-oyster grow-out operation in Deep Bay (49°27’37’’N, 24°44’30’’W), a semi-enclosed bay at the southern end of Baynes Sound, Strait of Georgia, BC (Fig. 2.1). Sampling was conducted weekly between May 17th and November 16th, 2007. During this study, the oysters on this farm were mechanically tumbled (i.e. a process used to break off new shell growth, leading to a deeper-cupped smaller oyster) and sorted by size at irregular intervals.

4.2.1 Environmental monitoring

A Stowaway Tidbit V2 temperature data logger (Onset Computer Corporation, Pocasset, Massachusetts, USA) was used to measure the temperature at 6 m depth every hour throughout the experiment. In addition, seawater temperature was measured at 1, 3, 6, 10, and 15 m depths every sample period (weekly) using a Clinefinder digital probe (Catalina Technologies Inc., Tucson, Arizona, USA). Other environmental parameters measured at each weekly sampling event (at 6 m depth) included: chlorophyll a, salinity, dissolved oxygen saturation level, nitrate concentration, and phytoplankton abundance and composition. Secchi depth was also measured at each sampling event with a standard Secchi disk. A water sample was obtained from 6 m depth using a JT-1 water sampler
(LaMotte Company, Chestertown, Maryland, USA) for salinity measurement with an
STX-3 refractometer (VEE GEE Scientific Inc., Kirkland, Washington, USA). Dissolved
oxygen was measured with a YSI 55 probe (YSI Inc., Yellow Springs, Ohio, USA).

An aliquot of the water sample obtained weekly at 6 m depth was preserved with
Lugol’s iodine solution for quantitative evaluation of phytoplankton abundance using
Utemöhl’s method with modifications by Hasle (1978). Phytoplankton biomass was
calculated at each time point using the abundance of each species and species-specific
measurements of biovolume (Haigh et al., 1992). These biovolumes were then converted
to carbon biomass using group-specific equations from Strathmann (1967) and
Montagnes and Franklin (2001). The phytoplankton biomass was used in the statistical
analysis as total biomass or separated into the following subgroups:

- Taxonomic groups: diatoms, dinoflagellates, other taxa identified.

- Toxic phytoplankton: all species with known toxins that can accumulate in
  shellfish based on Landsberg (2002) (e.g. Alexandrium spp.).

- Potentially-harmful phytoplankton: included all the species with known or
  suspected negative effects based on the descriptions by Cassis and Taylor (2006) and
  Landsberg (2002) (e.g. Rhizosolenia setigera, Heterosigma akashiwo).

- Non-harmful diatoms: all diatoms except the harmful Rhizosolenia setigera.

Chlorophyll and nitrate levels were obtained monthly from the water sample taken
at 6 m. Seawater samples were filtered through a 0.7-µm GF/F filter, collected in acid-
washed vials, and kept frozen (-20°C) until analysis for dissolved nitrate using an
Autoanalyzer 3 continuous flow autoanalyzer (Bran+Luebbe, Norderstedt, Germany).
The filters underwent chlorophyll extraction in 90% acetone and fluorescence
measurement in a 10AU fluorometer (Turner Designs, Sunnyvale, California, USA) using the methods of Parsons et al. (1984).

4.2.2 Oyster sampling

Six standard oyster culture trays (L × W × H: 56.25 × 56.25 × 21.25 cm), stocked with 339±26 (mean±SE, n=6) diploid Pacific oysters, were held suspended in random stacks under one raft at 6-m depth in Deep Bay. The oysters used in this experiment were two years old and had an initial shell height of 4.5±0.2 cm and wet weight of 24.8±0.8 g (means±SEs, n=6). At every weekly sampling event one oyster was randomly selected from each of the six experimental trays, measured for shell height, weighed, and carefully dissected to assess its reproductive state. The latter was noted qualitatively using the Mason scale (1958) based on five different reproductive states: 1) immature/non-reproductive state, 2) gonads start development and differentiation from other tissues, 3) follicles in gonads start maturation or recovery after spawning, 4) mostly mature follicles, 5) spawning.

The number of live oysters in each experimental tray was counted at the beginning of the experiment and at every sampling event. Instantaneous mortality rate was calculated as \([((N_1 - N_2) / N_1 \times 100) / D]\), where \(N_1\) and \(N_2\) are the numbers of live oysters at times 1 and 2, respectively, and \(D\) is the number of days between these two times. Cumulative mortality percent was calculated as \((N_i - N_1) / N_i \times 100\) where \(N_i\) is the number of live oysters at the beginning of the experiment.

Gill tissue for HSP determination was excised from each sampled oyster and frozen (-80°C) until further analysis. This sampling occurred every second week for most
of the study period – the exception being August when weekly samples were taken. Although no difference in HSP expression has been observed between gills and other oyster tissues, the gills are normally used for HSP determination due to their closer contact with the surrounding water (Boutet et al., 2004). The gill tissue of each selected oyster was ground with a homogenizer equipped with a teflon-coated pestle in a microcentrifuge tube over ice with a buffer solution of 100 mM ammonium bicarbonate and hydrochloric acid at pH 7.8 (following Brun et al., 2008). The lysed samples were then centrifuged at $1 \times 10^3$ rpm at 3°C for 5 minutes. The supernatant was kept at -80°C until analysis. The total dissolved protein concentrations of oyster gill lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Illinois, USA) and standardized before further analysis.

4.2.3 Orthologue-based multiple reaction monitoring of heat-shock proteins

Peptide sequences corresponding to HSP 69, an inducible protein of the HSP 70 family, and HSP 90 were used to quantify the changes in oyster HSP levels relative to heavy stable-isotope labelled (Lys-D4, Arg-13C6) Jurkat cells (Zhang et al., 2009). These peptide sequences were selected during initial experiments by compiling all available oyster inducible and constitutive proteins in the HSP 70 and HSP 90 families contained in the National Center for Biotechnology Information (NCBI) at the time (Table 4-1). These available proteins underwent in silico trypsin digestion, and sequence fragments unique to HSP 70 and HSP 90 were identified. The unique peptide sequence fragments selected were those of a 69 kDa inducible form of the HSP 70 family (and thus called HSP 70 hereafter) described by Hamdoun et al., 2003, and those of an HSP 90 described by Choi
et al., 2008. More specifically, the sequences chosen were: FEELNADLFR, IINEPTAAAIAAYGLDK, and LLQDFNNGK for HSP 70 and GVVDSEDLPNISR, ELISNASDALDK, and LGIHEDSTNR for HSP 90.

In order to check that the selected peptide sequences were found in our oysters, gill lysates from heat-shocked oysters were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); gel sections corresponding to the 70 and 90 kDa molecular weight (MW) ranges were excised and in-gel trypsin digestion was performed on the proteins retained in them (Shevchenko et al., 1996). Extracted peptides were then analyzed by tandem mass spectrometry (MS/MS) using a 2000 Q-TRAP system (Applied Biosystems, Framingham, Massachusetts, USA) coupled to a nano-LC system (LC Packings, San Francisco, California, USA). Mascot search without organism specification was performed to reveal orthologous peptides that identify HSP. With this information a list of oyster HSP peptides suitable for orthologue-based multiple reaction monitoring (MRM) was produced. This list included the six unique peptide sequences selected using the in silico trypsin digestion of the inducible HSP70 and HSP90 described above. Thus, these amino-acid sequences were selected to monitor and quantify HSP 70 and 90 in our oyster samples.

Following proteolysis of the selected field oyster samples, the complex peptide mixtures, containing both heavy and light peptides, were analyzed by MRM. The high-pressure liquid chromatography conditions and the parameters for MRM measurements were those of previous studies (Walsh et al., 2009). The correctness of the MRM-peptide assignment was confirmed by MRM-initiated detection and sequencing (MIDAS) experiments and database searches (Walsh et al., 2009). Data were acquired for a
minimum of two technical repeats per replicate oyster and processed using Analyst 1.4.2 software (Applied Biosystems, Carlsbad, California, USA).

Table 4-1 Protein sequences for various heat-shock proteins contained in the National Center for Biotechnology Information (NCBI), published source, reported total weight (kDa), and number of fragments larger than 500 Da. * = identical sequences. Sequences indicated by arrows used as basis for inducible forms for HSP 70 and 90.

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<th>Sequence</th>
<th>Protein</th>
<th>Study</th>
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4.2.4 Statistical analysis

One-way analyses of variance (ANOVA) were conducted to examine the effect of date on the instantaneous mortality rate, while two-way ANOVAs were used to examine the effects of date, sequence type, and interaction between these two factors on the HSP relative abundances. Significant differences ($P<0.05$) among treatment means were determined using Fisher’s least significant difference (LSD) tests. The datasets were
homoscedastic and normal as tested using Bartlett’s and Kolmogorov-Smirnov tests. The phytoplankton community was analyzed using agglomerative hierarchical clustering based on the variations registered in the biomass of each species along time. Correlations between the various environmental variables, instantaneous mortality rate, and the various HSP sequences were examined with Pearson correlations, considering a significance level of $P<0.05$. Several time-lags between relative abundance of stress proteins, instantaneous mortality, and the environmental variables monitored were tried for these correlations, but the best and most significant results were obtained with concurrent data. Bonferroni correction was considered and tried due to the high number of variables in the correlation matrix. All statistical analyses were performed with XLStat for Windows (Addinsoft, New York, New York, USA).

4.3 Results

4.3.1 Environmental variables

4.3.1.1 Temperature and salinity

The seawater temperature at 6-m depth at the study site varied between 9.3 and 19.2°C during the experimental period (Fig. 4.1). The daily temperature fluctuations at this depth averaged 1.7±0.2°C (mean±SE, $n=22$), with a maximum value in September 6th (4.0°C) and a minimum in November (0.2°C) (Fig. 4.1). The water column was well mixed during June and weakly stratified from July to September (stratified temperature data not shown). A wind event, with gusts up to 33 km h$^{-1}$ (Environment Canada), occurred on August 19 and 20, completely mixing the water column. The salinity at 6-m depth at the study site varied between 22.5 and 28.0, with an average throughout the
experiment of 26.0±0.3 (mean±SE, n=22) (Fig. 4.1). The salinity decreased from May to early August, with the lowest value recorded at the beginning of August. From this point on, the salinity increased to reach a stable plateau at 28 by late September.

![Graph showing daily mean salinity and temperature (°C) at 6 m depth at study site in Deep Bay between June and November 2007. Error bars indicate daily temperature range (n=24). Tick marks in x-axis correspond to the first day of the month.]

**Figure 4-1** Daily mean salinity and temperature (°C) at 6 m depth at study site in Deep Bay between June and November 2007. Error bars indicate daily temperature range (n=24). Tick marks in x-axis correspond to the first day of the month.

4.3.1.2 Transparency and chlorophyll

Secchi transparency was variable during May and June but set at ~ 3 m from July through September, increasing during the latter (Fig. 4.2). An isolated peak in transparency was detected August 20 when it reached 11 m as a result of a wind event. The high transparency period, starting at the end of September, was interrupted briefly at the end of October (3.5 m), but Secchi depth quickly returned to fall values thereafter.
(11.5 m). The chlorophyll at 6-m depth varied between 0.2 µg L\(^{-1}\) on November 16 and 15.9 µg L\(^{-1}\) on September 6, with an average of 5.4±0.8 µg L\(^{-1}\) (mean±SE, \(n=22\)) during the experimental period (Fig. 4.2). It followed an almost identical, although inverse trend, to that of Secchi depth.

**Figure 4-2** Chlorophyll concentration (µg L\(^{-1}\)) and Secchi depth (m) at 6 m depth at study site in Deep Bay between June and November 2007 (\(n=1\)). Tick marks in x-axis correspond to the first day of the month.

4.3.1.3 Nitrate

The concentrations of dissolved nitrate decreased throughout spring and summer, with a minor peak in mid-June (Fig. 4.3). Nitrate concentrations declined to levels typically below 1 µmol L\(^{-1}\) from June 27 to August 15, being partially replenished (up to 12.4 µmol L\(^{-1}\)) during the August 19/20 wind event. During the rest of the summer low
nitrate concentrations persisted, being replenished in the photic zone during the fall, with a maximum observed on November 16 (26.2 µmol L\(^{-1}\)).

**Figure 4-3** Dissolved nitrate concentration (µmol L\(^{-1}\)) and oxygen saturation (%) at 6 m depth at study site in Deep Bay between May and November 2007 (n=1). Tick marks in x-axis correspond to the first day of the month.

4.3.1.4 Oxygen

Oxygen saturation varied mostly between 72 and 140% during the experimental period (Fig. 4.3). Values above the saturation point were observed between mid May and the second week of September, averaging 112.8±3.7% (mean±SE, n=16). Lower levels of oxygen were observed between September 12 and the end of the sampling period, averaging 82.4±3.9% (mean±SE, n=6) saturation.
4.3.1.5 Phytoplankton

A total of 69 species were identified: 35 diatoms, 24 dinoflagellates, and 10 from other taxa. Total estimated biomass varied between 0.01 and 5.31 mg C L\(^{-1}\) during the experimental period, averaging 0.88±0.30 mg C L\(^{-1}\) (mean±SE, \(n=22\)) (Fig. 4.4A). The peak biomass values were observed during a bloom of the potentially-harmful diatom *Rhizosolenia setigera* at the end of June and a mixed bloom of dinoflagellates during August (Fig. 4.4A). Small flagellates (*e.g.* euglenoids and *Heterosigma akashiwo*) dominated the phytoplankton at the start of June, but were quickly replaced by diatoms, which achieved high values of biomass for the rest of that month (Fig. 4.4B). From July until mid-September, the phytoplankton community was dominated by dinoflagellates, especially *Alexandrium* spp. and *Ceratium fusus* (Fig. 4.4B). The phytoplankton at the end of the summer briefly had a higher percentage of small flagellates and ciliates (*i.e.* *Myrionecta rubra*) before diatoms gained dominance for the remainder of the sampling period (Fig. 4.4B).

The harmful algae identified during this study included both toxigenic species as well as algae potentially-harmful to shellfish. The toxic algae included the dinoflagellates *Alexandrium* spp. (paralytic shellfish poison), *Protoceratium reticulatum* (yessotoxin), *Dinophysis acuminata* (diarrheic shellfish poison), and the diatom *Pseudo-nitzschia* spp. (amnesic shellfish poison) (Landberg, 2002). The most prominent toxic genus at the site was *Alexandrium* spp., which was present from July 11 to September 12, composing >40% of the total biomass from August 2 to 20 (Fig. 4.5). This bloom, however, did not produce an increase in paralytic shellfish poison levels in the shellfish (Canadian Food Inspection Agency). Throughout August, however, a large amount of mucus was noticed.
accumulating on farm gear, the oysters were observed to be closed during sampling, and very little growth was reported by the farmers. At the end of the *Alexandrium* spp. bloom (September 6-12\textsuperscript{th}) a small bloom of the yessotoxin-producing dinoflagellate *Protoceratium reticulatum* reached 40\% of the total biomass. All other toxigenic species were only present in low abundance (<1±0.4 \%, mean±SE, \(n=22\)) in mixed phytoplankton communities.

Potentially-harmful algae included the raphidophyte *Heterosigma akashiwo*, the diatom *Rhizosolenia setigera*, the dinoflagellate *Ceratium fusus*, and the silicoflagellate *Dictyocha speculum*. A bloom of *H. akashiwo* was observed during the last week of May and start of June (Fig. 6), which subsided quickly, being replaced with a bloom of *R. setigera* for the rest of June. *Rhizosolenia setigera* was seen again in low numbers from the end of September until the beginning of November, when it combined with various diatom species to form a low biomass fall bloom (Fig. 5B, 6). *Ceratium fusus* was mainly present during the *Alexandrium* bloom, while *D. speculum* was present sporadically throughout the summer in its normal and skeleton-less forms.

The agglomerative hierarchical clustering analysis of the phytoplankton community placed all but six of the species in a single group. This analysis separated the populations of the potentially-harmful diatom *R. setigera*, the toxic dinoflagellate *Alexandrium* spp., the giant heterotrophic dinoflagellate *Noctiluca scintillans*, the potentially-harmful flagellates *H. akashiwo* and *D. speculum*, and the spring and fall bloom diatom *Skeletonema costatum* from the general summer planktonic community by their timing (Table 4.2).
Figure 4-4 (A) Phytoplankton biomass (mg C L\(^{-1}\)) and (B) percentage of the total biomass per taxonomic group at 6 m depth at study site in Deep Bay between May and November 2007. Tick marks in x-axis correspond to the first day of the month.
Diatom biomass, excluding the potentially-harmful *R. setigera* but including the spring and fall blooms of *S. costatum*, was used to gage the food available for the oysters based on the results by Cassis *et al.* (2011b). Non-harmful diatom biomass had three peaks throughout the monitored period: spring (May), mid-summer (late July), and fall (October and November) (Fig. 4.5). These diatoms reached 0.12 mg C L\(^{-1}\) (15% of total biomass) during late spring, 0.20 mg C L\(^{-1}\) (34% of total biomass) in their summer peak, and 0.22 mg C L\(^{-1}\) (83% of total biomass) during the fall bloom of diatoms.
Figure 4-5 Percentage of total biomass represented by toxic and potentially-harmful algae and non-harmful diatom biomass (mg C L$^{-1}$) at 6 m depth at study site in Deep Bay between May and November 2007. Tick marks in x-axis correspond to the first day of the month.

4.3.2 Oysters

4.3.2.1 Growth, mortality, and reproductive state

The oysters of this study are considered a specialty product, as they underwent an irregular but roughly biweekly regime of mechanical tumbling to produce a small, but deeply cupped, product. Although the oysters exhibited new growth on the edge of the shells in most of the sampling events, the shell height and wet weight did not change considerably throughout the studied period. The oysters reached a maximum shell height of 4.6±0.1 cm (initial 4.5±0.2 cm) (mean±SE, $n=6$) and wet weight of 24.3±0.8 (initial 24.8±0.8 g) (mean±SE, $n=6$),
The mean accumulated oyster mortality percent over the sampling period was 8.5±0.8% (mean±SE, n=6) (Fig. 4.6A). The cumulative mortality percent increased sharply from June until the first week of September, but levelled off during October and November (Fig. 4.6A). Over the entire sampling period, the instantaneous mortality rate averaged 0.07±0.006% d$^{-1}$ (mean±SE, n=144), with a maximum of 0.17±0.01% d$^{-1}$ (n=6) (June 7) and a minimum in August 22$^{nd}$, when no mortalities were detected (Fig. 4.6A). There were three general sub-periods in the instantaneous mortality rates: late spring, characterized by high variability and large peaks during June and July (0.0814±0.014 % d$^{-1}$, mean±SE n=42); summer, characterized by slightly lower and more consistent values (0.065±0.009 % d$^{-1}$, n=54); and fall, which had consistently low levels (0.0214±0.004% d$^{-1}$, mean±SE n=48). The average instantaneous mortality rates for the late spring and summer periods were not statistically different from each other ($P=0.709$), but were significantly higher than the average for the fall period ($P=0.006$ and 0.014, respectively).

The oysters’ gonads developed rapidly during the late spring, reaching maturity at the end of May, remaining mostly mature throughout the summer (Fig. 4.6B). Little change was observed until the second week of September, when the oysters started the recovery and reabsorption of the unspent ova. A small maximum in gonadal development was observed at the end of October, although the oysters quickly returned to the non-reproductive state (Fig. 4.6B).
Figure 4-6 (A) Instantaneous (\% d\(^{-1}\)) and cumulative (\%) mortality in *Crassostrea gigas* at 6 m depth at study site in Deep Bay between June and November 2007. Letters indicate results of Fisher’s LSD groupings where dates with different letters differ significantly \((P<0.05)\). (B) Mean reproductive state in *Crassostrea gigas* at 6 m depth at study site in Deep Bay between June and November 2007. Scale based on Mason (1958). Error bars indicate standard error \((n=6)\). Tick marks in x-axis correspond to the first day of the month.
4.3.2.2 Heat-shock Proteins 70 and 90

A two-way ANOVA on the variance of the relative abundances of the three HSP 70 peptide sequences across time indicated that both date and sequence had a significant effect, while the interaction between these factors was not significant (Table 4.3). The relative abundance of the three sequences was significantly lower on August 15 and September 26 and significantly higher on July 25 and October 17 relative to most other sampling dates (Fig. 4.7A). The relative abundances of the sequences FEELNADLFR and IINEPTAAAAYGLDK were not significantly different from one another, but were significantly different to LLQDFFNGK (Fig. 4.7A).

A two-way ANOVA on the variance of the relative abundances of the three HSP 90 peptide sequences across time indicated that only date had a significant effect (Table 4.3). The relative abundances of the three sequences were lowest on October 31, being significantly lower ($P<0.001$) than all other dates except October 17 and September 12, and highest on September 26 (but only significantly ($P=0.017$) higher than August 15, September 12, October 17, and October 31) (Fig. 4.7B).

4.3.3 Correlations

4.3.3.1 Instantaneous mortality rate

Instantaneous mortality rate was significantly positively correlated with total phytoplankton biomass, the combined biomass of toxic phytoplankton and algae potentially-harmful to shellfish, temperature, and oxygen saturation (Table 4.4). In contrast, instantaneous mortality rate was significantly negatively correlated with non-
harmful diatom biomass, nitrate concentration, Secchi depth, salinity, and HSP 70 sequence LLQDFFNGK (Table 4.4).

Bonferroni correction of multiple correlation coefficients was tried in order to reduce possible type I statistical errors. This correction resulted execively conservative as only the correlation between oyster instantaneous mortality and non-harmful diatom biomass was retained.

Table 4-3 Results of two-way ANOVAs (factors: date and sequence type) on for the relative abundance of HSP 70 and HSP 90 sequences. n=6. Significant P values (P<0.05) are in bold.

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4.3.3.2 Heat-shock proteins

All three HSP 70 sequences were significantly positively correlated with non-harmful diatom biomass and nitrate concentration and significantly negatively correlated
with temperature and reproductive state (Table 4.4). LLQDFNGK was also significantly negatively correlated with instantaneous oyster mortality rate. Aside from a negative correlation between GVVDSEDLPNISR and non-harmful diatom biomass, the only HSP 90 sequence to show significant correlations with any environmental parameter was ELISNASDALDK which was positively correlated with the biomass of algae potentially-harmful to shellfish and negatively correlated with non-harmful diatom biomass and nitrate concentration (Table 4.4).
Figure 4-7 Relative abundance per peptide sequence of (A) HSP 70 and (B) HSP 90 in *Crassostrea gigas* at 6 m depth at study site in Deep Bay between June and November 2007. Error bars indicate standard error (n=6) and letters indicate results of Fisher’s LSD groupings where dates with different letters differ significantly (P<0.05). Tick marks in x-axis correspond to the first day of the month.
Table 4-4 Pearson correlations between *Crassostrea gigas* heat-shock protein (HSP 70, HSP 90) relative abundances per sequence, instantaneous mortality rate (% d\(^{-1}\)), and various phytoplankton components [chlorophyll concentration (µg L\(^{-1}\)) and biomass (mg C L\(^{-1}\)) of total phytoplankton, diatoms, dinoflagellates, other phytoplankton groups, potentially-harmful phytoplankton, toxic phytoplankton, and non-harmful diatoms]; dissolved nitrate (µmol L\(^{-1}\)); environmental variables [temperature (C°) at 6m, Secchi depth (m), salinity at 6 m, and oxygen saturation (%) at 6 m]; and reproductive state of *C. gigas* (arbitrary units). P-values in brackets, significant (P<0.05) in bold. ns=not significant (P>0.05).
4.4 Discussion

4.4.1 General characteristics of the environment and phytoplankton

Deep Bay represents a typical environment of semi-enclosed bays around the Strait of Georgia, BC. Some of the normal features are summer and fall salinity-driven stratification, temperatures that peak at around 19°C, low transparency in the summer and high transparency in the winter, high levels of oxygen saturation, and depleted dissolved nutrients in the photic zone during the summer which are replenished during the fall (Harrison and Yin, 1998). The phytoplankton community was composed of species typical of temperate estuaries (Harrison and Yin, 1998) and presented the usual species succession found in the Strait of Georgia: dominance by diatoms (*R. setigera*) during late spring, followed by small flagellates (*H. akashiwo*) and dinoflagellates (*Alexandrium* spp.) during the height of summer, and diatoms again in the fall (Harrison and Yin, 1998).

Several environmental variables were interlinked in expected patterns by the correlations observed (not shown) (Harrison and Yin, 1998). The chlorophyll *a* was closely correlated with the biomass of several groups of the phytoplankton, with both being negatively correlated to nitrate and positively with oxygen saturation.

4.4.2 Oyster mortalities and environmental variables

The oyster mortality rates observed throughout this study were close to what could be considered baseline or average for second-year oysters during a year in a suspended culture system without large mortality events. This was true for both instantaneous mortality rate (0.07±0.006% d⁻¹, mean±SE, *n*=144), which was comparable
to that observed by Royer et al. (2007) for C. gigas grown in Normandy (i.e. 0.08 to 0.77% d\(^{-1}\)), as well as for accumulated mortality percent (8.5±0.8% this study, mean±SE, \(n=6\)) which was similar to that recorded over a year by Pauley et al. (1988) in Washington, USA (~3%) and Katkansky and Warner (1974) in California, USA (6–12.1%). Despite the parts of the year not considered in this study, our results are comparable to these previous studies as most of the mortalities were registered from late spring to early fall.

The results of the correlations indicated that instantaneous mortality rates were higher during periods of high temperature, low salinity, high algal biomass (and especially high potentially-harmful and toxic algae biomass), and low non-harmful diatom biomass (food). These conditions were associated with low transparency and nitrate concentrations, and high oxygen saturation. Similar results were obtained by Cassis et al. (2011b) in a previous study conducted in Metcalfe Bay, an area 4 km apart from Deep Bay which shares the same environment prevalent in Baynes Sound. The temperatures and salinities generally observed during the summer in Deep Bay should not be conducive to large oyster mortalities, as they fall within acceptable ranges for adults of this mollusc (temperature: 4-24 °C with lethal effects at -4 and 43 °C, salinity: 20-35 with lethal effects below 10.5, Pauley et al., 1988, Hamdoun et al., 2003). However, instantaneous mortality rates were significantly correlated with both high temperature (\(r=0.435\)) and low salinity (\(r=-0.440\)). The effects of sub-lethal temperatures and salinities in the summer are most likely compounded by reduced oyster feeding (Cassis and Taylor, 2006) [due to blooms of toxic and potentially-harmful algae (\(r=802\)) and lowered concentrations of non-harmful diatoms (\(r=-0.410\))], leading to a suite of factors that
culminate in increased instantaneous mortality rate, as has been previously observed for C. gigas in other parts of the world (Pauley et al., 1988; Soletchnik et al., 2007). The negative correlation between instantaneous mortality rates and the HSP 70 sequence LLQDFNGK needs to be further investigated as it may indicate that a low expression of members of the HSP 70 family might be linked to oyster deaths, as described by Luna-González et al. (2008), although the other two sequences used to identify HSP 70 did not follow this correlation (see below).

4.4.3 Stress biomarker sequences

The two-way ANOVA on the variance of the relative abundances of the three HSP 70 peptide sequences across time indicated that both date and sequence had a significant effect (Table 4.3). While the effect of date was expected—given that the relative abundance of the selected peptide sequences is expected to change over time—, the effect of sequence was not expected. This sequence effect indicates that the three selected peptide sequences for the inducible HSP70 protein were not unique to this protein. More specifically, the LLQDFNGK peptide sequence seems to be associated with another protein, and thus it does not follow the same abundance pattern as the other HSP70 peptide sequences: FEELNADLFR and IINEPTAAIAYGLDK (Fig. 4.7A).

The LLQDFNGK peptide sequence might be part of undescribed members of the HSP 70 family, such as HSC 77 (Hamdoun et al., 2003), or other proteins of similar molecular weight.
4.4.4 Stress biomarkers and environmental variables

4.4.4.1 Heat-shock protein 70

The relative abundance of HSP 70 throughout the study period reached two peaks of about 1.5 fold increase over the lowest detected levels. This contrasts sharply with other studies where an increase in abundance of 9–10 fold has been observed (e.g. Hamdoun et al., 2003, Rossi et al., 2006). However, other studies have shown similar modest increases in HSP 70 mRNA (e.g. Farcy et al., 2007). The discrepancy among studies may be due to the use of different quantification methods and/or the lack of extreme stress events registered during the current study. Nevertheless, the pattern of expression through time was similar to other studies in which peaks were observed at the height of the summer and at the start of the fall (Farcy et al., 2007).

The expression of HSP 70 appears to be controlled by both physiological and environmental variables, as evidenced by the correlations obtained. The variables in the present study that modulated HSP 70 concentrations in oysters throughout the summer were temperature, reproductive effort, and food availability.

4.4.4.1.1 Temperature

Seawater temperature has been described as an important factor linked to oyster mortalities (Pauley et al., 1988; Moal et al., 2007) and thus may be an inductor of stress proteins under certain circumstances (Hamdoun et al., 2003). The correlation between temperature and mortality rate was corroborated by the results of the present study, although temperature in Deep Bay did not reach damaging thresholds for Pacific oysters. The temperature range observed in Deep Bay in the present study (9.3–19.2°C) was also
lower than the induction levels for HSP 70 expression in Pacific oysters \([i.e. \, 33–37^\circ\text{C for winter and } 37–40^\circ\text{C for summer}] (\text{Hamdoun et al.}, 2003)\). Contrary to previously proposed mechanisms, temperature was correlated negatively with the levels of all HSP 70 sequences in the current work which may indicate a different effect than that previously described. One hypothesis for this finding is that temperatures observed at the height of the summer were close to the oyster’s physiological optimum and maximum oxygen consumption point (Bougrier et al., 1995), and coincidental with a high period of reproductive effort, thus this peak in energy consumption may have deprived the oysters from the energy necessary to produce an HSP 70 response.

Although the temperatures that trigger HSP 70 expression are close to 30 °C, depending on the season, (Clegg et al., 1998; Hamdoun et al., 2003), HSPs were detected during this study at much lower temperatures possibly due to the effect of combined stressors.

4.4.4.1.2 Reproductive effort

The oysters in this experiment underwent much of their reproductive cycle throughout the study period. This included the production of gametes and their release during the summer, recovering throughout the late summer and fall. The precedence that the reproductive effort has over growth and immune response has been cited as a possible drain for oyster energy reserves to such an extent that it may lead to a reduced expression of HSP 70 (Werner and Hinton, 1999; Rossi et al., 2006). This was supported in our study by the negative correlations between reproductive state and the three HSP 70 sequences.
4.4.4.1.3 Phytoplankton

The third variable that affected HSP 70 relative abundance in the present study was phytoplankton abundance and composition. Total phytoplankton biomass (as chlorophyll) has been used to measure the abundance of food in oyster leases (e.g. Brown and Hartwick, 1988a). As demonstrated by Cassis et al. (2011b), however, not all taxonomical groups that form the phytoplankton are beneficial to oysters, with some producing detrimental rejection reactions when in high abundance (Cassis and Taylor, 2006). In contrast, however, diatoms are considered the main energetic input for oysters and a good source of nutrients for a healthy immune response (Rivero-Rodríguez et al., 2007, Marshall et al., 2010). The statistical analysis indicated that non-harmful diatom biomass was positively correlated with HSP 70 levels and negatively with instantaneous mortality rates. This suggests that the abundance of non-harmful diatoms may lessen the impact of a heightened energetic demand, allowing some expression of HSP 70 and thus better preparing the oysters to face other stressors.

Overall, the relative abundance of HSP 70 seems to be curtailed by a high energy demand associated with a high metabolism (due to high seawater temperature) and reproductive effort. In contrast, high abundance of non-harmful diatoms provides extra energy and enhances the expression of HSP 70. Periods of low abundance of these nutritive particles could be exacerbated by the presence of harmful algal blooms, which further hinders the oyster’s access to nutritive phytoplankton (Cassis and Taylor, 2006; Hégaret et al., 2007).
4.4.4.2 Heat-shock protein 90

The relative abundance of HSP 90 was higher at the start of the summer and decreased towards the fall. However, a large peak (close to 2.5 fold higher than the lowest levels detected) was observed at the start of the fall. This increase was half of that found by Farcy et al. (2007) (up to 4 fold increase) in Normandy, and 3 times smaller than that described by Rossi et al. (2006) (up to 9 fold increase) in the Mediterranean. In the present study, aside from one correlation with nitrate concentration, the concentrations of this protein were only significantly correlated with the biomass of potentially-harmful algae (positively) and non-harmful diatoms (negatively), indicating a possible inverse link to the availability of food. This protein may be expressed in response to starvation, especially of carbohydrates, as proposed by Rossi et al. (2006).

The correlations obtained during this study showed that the two families of stress proteins can behave quite differently and in an almost opposite way in the field. This differential pattern of expression of these proteins was described by Rossi et al. (2006) for a cnidarian and by Manitašević et al. (2007) for a terrestrial plant. One explanation for this differing behaviour pivots on the particular role that HSPs have in protein synthesis, with their expression being highly sensitive to energetic reserves and availability, especially that of carbohydrates (Rossi et al., 2006). It has been indicated that HSP 90 is expressed during tissue differentiation and gametogenesis, which are stronger during spring and early summer. We also observed that expression of this protein was positively correlated with harmful algae biomass. The levels of HSP 70 should respond mostly to stress, thus being expressed in higher quantities during the height of the summer (Csermely et al., 1998; Manitašević et al., 2007).
4.4.5 Recommendations for the use of stress biomarkers under field conditions

The use of finger printing amino acid sequences and MRM techniques offers the advantage of better predictable values than those obtained with Western blot and antibody-based techniques. Nevertheless, the cost of MRM analysis is a strong drawback for routine use.

Although stress proteins have been validated as stress biomarkers under controlled conditions (e.g. Clegg et al., 1998), their expression in the field and interpretation requires further study. As their expression is elicited by multiple stressors (Clegg et al., 1998), is dependent on the recent environmental history of the animals (Hamdoun et al., 2003), and may be down-regulated under nutritionally deficient periods (Luna-González et al., 2008), the interpretation of stress proteins from field samples can be problematic. Multiple biomarkers such as HSPs (e.g. 27, 60, 70, 90) or immunoactive compounds (e.g. noradrenaline, dopamine) (Lacoste et al., 2002) as well as condition index, reproductive state, and biochemical composition (lipids, glycogen) should all be considered, if possible, when undertaking shellfish stress-related field studies. This is required to help account for the effect of multiple stressors and seasonal variations on the levels of stress. During these studies attention should be given to phytoplankton composition, especially the abundance of non-harmful diatoms and the presence of toxic and potentially-harmful algae. The study of multiple cellular responses to different stresses at the genomic level is also an emerging technique that could be useful in characterizing and measuring the stress response in Pacific oysters (Lang et al., 2009).
5.1 Pacific oyster culture in British Columbia: helping solve current problems and optimizing traditional methods

Pacific oysters have adapted well to the general environment of the Strait of Georgia since their introduction in 1912-13 (Lavoie, 2005), being successfully grown in suspended systems and on beaches throughout the strait (Quayle, 1971, 1988; Bourne, 1979). Recent developments in international markets and stronger foreign competition have driven this industry to search for solutions to new challenges. Some of the main issues currently facing oyster growers in British Columbia (BC) are high oyster cadmium (Cd) levels, low growth rates, high summer mortality rates, and sublethal stress. These challenges may be addressed by either introducing new grow-out methods or by the modification of existing techniques based on key environmental parameters such as temperature, salinity, and algal community dynamics. Simple modifications might be made to existing oyster farm culture protocols; these modifications may include changing the culture depth, reducing handling of the oysters when harmful algae or peak seawater temperatures are present, or changing the season when the oyster seed is introduced to the farm and/or the timing of the harvest of the final product. All of these possible improvements in grow-out technology, however, require a better understanding of the effects of the environment on the oysters and specific on-site trials.

This study was aimed at improving the BC oyster culture industry by providing a better understanding of environment-oyster interactions. Environmental parameters studied included temperature, salinity, nutrient concentration, oxygen saturation, and
Various approaches were used to study these interactions including observational, experimental, and, when possible, predictive. This study included research on three main areas: 1) the role of various phytoplankton taxonomic groups in modulating Cd in the environment and in oysters, 2) the effect of environmental variables (i.e. temperature, salinity, depth, algal taxonomic groups) on growth and mortality in oysters during their first year in culture, and 3) stress levels in second-year oysters and the environmental variables that affect stress. The main results obtained for each of these research areas are detailed below.

5.2 Key findings of this study

5.2.1 Modulation of dissolved, particulate, and oyster cadmium by phytoplankton

An emerging problem that has limited the international markets available for BC oysters is the adoption of stringent limits of Cd levels in oyster tissue by European and Asian nations in the year 2000 [1 and 2 ppm, respectively (Kruzynski, 2004)]. As BC oysters have naturally high Cd concentrations relative to those in eastern Canada (due to relatively higher Cd levels in the Pacific than in the Atlantic Ocean), options to reduce their Cd content have been sought through the study of Cd geographical distributions and biochemical cycling in BC (Kruzynsky, 2004; Lekhi et al., 2008). Initial research at Deep Bay, BC, indicated that dissolved Cd (Cd$_{diss}$) was the main source to the oysters. Seasonal variation of Cd$_{diss}$ largely determined the oyster’s concentrations of this metal (Cd$_{oys}$) throughout the year, being higher during the winter and lower in the summer (Lekhi et al., 2008). The particulate Cd (Cd$_{part}$), often cited as a possible trophic source of this metal to bivalves (Ettajani et al., 2001, Bendell and Feng, 2009), had a strong negative
correlation with the concentrations of both $\text{Cd}_{\text{diss}}$ and $\text{Cd}_{\text{oys}}$ (Lekhi et al., 2008). As a result, it was deemed important to further investigate the environmental variables that control Cd levels in BC oysters. Special attention was given to the role of phytoplankton, as the main component in suspended particulate material and food supply for the oysters, in modulating the concentrations of $\text{Cd}_{\text{diss}}$, $\text{Cd}_{\text{part}}$, and $\text{Cd}_{\text{oys}}$. This information may help the oyster growers understand and be able to manage Cd concentrations in their products.

The study site, Deep Bay, is a protected meso-tidal estuary located on the south-western part of Baynes Sound, one of the main oyster culture areas in BC. Several small shellfish farms and a shellfish research and education field station operate on the western side of the bay, while a small harbour is located on the eastern shore of the basin. This bay has small seasonal sources of freshwater (Braybrook et al., 1995), which help drive the surface salinity to a minimum of 23 during the summer and which produce a weak haline stratification.

This study used data obtained by Lekhi et al. (2008), including environmental parameters, as well as cadmium concentrations in the dissolved phase, in suspended particulate matter, and in oysters. In addition, these data were complemented with dissolved nutrient data, as well as detailed phytoplankton abundance and composition. This information was obtained at Deep Bay during a year (July, 2004 to August 2005) of biweekly sampling.

The objectives of this research were 1) investigating the role of phytoplankton in modulating Cd concentrations in the environment and oysters and 2) designing mathematical models to predict Cd levels in cultured oysters throughout the year.
The results presented in this study and those of Lekhi et al. (2008) point towards a reduction of $\text{Cd}_{\text{diss}}$, and ultimately of $\text{Cd}_{\text{oys}}$, concentrations by phytoplankton during the summer. Phytoplankton are known to concentrate dissolved metals from seawater, reducing their concentrations in this medium (Dixon et al., 2006). During the fall, $\text{Cd}_{\text{diss}}$ was replenished in the photic zone by various oceanographic processes including upwelling, increased entrainment, and water mixing (Lekhi et al., 2008). At this particular site, phytoplankton accounted for most of the drawdown of $\text{Cd}_{\text{diss}}$, uptaking close to $\sim 37\%$ of the winter concentrations of this metal throughout the spring and summer. Although phytoplankton ($i.e.$ $\text{Cd}_{\text{part}}$) are believed to be a significant source of Cd to bivalves in some areas through trophic interactions (Ettajani et al., 2001), such was not the case in Deep Bay. The correlations of all the studied taxonomic and functional groups of the phytoplankton community ($i.e.$ diatoms, dinoflagellates, other taxa, toxic species, potentially-harmful algae) were negative with both $\text{Cd}_{\text{oys}}$ and $\text{Cd}_{\text{diss}}$, indicating that accumulation of $\text{Cd}_{\text{diss}}$ by phytoplankton reduced its availability to oysters. The relatively slow sedimentation of up to half of the phytoplankton biomass out of the photic zone (Skjoldal and Wassmann, 1986; Sancetta, 1989) may create a sink of Cd, effectively taking it out of the reach of cultured oysters in the upper water column. Alternatively, the removal of $\text{Cd}_{\text{diss}}$ from the photic zone can be a relatively fast process; such was the case for algal blooms that created short-lived, but strong pulses of $\text{Cd}_{\text{diss}}$ reduction and burial (Garcia-Hernandez et al., 2005; this study). As well, HABs or pHABs may limit the acquisition of $\text{Cd}_{\text{part}}$ by oysters via filter feeding reduction (Cassis and Taylor, 2006).

Two descriptive models developed from our environmental and oyster data explained 81–87% of the variability observed in $\text{Cd}_{\text{oys}}$ throughout the year. One of these
models was specifically designed for the oyster growers of Deep Bay and was based on simple oyster and environmental variables (i.e. oyster age, oyster meat weight, seawater temperature, and salinity). The second model was designed to explain the maximum variability in Cd\textsubscript{oys} using oyster age and meat weight, seawater temperature, Cd\textsubscript{diss} and Cd\textsubscript{part} (>20\textmu m) concentrations, as well as total phytoplankton biomass and that of toxic algae. The results obtained indicated that the oyster growers need to harvest smaller oysters, or during the summer to obtain the lowest Cd levels possible in their products.

5.2.2 Pacific oyster growth and mortality in the Strait of Georgia

The two main factors that have been identified as being crucial for oyster growth and survival in suspended cultured in BC are seawater temperature and food availability (Brown and Hartwick, 1988b,c; Pauley \textit{et al}., 1988; Clegg \textit{et al}., 1998; Hamdoun \textit{et al}., 2003; Pouvreau \textit{et al}., 2006). Periods of high water temperature and harmful-algal blooms, in contrast, have been linked to oyster mortality events during the summer (Brown and Hartwick, 1988c; Burge \textit{et al}., 2007), although these variables rarely reach damaging levels in the Strait of Georgia. Oyster mortalities during the second year of grow-out typically range from 3 to 40\% in suspended tray operations (Bodoy, 1986; Burge \textit{et al}., 2007), but extreme cases of mortalities sometimes occur and can affect greater than 50\% of the cultured oysters (e.g. Pauley \textit{et al}., 1988; Burge \textit{et al}., 2007).

The effects of temperature on Pacific oysters have been thoroughly studied in both laboratory and field studies (e.g. Brown and Hartwick, 1988b,c; Bougrier \textit{et al}., 1995) and this variable has been highlighted as an important factor in oyster culture site selection (Brown and Hartwick, 1988a; Pouvreau \textit{et al}., 2006). In contrast, however, food
availability is still being estimated as bulk chlorophyll or suspended solids (Brown and Hartwick, 1988a; Pouvreaux et al., 2006) without any information on its species composition and variation throughout the year. The diversity in algal species (Haigh and Taylor, 1991) and in the responses exhibited by the oysters when faced with these algae (Cassis and Taylor, 2006), however, makes bulk chlorophyll estimations inadequate as predictors of good food quality for bivalves. Certain phytoplankton groups constitute preferred food for oysters, providing them with energy and essential nutrients for growth and survival. Diatoms have been identified as the best phytoplankton food for oysters (Ma’ruf and Hiroshi, 2009, Marshall et al., 2010). Diatoms, such as Chaetoceros calcitrans, can provide all the nutrients required by Pacific oyster larvae within a realistic daily dose and, unlike other taxa (i.e. Pavlova lutheri), a monospecific diet of diatoms is able to sustain small oysters (Marshall et al., 2010). Thus, the study of the effect of the environment on oyster culture should include a detailed analysis of the phytoplankton community composition and succession.

The two main variables likely to cause mortalities in oysters during the summer are harmful-algae blooms and high temperature spikes, which normally present their highest levels and largest variations close to the sea surface (Thomson, 1981; Masson and Cummins, 2007; Cassis et al., 2011b). In order to prevent exposure to potentially-harmful algae and high temperature, an experiment was undertaken in four oyster culture areas around the Strait of Georgia. This experiment consisted in lowering the oysters from their normal culture depth (3 m) to 10 or 15 m deep whenever the temperature exceeded a predetermined trigger point (14, 16, or 18°C). Fixed controls were kept at 3, 10, and 15 m for comparison.
The sites selected for this experiment presented a variety of environmental conditions. The study site in Metcalf Bay (MB) is located within Baynes Sound, an area characterized by strong tidal flows, weak stratification, and an abundance of diatoms over other phytoplankton taxonomic groups. Two sites were located within the Desolation Sound system: Trevenen Inlet (TI) and Thor’s Cove (TC). These sites were located in bays with loosely restricted water flow and intensive aquaculture usage. At these two sites dinoflagellates constituted a large proportion (45%) of the overall phytoplankton biomass, with diatoms and other groups being less abundant. The fourth site, Sykes Island (SI), was located in Jervis Inlet and had the largest fresh water influx and strongest stratification of all the studied sites. At this site the phytoplankton was dominated by blooms of various flagellates (*Heterosigma akashiwo*, *Ceratium fisus*, *Protoceratium reticulatum*, *Dictyocha speculum*, *Rhizosolenia setigera*) potentially-harmful to oysters.

The objectives of the research were to: 1) establish optimal culture depth for maximal growth and minimal mortality rates, 2) test a depth manipulation technique as a possible mechanism to reduce oyster mortality rates during the summer, and 3) study the interactions between oyster growth/mortality and various biological, physical, and chemical parameters in a variety of environments and under two distinct oyster growing methodologies (*i.e.* different initial oyster seed size).

The research identified 3 m as the best culture depth (in comparison to 10 and 15 m) as it produced the best oyster yields despite having slightly higher mortality rates than the other depths. The depth manipulation experiment failed to significantly reduce oyster mortality rate, based on a temperature trigger. The information gathered to answer the third objective of this research indicated that the smaller sized oysters (5 mm initial shell
height) were more affected by harmful-algal blooms than the larger sized oysters (27 mm initial shell height). These smaller oysters registered high mortality rates and little growth under adverse conditions, such as those encountered at SI. Oysters of the same size (5 mm) grew well and presented only low level mortality rates at the diatom-dominated MB site. The best oyster-growing conditions were attained in Baynes Sound (MB), having a well-mixed water column, high salinity, low temperature, and dominance of diatoms. The worst conditions for raising small seed were observed in Jervis Inlet (SI) – strong and enduring stratification, a long period of low salinity, high temperature, and pHABS which can result in increased mortality and reduced growth rates. The characteristics of this latter site should be particularly avoided for the culturing of small juveniles oyster.

The smaller seed (~5 mm shell height) was highly sensitive to the presence of harmful algae, while the larger seed (~27 mm shell height) had much higher survival rates under any culture conditions. The main difference between these seed sizes was hypothesized to be their particle processing capabilities, as oysters above 24 mm shell height had reached a level close to that of adult oysters (Cannuel and Beninger, 2007). The presence of harmful-algae blooms, and the area’s propensity for these events, were driven by strong fresh water influxes, which produced haline stratification and high surface temperatures. This environment was ideal for the formation of dense and persistent blooms of *Heterosigma akashiwo* and other flagellates potentially-harmful to shellfish and linked in this study to high mortality rates of small seed.

In order to optimize the balance between oyster growth and mortality rates, oyster culture sites should be studied in terms of temperature and salinity regimes, as well as phytoplankton composition and species succession. Temperature and salinity are good
indicators for water-column stratification, which in turn defines phytoplankton species composition. Chlorophyll estimates are not sufficient to describe the potential for oyster production, as some of the highest chlorophyll values obtained throughout this study were caused by potentially-harmful species such as *Heterosigma akashiwo* and various dinoflagellates which were detrimental to the oysters.

5.2.3 Effects of environmental variables and phytoplankton on stress and mortality in oysters

As oysters present no visible signs of stress before they are overcome by unfavourable conditions and die, biochemical proxies for stress (e.g. stress or heat-shock proteins) have been investigated (Hamdoun *et al*., 2003; Encomio and Chu, 2005; Ivanina *et al*., 2009). Heat-shock proteins are an energetically expensive repair mechanism normally reserved for acute, close-to-lethal, damage produced by spikes of stressors (Hofmann and Somero, 1995; Hamdoun *et al*., 2003). However, HSPs can also be found under less severe and more environmentally relevant levels of stress (Hofmann and Somero, 1995). Stress may produce a significant energetic drain, causing periods of reduced oyster growth and higher sensitivity to opportunistic parasites and diseases or other stressors, which may ultimately result in mortalities (Li *et al*., 2009). Phytoplankton, despite being among the most important factors in oyster growth and the main source of energetic reserves, have been among the least studied variables in relation to growth, mortality, and stress protein expression.

At Deep Bay (site previously described), throughout the summer and fall of 2007, we sampled oysters and phytoplankton, and monitored oyster mortalities and several
environmental variables (temperature, salinity, oxygen saturation, dissolved nutrients, chlorophyll $\alpha$, and transparency). The oysters were measured and dissected to establish their reproductive state and to obtain a gill tissue sample for HSP 70 and 90 determinations. The stress proteins were quantified using multiple reaction monitoring techniques based on the differential expression of fingerprinting peptides (Walsh et al., 2009). The objectives of this study were 1) to use a new proteomic method to fingerprint, detect, and quantify two stress biomarkers (HSP 70 and 90) in oysters during their second year in culture to determine their summer stress levels, and 2) study the effects of environmental variables and phytoplankton on stress indicators and their relationship with oyster mortalities.

The HSP 70 levels in the oysters at Deep Bay had modest variations throughout the summer, only peaking at the height of the summer and start of the fall with a maximum 1.5 fold higher level than the lowest level detected throughout the study period, similar to the results obtained by Farcy et al. (2007) for Pacific oysters grown in Normandy. The levels of these proteins during this study were lower than those previously reported by other studies (i.e. Hofmann and Somero, 1995; Hamdoun et al., 2003; Rossi et al., 2006), but this could be an effect of the method used (proteomic versus colorimetric antibodies) or the lack of stressing spikes in the environmental variables during this specific year of study. Potential environmental stressors did not reach HSP 70 induction levels, thus the expression of HSPs was modulated by a combination of environmental variables (seawater temperature and the food availability in the form of non-harmful diatoms) and the physiological state of the oysters (metabolic rate and reproductive state). Temperature did not reach HSP-induction levels previously described
for Pacific oysters [40–43°C (Clegg et al., 1998; Hamdoun et al., 2003)] and, in fact, had negative correlations with HSP 70 levels in my study. This may indicate that the increase in metabolic activity produced by higher temperatures (Bougrier et al., 1995) could deduct energetic resources necessary for HSP 70 expression. The energy expended during the reproductive effort could have a similar effect on HSP 70 (as seen in Rossi et al., 2006). This was supported by our findings, showing a strong negative correlation between HSP 70 sequences and reproductive state.

Subgroups within the phytoplankton, particularly non-harmful diatoms, were also correlated with the expression of HSPs, although with opposite results between the stress biomarkers used. Food availability, in the form of non-harmful diatoms, may be able to supply the oysters with sufficient energy for the expression of HSP 70. This phytoplankton functional group presented small abundance peaks throughout the summer which were accompanied with heightened values of HSP 70. Taken as a whole, these correlations suggest that HSP 70 seems to be controlled by the energetic balance between demand (reproductive effort, immune response) and the abundance of food (non-harmful diatoms), in that oysters are able to produce HSP 70 only when provided with enough energy (food) to do so. Despite the expected reduction of the immune system caused by the summer energetic imbalance, the oysters should be able to produce HSPs in response to acute stresses, such as temperature shock [as shown by Hamdoun et al. (2003)], provided they have access to suitable food at some point.

The abundance of HSP 90 was high during late spring and decreased towards the fall. However, the largest peak (almost 3 fold higher than the lowest levels detected) was observed at the start of the fall. The concentrations of this protein were only correlated
positively with the presence of potentially-harmful algae, while negatively with the biomass of non-harmful diatoms, indicating a possible inverse link to the availability of food. This protein may be expressed in response to starvation, especially of carbohydrates, as proposed by Rossi et al. (2006).

Although HSP 70 and 90 are typically expressed in response to stress in a similar way in some laboratory experiments (Tomanek and Somero, 2002; Farcy et al., 2009), the correlations obtained during this study showed that the two families of stress proteins behave quite differently and in an almost opposite way to each other in the field. This differential pattern of expression for HSP 70 and 90 was described for a cnidarian (Rossi et al. 2006) and a terrestrial plant (Manitašević et al. 2007). In the present study, the abundance of good quality phytoplankton species for oysters’ growth was seen as a determining variable, being positively correlated with HSP 70 levels and negatively with HSP 90 levels and oyster mortality rates. This differing behaviour pivots on the particular role that HSPs have in protein synthesis, with their expression being highly sensitive to energetic reserves and availability, especially that of carbohydrates (Rossi et al., 2006). It has been indicated that HSP 90 is expressed during tissue differentiation and gametogenesis, which are stronger during spring and early summer, while HSP 70 would respond mostly to stress, thus being expressed in higher quantities at the height of the summer (Csermely et al., 1998; Manitašević et al., 2007).

Although stress proteins have been validated as stress biomarkers under controlled conditions (Clegg et al., 1998), their expression is elicited by multiple stressors (Clegg et al., 1998), is dependent on the recent environmental history of the animals (Hamdoun et al., 2003), and may be down-regulated under nutritionally deficient periods (Luna-
González et al., 2008). Due to this, environmental monitoring and multiple stress biomarkers should be used to assess shellfish stress under field conditions. The inclusion of detailed phytoplankton taxonomic information and the use of two stress biomarkers in this study offered some insight into the workings of the stress response under field conditions. The use of protein stress biomarkers and proteomic detection and quantification methods proved to be useful to study the sublethal effects of environmental variables on the oysters.

The oyster mortalities observed during this part of the study only reached an accumulated 8.5%, similar to those described for a full year by Katkansky and Warner (1974) (6–12.1%) for Pacific oysters in California. The instantaneous mortality rates recorded throughout our study were positively correlated with temperature, oxygen saturation, and phytoplankton biomass (and especially potentially-harmful and toxic phytoplankton biomass) and negatively correlated with salinity, Secchi depth, nitrate concentration, and non-harmful diatom biomass (food). Similar correlations have been described for other sites in the Strait of Georgia for first-year oysters (Cassis et al., 2011b). The mortalities in this study were likely produced by an energetic imbalance that compromised the oyster’s immune response, which limited the expression of stress-response proteins in favour of reproduction and increased metabolism (as shown in Moal et al., 2007; Li et al., 2009). This may have exposed the oysters to heightened physiological stress produced by environmental stressors (high temperatures, low salinities) and/or possible exposure to opportunistic diseases (as in Friedman et al., 1991; Burge et al., 2007).
5.3 Summary

The results of this study indicate that phytoplankton abundance and composition and environmental variables (e.g. temperature, salinity) can have a strong effect on oyster Cd concentration, survival, growth, and stress response during the summer. Phytoplankton play a role in reducing the Cd concentrations in seawater and in the oysters, and fuel oyster growth and their stress response mechanisms. Harmful and potentially-harmful algae, common in the summer, can also cause a reduction in dissolved Cd, and thus in oysters. Harmful-algal blooms can also produce periods of reduced oyster growth and enhanced mortality rates (as in Landsberg, 2002). The deleterious effects of these blooms are stronger in small oysters due to their ineffective particle processing capabilities (Cannuel and Beninger, 2007).

High temperature and low salinity values have been identified as a possible factor in oyster mortalities (Brown and Hartwick, 1988c; Burge et al., 2007), although these variables rarely reach damaging levels in the Strait of Georgia. In this study, high seawater temperature in the upper water column, caused by haline stratification, was a significant factor in oyster mortalities and stress response (although negatively in the latter). These two environmental variables determined phytoplankton species composition and succession, which were distinct from one site to another. Environments that have a high input of fresh water have been identified as ideal for the formation and persistence of potentially-harmful blooms, and detrimental for diatoms (Haigh et al., 1992).

During the winter, physical and chemical oceanographic processes had a larger role in oyster Cd concentration. Low seawater temperature, high salinity, and high nutrient concentrations signalled the breaking of the thermocline, and thus renewal of the
dissolved Cd in surface waters, which was the main source of this metal for oysters. Phytoplankton was scarce during this season and did not have a significant impact in dissolved or oyster Cd concentrations.

The knowledge acquired in this study on the interactions between cultured oysters and the environmental physical, chemical, and biological characteristics could help in the optimization of oyster culture techniques. These improvements could include, but are not necessarily limited to: a) selection of ideal culture depth for grow-out, b) selection of optimum seed size and timing for out planting, c) optimal timing of size screening and other processes (i.e. mechanical tumbling) to reduce added stress in adults, d) optimal timing of harvest to reduce Cd concentrations, and e) identifying site selection criteria for nurseries, new culture areas, and the repurposing of the existing leases (e.g. relocation of nurseries to diatom-rich areas).

5.4 Limitations of this study

1) The research conducted during this study was limited to the Strait of Georgia and, although similar to other large estuarine systems [e.g. Reloncavi estuary, Chile, Bay of Fundy, Canada, Gironde estuary, France], the relationships described here between phytoplankton and the various forms of Cd, oyster growth/mortality, and stress response, as well as the models produced during this study, may only be applicable to this geographical area.

2) The results on growth, mortality, and stress were limited to the summer and spring, and did not take into account winter events, as oyster mortalities are normally negligible during this time (Royer et al., 2007). None of the work was conducted over
multiple years and, hence, does not account for possible annual variations. The lack of knowledge on the inter-annual variability limits the usefulness of the models, which may require further research to account for possible yearly variations.

3) Current knowledge on the effects of different components of the plankton (either phytoplankton or zooplankton) community on the growth, mortality, and stress response of shellfish is limited and thus requires further study for a more comprehensive understanding.

4) The analysis of phytoplankton samples to a high taxonomical degree was expensive and time demanding which may be a limitation for future studies. Further research should be conducted with automatic identification devices (e.g. Rodenacker et al., 2006) that can subtract harmful algae from overall phytoplankton biomass estimations. Remote sensing may also be integrated into these studies to provide large-scale synoptic information on chlorophyll and temperature levels, but ground-truthing for algal species identification, through in situ phytoplankton sampling, is needed to effectively use this tool.

5) Drawing direct correlations between HSP levels in oysters and specific environmental variables has been challenging, being hampered by the effects of preconditioning (Hamdoun et al., 2003), chronic stress (Farcy et al., 2007), and the expression of these proteins in response to multiple stressors (Werner and Hinton, 1999; Rossi et al., 2006). The differential behaviour of HSP 70 and 90 in response to environmental variables also makes the interpretation of field results more complex. Despite these drawbacks, the inclusion of detailed phytoplankton community dynamics
and the simultaneous measurement of two stress biomarkers offer more information into the causes and management of stress in oysters.

6) The results of this research were limited to one species, the Pacific oyster (Crassostrea gigas), although several other species of bivalves are currently cultured in BC (clams, scallops, mussels). Although this study may set some basic precedents, the response of other shellfish species to environmental variables may be different than that recorded in the present study.

5.5 Possible future applications and research

5.5.1 Site optimization

Current site selection techniques for oyster leases are based on habitat suitability indices such as those described by Brown and Hartwick (1988a). These techniques define the environment suitable for oyster culture based on biological (parasites and diseases, chlorophyll concentrations) and oceanographic characteristics (temperature, salinity, water flow, oxygen saturation). Despite their usefulness, these site selection techniques fail to consider changes in requirements due to the oyster’s age, and the large diversity that exists in the phytoplankton. The results of this study may aid in improving site selection for new oyster farms and/or the repurposing of existing leases by addressing these two weaknesses.

In the longer term, research should continue into the optimization of oyster culture in estuarine areas in terms of culture depth, site selection, and management options to reduce Cd concentrations, mortalities, and stress and to increase growth. This research would be greatly enriched by the addition of phytoplankton taxonomic information into
all aspects of oyster culture as a determining factor in defining the use of leases, the
timing of seed introduction, and product harvest. Due to the dominance of diatoms in
Baynes Sound, this area is ideal for oyster nursery and grow-out operations. The diversity
of phytoplankton in Okeover Inlet makes it a good grow-out area for larger seed (~24 mm
shell height). The large and persistent blooms of potentially-harmful algae present in
Jervis Inlet make this area unsuitable for nurseries and the introduction of seed smaller
than 24 mm shell height. The conclusions presented in this study could be used to
complement current site selection tools (Brown and Hartwick, 1988a), and coupled with
future research using novel tools (e.g. geographic information systems, remote sensing)
could be used to produce a map of the best areas for prospective oyster farms throughout
the Strait of Georgia.

Practical management measures, such as the depth manipulation technique, should
be investigated further to reduce oyster mortality rates. Alternative triggers to our
temperature trigger should be considered, such as phytoplankton composition and haline
stratification.

Efforts to elucidate the effects of multiple environmental variables on the stress
response of cultured oysters should continue with the help of new techniques including
proteomics and genomics. Further field studies should include multiple stress markers
[i.e. HSPs (e.g. 27, 60, 70, 90), immunoactive compounds (e.g. noradrenaline,
dopamine)] and physiological variables [condition index, reproductive state, biochemical
composition (lipids, glycogen)] of the oysters. As well, detailed environmental profiling
and phytoplankton composition and biomass should be considered. The physiological
stress response of oysters to farm practices, such as grading and tumbling, should also be
measured to reduce their impact. Laboratory studies could also be conducted to shed light into the immunoactive effect of harmful and non-harmful plankton species at various developmental stages of cultured oysters.

5.5.2 Cadmium reduction

As the Cd levels in the dissolved and in oysters are higher in the winter than in the summer, the harvest of oysters for markets with stringent limits on this metal (i.e. Hong Kong, European Union) should be avoided during the winter (Rasmussen et al., 2007; Lekhi et al., 2008; this study). This may present a challenge, as oysters normally prepare for reproduction during the summer, decreasing the meat quality. The use of triploid oysters is also an interesting possibility that should be investigated for a low Cd summer product without the problems of reproduction. Another possibility would be to select sites that present low dissolved Cd, although this is rather difficult as many existing farms in BC count with only one or a small number of leases (Rasmussen et al., 2007). The harvest of oysters as young as possible could also contribute by reducing the Cd bioaccumulated due to age. A refinement of this idea could be the selection of oyster families for very fast growth, which could then be harvested younger, reducing the accumulation of Cd in their tissues due to age. Processing the oyster meat also produced a small reduction in Cd concentrations (Rasmussen et al., 2007).

The results of this study present a challenge to traditional views on Cd acquisition routes for shellfish. To confirm our results and study their generality, field-based research in other geographical areas across multiple years is necessary. Further research is needed to elucidate the role of various phytoplankton taxonomic groups in modulating both
dissolved and oyster cadmium levels. Laboratory-based research with techniques similar to those used by Strady et al. (2011) is also needed to elucidate the role of different algal species and their combinations, as well as the effect of different temperatures, shellfish species and sizes or developmental stages. Based on these results, predictive models for Cd in oysters could be created.

5.5.3 Recommendations to lower mortalities and enhance growth

The following recommendations should be considered to lower mortalities and enhance growth in cultured, suspended oysters. These guidelines are for oysters grown between the surface and 10 m deep. All the measurements should be performed at the depth normally used for oyster culture. Phytoplankton blooms described in this set of recommendations are those in which one species covers 95% or more of the total abundance. These blooms are normally characterized by abundances above 2-5 million cells per litre (2000-5000 cells per ml) and chlorophyll values above 7 mg m\(^{-3}\).

5.5.3.1 Conditions ideal for nursery systems in the Strait of Georgia.

a) Areas dedicated to the culture of oyster seed from 5 to 24 mm in shell height should have the following characteristics:

   o Salinity of 24 ppt or higher from February to October at the culture depth.

   o Temperatures that do not surpass 19\(^\circ\)C at the culture depth.

   o Phytoplankton dominated by diatoms (above 90% in overall abundance).
- Lack of blooms of potentially harmful *Heterosigma akashiwo*, or only a short period (2 weeks maximum) of the presence of this species. If blooms of this species (>1500 cells per ml) are present, consider a fall introduction for this size of seed.

- Other species of concern are the potentially harmful *Rhizosolenia setigera, Ceratium fusus* and *Dictyocha speculum*. These species should be absent or present only in low abundance (<20% total abundance) relative to other components of the phytoplankton in each sample. According to our experience, harmful concentrations for *Rhizosolenia setigera* start at 500 cells per ml, while *Ceratium fusus* may be harmful from 200 cells per ml, and *Dictyocha speculum* at 60 cells per ml in any of their forms.

- Chlorophyll values should remain between 1 and 6 mg m\(^{-3}\), except during the diatom-dominated spring bloom, which is normally observed between February and April.

- Areas with high values of chlorophyll during late spring and throughout the summer (>10 mg m\(^{-3}\)) should be avoided as in this region these values normally indicate blooms of *Heterosigma* or other potentially harmful algae.

b) Areas dedicated to the culture of 1\(^{st}\) year oysters above 24 mm in shell height should have the following characteristics:

- Salinity of 20 ppt or higher during the spring and summer at a depth of 3m.

- Temperatures that do not surpass 19°C at a depth of 3m.

- Phytoplankton rich in diatoms and other non-harmful phytoplankton (above 50% of diatoms and dinoflagellates).
Areas with high values of chlorophyll during late spring and throughout the summer (>10 mg m\(^{-3}\)) should be avoided as these values normally indicate blooms of *Heterosigma* or other potentially harmful algae.

5.5.3.2 Conditions ideal for rearing adult 2nd year oysters in the Strait of Georgia.

- Salinity should not be below 20 ppt at the culture depth, throughout the spring and summer.
- Phytoplankton with at least 30% biomass of diatoms throughout the spring and summer.
- Lack of toxic species, especially *Alexandrium* spp. (main cause for Paralytic Shellfish Poison).
- Low proportion (maximum of 20% of total abundance at any time) of potentially harmful *Heterosigma akashiwo*, *Ceratium fusus*, *Rhizosolenia setigera*, and *Dictyocha speculum*.
- If a lack of growth, in the form of new shell, is detected during the summer, discontinue all aggressive management such as tumbling and grading.

5.5.3.3 Methodology

To determine the parameters included in these selection criteria the following measurements should be conducted for at least the period between February and October. Weekly measurements would be ideal, or biweekly at least. In any case, 2 years should be monitored to account for variations between years.
**Temperature** should be measured using a probe that can be submerged to the depth where the oysters will be grown (3-6m), such as *Clinefinder* digital probe (Catalina Technologies).

**Salinity** should be measured at a depth of 3m either through the use of a probe or with the help of a sampling bottle that can retrieve a sample at depth, such as the Lamotte JT-1. In the latter case a refractometer such as Vee Gee Scientific’s *STX-3* can be used.

**Phytoplankton** should be sampled in vertical hauls from 15m to surface using a 20µm mesh net, with 15 cm diameter at the mouth. Preservation of phytoplankton samples should be done using non-volatile or harmful Lugol’s iodine solution and stored at room temperature in dark (amber) glass bottles. Lugol’s iodine solution recipe can be found here as well as cell counting technique details: [www.marine.csiro.au/microalgae/methods/haemacytometer%20counting.htm](http://www.marine.csiro.au/microalgae/methods/haemacytometer%20counting.htm)

Phytoplankton identification and enumeration should be conducted using taxonomic guides such as Rita Horner’s “A taxonomic guide to some common marine phytoplankton” published in 2002, or using the harmful algae monitoring manual created by Nicky Haigh (Centre for Shellfish Research, Vancouver Island University, Harmful Algae Monitoring Program, ph.: 250-753-3245 extension 6354). Enumeration should be conducted using a *Sedgewick-Rafter* chamber under a magnification of 200x. These tasks are relatively simple with the materials and taxonomic guides detailed and can normally be performed by farm personnel with the instructions provided in the guides. Consultation with a specialist should be considered for initial training, in more complicated cases or in blooms of species not described in the recommended guides.
**Chlorophyll** Samples should be obtained at the normal culture depth using a sampling bottle to ensure no dilution or changes in chlorophyll concentration. The samples should be kept frozen in dark plastic bottles until analysis at a lab equipped with a refractometer or fluorometer.

**Costs** for the monitoring equipment described are relatively modest, in the area of USD 400 for a sampling bottle, temperature probe, refractometer and *Sedgewick-Rafter* chamber. Simple microscopes useful to on-lease phytoplankton identification cost less than USD 500 (for example model B100B-MS at http://www.amscope.com/). Most materials can be found at general environmental supply stores such as Dynamic Aqua-Supply.

5.5.3.4 Brief review of common harmful species in the Strait of Georgia

![Figure 5-1](image.png) *Alexandrium catenella* at 200 and 400x magnification.

Toxic *Alexandrium catenella* may cause Paralytic Shellfish Poison (PSP) at any concentration. Presents a typical “hamburger” shape and normally is found in chains of 2-16 cells.
Figure 5-2 Lugol-fixed and live cells of *Heterosigma akashiwo* at 400x magnification.

Potentially harmful *Heterosigma akashiwo*, normally kidney-shaped flagellated cell acquires a unique “raspberry” shape when preserved in Lugol’s iodine solution. This species is harmful to oysters when in high concentrations above 1500 cells per ml, and can cause mortalities especially in smaller seed (5-24mm shell height).

Figure 5-3 *Ceratium fusus* at 200x magnification.

Potentially harmful *Ceratium fusus*, possible cause of oyster seed mortalities and decreased water quality when in concentrations above 200 cells per ml.
Figure 5-4 *Dictyocha speculum* in its normal and skeleton-less forms at 400 and 200x magnification, respectively.

Potentially harmful *Dictyocha speculum* can cause mortalities especially in smaller seed (5-24mm shell height) when in concentrations above 60 cells per ml.

Figure 5-5 Bloom of *Rhizosolenia setigera* at 200x magnification.

Potentially harmful *Rhizosolenia setigera* can reduce feeding efficiency when in high abundance (blooms) of 500 cells per ml or more.

All images by David Cassis unless specified.
Bibliography


**Canadian Food Inspection Agency** (CFIA) reports on PSP closures accessed throughout the monitored period at http://www.pac.dfo-mpo.gc.ca/fm-gp/contamination/index-eng.htm


Environment Canada’s National Climate Data and Information Archive ([http://www.climate.weatheroffice.gc.ca/](http://www.climate.weatheroffice.gc.ca/)) accessed throughout the monitored period.


Crassostrea gigas (Thunberg) in Marennes-Oleron Bay (France). *Aquaculture* 252, 328–338.


