Micro contamination routes and feed quality in hatchery culture of *Ostrea edulis* larvae

Department of Biological and Environmental Sciences
Supervisor: Susanne Lindegarth
Co-Supervisor: Alyssa Joyce
Examiner: Kerstin Johannesson

2013

Daniel Simonsson
Master’s thesis in Marine Ecology 60 hp
Abstract

Potential causes for mortality of *Ostrea edulis* larvae were examined in order to optimize hatchery production of juvenile oysters. The nutritional value and bacterial loading of the microalgae *Chaetoceros calcitrans*, *Chaetoceros muelleri* and *Thalassiosira pseudonana* were measured in the first section of the present thesis. Fatty acids were identified as methyl ester derivatives using gas chromatography. Bacteria were enumerated with epifluorescence microscopy after staining with 4’6-diamidino-2-phenylindole (DAPI). *Vibrio* spp. bacteria were detected with fluorescence in situ hybridization (FISH). Bacteria levels in all trial cultures were above the chosen reference value of 2.0 x 10^6 ml^-1. *C. muelleri* were identified as best suited for larval feeding in terms of bacteria loading. Conversely, *C. muelleri* contained significantly lower relative amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to *C. calcitrans* and *T. pseudonana*. ω-6 fatty acids were most prominent in *C. muelleri*.

The second study of this thesis investigated possible contamination routes at the oyster hatchery Ostrea Sverige AB (OSH). Bacteria levels were measured at key points by plating on marine agar and thiosulfate-citrate-bile-sucrose (TCBS) agar as selection for *Vibrio* spp. bacteria. The results demonstrated that water treatment at OSH significantly reduced bacteria abundance in production water, while completely removing presumptive *Vibrio* spp. Presumptive *Vibrio* spp. were frequently detected in microalgae cultures of *Isochrysis* sp. (clone T-iso), oyster larvae and the oyster broodstock. The results point towards introduction of *Vibrio* from biological sources at OSH, i.e. the broodstock and cultures of T-iso.
Introduction

Background

The European flat oyster *Ostrea edulis* is by many considered a top delicacy. Collecting wild oysters by hand-picking is the traditional method of bringing them to the table in Sweden. Cultivation by seed collection is another method that has been done for a long time in southern Europe, dating back to the 17th century France. With this technique, newly settled oysters are collected from the sea bed or from artificial collectors such as PVC dishes. They are then moved to areas with a good supply of phytoplankton and strong currents, which provides the oysters with the clean water and nutrition needed to develop into adults.

Fishing for oysters or cultivating them by spat collection are both methods that depend on the natural recruitment from wild populations. Recruitment has historically been irregular and infrequent in Sweden. This has been explained by the hydrographical fluctuations between seasons along the west coast, which is the habitat of *O. edulis* in Swedish waters (Rödström 1999). An alternative method to produce oysters is by cultivation in hatchery. The recruitment process is more or less controlled in a hatchery, from spawning of selected adult oysters to metamorphosis of the larvae into settled juvenile oysters. The advantage with this method is that the breeding season is prolonged, resulting in more spat without affecting the wild populations.

Production and fishery of *O. edulis* have for the last centuries primarily been located to the western and southern coasts of Europe. The natural populations have seen major declines during this time, with probable causes being overfishing, habitat loss and diseases (Beaumont et al. 2006). Production in the last decades has been highly limited by outbreaks of the protozoan parasites *Bonamia ostrea* and *Marteilia refringens* (Baud et al. 1997). Oyster cultivation has in western and southern Europe moved focus from *O. edulis* to the more resistant pacific oyster *Crassostrea gigas* because of these problems. As of today, there is no evidence of *B. ostrea* or *M. refringens* in Norwegian or Swedish waters (Mortensen 1993; Höglund and Christensson 1997; Valero and Loo 2008).

The Nord-Ostron project

The Nord-Ostron project is a transnational collaboration between Sweden, Denmark and Norway with the ambition of developing a knowledge and innovation platform for the culture of *O. edulis*. The present thesis focuses primarily on analyses performed at two of the project partners, the University of Gothenburg and the hatchery Ostrea Sverige AB located at Sydkoster in Northern Bohuslän (hereafter called “OSH”).

Cultivation of *Ostrea edulis* in hatchery

A hatchery is in short a producer of oyster spat (i.e. juveniles). The process starts with adult oysters
that are brought into the hatchery after being selected to become the broodstock. These oysters will produce the larvae and to do so they will need the appropriate environmental settings (fig. 1).

*O. edulis* are sequential hermaphrodites which mean that they alternate between reproducing as males and females. Oysters can spawn several times a year if the temperature is favourable (Walne 1979). They commence spawning when the water temperature reaches approximately 16°C (Laing et al. 2005). The main advantage with a hatchery is that the breeding season can be prolonged and controlled. Temperature and food availability in broodstock tanks is increased early on in the season to initiate spawning. This process is normally termed broodstock conditioning and is recognized as vital for the future offspring viability (Helm et al. 1973; Helm et al. 1991; Berntsson et al. 1997; Utting and Millican 1997). Male oysters release sperm into the surrounding water while females transport their eggs to the surface of the gills. *O. edulis* is, unlike many other bivalve species, larviparous. Fertilized eggs are brooded inside the females’ mantle cavity. The larvae are released as shelled veligers after approximately 10 days of development inside the females’ mantle cavity (Walne 1979). The larvae will then begin their planktonic life stage.

In a hatchery at this point, the larvae are transferred to separate nursing tanks where they will feed on microalgae and continue to develop. If properly fed and unhindered by disease, the larvae will be ready for settlement within two weeks. The pediveliger larvae settle when they find an appropriate substrate. In a hatchery this can take the form of sand or shell gravel. When settled, the larvae will metamorphose into juvenile oysters, or more commonly termed, oyster spat.

**Challenges in the hatchery setting**

Within the Nord-Ostron project it has become clear that one of the major challenges in hatchery culture of *O. edulis* is larval mortality. Possible explanations behind the high levels of larval mortality seen in Sweden and Denmark were believed to be bacterial or viral diseases and poor feed quality.

**Feed quality**

Starvation studies have demonstrated that lipid is the main energy reserve for *O. edulis* larvae (Millar and Scott 1967; Holland and Spencer 1973; Gallager et al. 1986). Further experiments indicated that the diets’ composition of lipids is more important than the caloric amount (Enright et al. 1986). *O.
edulis larvae are unable to feed at the beginning of metamorphosis and at this stage they use neutral lipids as energy reserve (Holland and Spencer 1973).

Fatty acids (FA) are fundamental components of most lipids and it is known that the essential FA eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA) are important for the growth and development of O. edulis larvae (Langdon and Waldock 1981; Webb and Chu 1983; Enright et al. 1986). Both EPA and DHA is needed for optimal growth of O. edulis larvae (Enright et al. 1986). The ω-3 fatty acids have been assigned as critical components for maintaining the cell membrane fluidity in cold-blooded animals (Langdon and Waldock 1981). Other findings indicate that EPA provides energy during metamorphosis while DHA has more of a structural role (Labarta et al. 1999). In another study, growth and settlement rate of O. edulis larvae was significantly correlated with C18:3n-3, C18:4n-3 and C22:6n-3 (Jonsson et al. 1999).

It is known that the fatty acid composition in O. edulis larvae reflects that of the diet (Jonsson et al. 1999). Bivalve’s ability to synthesize essential fatty acids (EFA) from shorter chained FA, such as 18:3n-3, is highly limited, consequently they must be supplied with EFA from the feed (Wallock and Holland 1984).

Species of diatom microalgae used in bivalve aquaculture are generally rich in EPA, while flagellate species such as Isochrysis galbana (T-Is) and Pavlova lutheri usually have a higher proportion of DHA (M. R. Brown et al. 1997; Marshall et al. 2010). Hatcheries that culture bivalves typically have a larval feeding regime with at least one diatom and one flagellate species. This provides larvae with the essential ω-3 fatty acids and other nutrients needed to grow and develop into adults.

Insufficient levels of ω-6 and ω-3 FA can be detrimental to oyster larvae (Enright et al. 1986). The effect is believed to be enhanced if these FA are diluted by other FA in the feed, which suggests that it is the relative composition of FA that is key. It has been found that bivalve diets with ω-6 : ω-3 ratios > 0.5 has a negative effect on larval growth (Webb and Chu 1983; Enright et al. 1986).

Manipulating with nutrients, temperature and light intensity in cultures can alter the biochemical composition of microalgae, thus improving the nutritional value (Webb and Chu 1983; Utting 1985; Enright et al. 1986; Thompson et al. 1990; Lombardi and Wangersky 1991; Thompson and Harrison 1992; Ponis et al. 2006). When phytoplankton are deprived of nutrients, the usual response is a halt in cell division rate and diversion of the cells metabolism into production of storage products (Enright et al. 1986). EPA is believed to be an important component of the chloroplast’s lipids in some marine phytoplankton. This has been the explanation of observed increases in phytoplankton EPA proportion when decreasing the light intensity (Thompson et al. 1990). It is also known that FA composition of microalgae varies depending on season and growth phase (Pernet et al. 2003).

The production process of microalgae typically starts from static stock cultures that are inoculated and scaled up into aerated starter cultures. These will usually provide inocula for intermediate-scale cultures. Microalgae cultures used as feed in a hatchery are likely to contain bacteria, if the culture medium is not completely sterile. Larger cultures are difficult to produce axenically but the preference of hatcheries is usually to keep at least stock- and starter cultures bacteria free. Thorough care is needed to keep them in the axenic state. Inoculations pose major infection risks which is why working in a laminar flow cabinet and using a sterile medium is advisable to minimize the chance of
infection. Lewis et al. (1986) provide more details on the sterilization of equipment and culture medium.

Bacterial infections

Periods of high mortality are common within bivalve aquaculture. Bacterial pathogens are in many cases found being the cause of these epizootics. For a hatchery it can be financially devastating if a disease spreads throughout the system and depletes the stock. There are a number of bacterial species identified with a pathogenesis (description of host syndrome) on Ostrea edulis (Tubiash et al. 1970; Jeffries 1982; Lodeiros et al. 1987).

Bacterial effects on larval development in oysters have been widely studied and reviewed (Paillard et al. 2004; Beaz-Hidalgo et al. 2010; Romalde and Barja 2010). Walne (1958) was the first to report evidence of pathogenic bacteria on bivalve larvae. In his study, significantly more larvae of O. edulis developed into spat when the bacterial fauna was treated with antibiotics. Guillard (1959) was able to isolate bacterial strains from a deceased Mercenaria mercenaria larva. Cultures of two of these strains, namely Pseudomonas sp. and Vibrio sp., proved to be pathogenic when tested on healthy M. mercenaria larvae. “Bacillary necrosis” is a term that has been associated with bivalve larvae and Vibrio spp. since it was first described in the 1960s by Tubiash et al. (1965). Larvae of O. edulis, Crassostrea virginica, M. mercenaria, Argopecten irradians and Teredo navalis were challenged with Vibrio spp. bacteria, which resulted in complete mortality. The first sign of infection was visible within 4-5 hours. Abnormal swimming, motility reduction, extension of the velum and bacterial “swarming” around the larvae are all typical indicators of the disease. DiSalvo et al. (1978) noticed another characteristic sign called “spotting”, a phenomenon where large numbers of moribound larvae aggregate at the bottom of culture tanks.

Elston and Leibovitz (1980) categorized bivalve larvae disease caused by Vibrio spp. into three patterns, named Pathogenesis I, II and III. Pathogenesis I affects all larval stages, the larvae become inactive while bacteria can be seen colonizing the mantle. Abnormal swimming and extension of the velum is categorized into Pathogenesis II, which affects the early stage of the veliger larvae. Pathogenesis III affects the pediveliger larvae, which become inactive with lesions in the organs of the digestive tract and extensive visceral atrophy (Elston and Leibovitz 1980; Beaz-Hidalgo et al. 2010).

Hasegawa et al. (2008) examined virulence factors for the pathogenicity of Vibrio tubiashii to Pacific oyster larvae (Crassostrea gigas) and identified the metalloprotease VtpA as a critical factor. This protein belongs to a family of zinc metalloproteases which is widespread among Vibrio species. The gene encoding for VtpA is activated at high cell densities and the authors suggest that VtpA contributes to pathogenicity by degrading tissues, which may provide bacteria with nutrients under poor growth conditions. Purified VtpA was found to be much more toxic for 6 day old larvae than for 16 day old larvae (Hasegawa et al. 2009). Treatment of Vibrio cultures with metalloprotease inhibitors in the form of EDTA (Hasegawa et al. 2008; Hasegawa et al. 2009) and Ovoglobulins (Takahashi et al. 2000) significantly improved survival of C. gigas larvae while suppressing growth of the pathogens.
The species *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio pectinicida*, *Vibrio neptunius* and *Vibrio tubiashi* have all been assigned as being causal agents of bacillary necrosis (Tubiash et al. 1970; Jeffries 1982; Tubiash and Otto 1986; Lodeiros et al. 1987; Nicolas et al. 1996; Lambert et al. 1998; Sugumar et al. 1998; Prado et al. 2005; Torkildsen et al. 2005). It has also been suggested that different bacterial species may act synergistically, as pathogens or opportunists, during the development of bacillary necrosis in bivalve larvae (DiSalvo et al. 1978; Prado et al. 2005).

The major sources of bacteria in shellfish hatcheries have previously been located to: the intake seawater used in the culture process; surfaces of pipes, tanks and equipment; the broodstock used for spawning and the microalgal feed (C. Brown 1983; Elston 1984; Lewis et al. 1986). Bacteria pathogenic on *Pecten maximus* (Torkildsen et al. 2005) and *Argopecten purpuratus* (Riquelme et al. 1996) have previously been found to proliferate in algal cultures, with the feed consequently acting as an entry point for pathogens into the hatchery systems.

Lewis et al. (1988) pointed out that completely axenic microalgal feed would be stressful to oyster larvae, by introducing a large amount of dissolved organic carbon into larval culture tanks. The authors found a threshold value of a tolerable bacteria concentration in microalgal cultures fed to *C. gigas* larvae, which was $< 2.0 \times 10^6$ ml$^{-1}$. Bacteria levels above this threshold resulted in sick larvae. Several other attempts have been made to find bacteriological reference values for a safe culture of larval *Crassostrea* spp. (Murchelano et al. 1975; Garland et al. 1986; Lewis et al. 1986). The mechanisms behind these reference values could possibly be explained by later findings such as Hasegawa and colleagues (2008) previously mentioned discovery: that the toxic protease VtpA was regulated in bacteria by quorum sensing.

Several known bivalve pathogens had been identified from larval tanks at OSH prior to this study (Unpublished results). All belong to the genus *Vibrio* (*V. alginolyticus*, *V. splendidus*, *V. vulnificus* and *V. fluvialis*). Signs of bacillary necrosis had been observed at OSH and it was believed that this could be implicated in larval mortalities (K. Berntsson, OSH, pers. comm.).

**Aim of present study**

This thesis was performed as part of the Nord-Ostron project, with the ambition of optimizing hatchery production of juvenile oysters. Research conducted for the present thesis was focused on microbial contamination routes and the nutritional value of microalgae as potential causes for larval mortality.

The first aim was to examine the nutritional value and bacterial loading of three Bacillariophyceae (diatom) species cultured as feed for *O. edulis* larvae. Strains of *Chaetoceros calcitrans*, *Chaetoceros muelleri* and *Thalassiosira pseudonana* from OSH’s collection were chosen as study organisms. Measured variables were: algal densities/growth rate, fatty acid composition with emphasis on EPA and DHA proportion, bacterial count and presence of *Vibrio* spp. bacteria. The objective was to evaluate the suitability of these microalgae from OSH as feed to oyster larvae. It is known that culture conditions such as light intensity (Thompson et al. 1990) and temperature (Thompson et al. 1992) influence the fatty acid composition of phytoplankton. Previous results on biochemical values of selected study organisms (Thompson et al. 1990; Thompson et al. 1992) are therefore predicted to
differ from results of microalgae cultured at OSH. A bacteriological threshold value of a tolerable bacteria concentration of 2.0 x 10^6 ml\(^{-1}\) was chosen from Lewis et al. (1988) as comparison to the measured bacteria concentrations.

The second aim was to investigate bacteria levels at potential point sources within OSH and to determine possible contamination routes. The abundance of total bacteria and *Vibrio* spp. were measured at key points in the hatchery production process in order to achieve the second aim. Hypotheses were:

- that point sources of bacteria would be located to the broodstock and microalgal cultures, since other potential sources (C. Brown 1983; Elston 1984; Lewis et al. 1986) were meticulously treated with antimicrobial measures.
- that larval survivability could be predicted by detection of *Vibrio* on TCBS agar.
- that the species of microalgae cultured as larval feed would differ from each other in bacterial counts and presence of *Vibrio* spp.
- that the water treatment at OSH would have reduced bacteria concentrations in production water compared to untreated intake water.

**Materials and Methods**

**Study 1: The Diatom evaluation**

**Experimental design**

Microalgae were obtained from Ostrea Sverige AB. *Chaetoceros calcitrans* (Takano; CCAP 1010/11), *Thalassiosira pseudonana* (Hasle & Heimdal; CCAP 1085/12) and *Chaetoceros muelleri* (Lemmermann; CCAP 1010/3) were batch-cultured in 20 l bags, starting from 250 ml Erlenmeyer flasks before inoculation into 5 l borosilicate bottles (fig. 2). Stock cultures were inoculated after 10 days and starter cultures after 7 days. Oceanic water from 35 m depth (approx. 32 ppt) enriched with F/2 medium (R. R. Guillard 1975) was used for all species. Water was particle-filtered in 7 steps down to 0.2 µm, then UV-treated and sterilized in autoclave, apart from water used in the bags, which was sterilized by pasteurization only. Bags and starter cultures were kept at 17-18°C with 24h light, stock cultures at 15-16°C with 16h light / 8h darkness. Light intensity ranged between 84-87 µmol. photons m\(^{-2}\) s\(^{-1}\).
Trial cultures were replicated in three per species. Starter- and stock cultures were not replicated since only one inoculator was used per step in the culture process (fig. 2). On each sampling occasion, 200 ml of algal culture was transferred aseptically to 50 ml polypropylene centrifuge tubes and kept in darkness at 4°C until further analysis. Samples were stored this way for a maximum of 2 hours before arriving at the laboratory.

Trial cultures crashed before the scheduled lipid analyses. A decision was made by the hatchery to discard strains of algae currently in use. Consequently, data on lipid composition from trial cultures are from a new set of algal strains, cultured in the same manner and sampled on the 7th day of the starter culture. These strains are: Chaetoceros calcitrans CCMP 1315, Thalassiosira pseudonana CCMP 1335 and Chaetoceros muelleri CCMP 1316. Experiments were run between January – March 2011.

Determining concentration of algae

Upon arrival at the laboratory, a fraction of each algal sample was fixed with glutaraldehyde (2.5% v/v final concentration) and stored in 4°C not longer than 3 weeks. The concentration was determined with a Coulter Counter after diluting samples 20-fold. To verify the accuracy of the particle counter, the results were compared with those obtained from counting the algal cells in a microscope using a hemacytometer.

Bacteria counting using DAPI

18 ml of algal sample was filtered through a 5 µm syringe-filter before fixation in solution with glutaraldehyde (2.5% v/v final concentration). Equipment was sterilized by autoclave pre-analysis, with ethanol and flame during analysis. The method used to stain bacteria was developed by Porter and Feig (1980). Black polycarbonate membrane filters (Millipore, 0.2 µm) were mounted on top of backing filter (Whatman GF/F, 25 mm diam, 0.7 µm) and placed in a glass filter holder (25 mm). A glass funnel covered with black insulating tape was attached with a clamp. Sterile millipore H₂O was rinsed through filters by vacuum filtration to open up the pores. The sample was injected with 4’6-diamidino-2-phenylindole (DAPI) to a final concentration of 2 µg ml⁻¹. Bacteria were stained in darkness for 8 min, followed by cautious vacuum filtration. Moist membrane filters were mounted on glass microscope slides (25 x 75 mm) with a drop of immersion oil and coverslip.

Bacteria were counted with Olympus BX51 epifluorescence microscope (10 x 100 magnification). Three fields of sight were counted with a total of 57 grids per slide. The aim was to count at least 500 bacteria per slide. If needed, this procedure was repeated with different amounts of algal sample until 500 bacteria per sample was reached.

Detection of *Vibrio* bacteria with FISH

Genera specific bacteria were detected with Fluorescence In Situ Hybridization (FISH) of fixed cells on
membrane filters according to Glöckner et al. (1996). An updated methodological protocol is available in Fuchs et al. (2007).

Vacuum filtration was used to attach bacteria onto membrane filters. Equipment was sterilized by autoclave pre-analysis, with ethanol and flame during analysis. Support filters (Whatman GF/F, 25 mm, 0.7 µm) were placed on glass filter holders (25 mm). Black polycarbonate membrane filters (Millipore, 0.2 µm) were mounted on top and glass funnels attached. Sterile millipore H2O was filtered through. Two filters were used per sample and between 10-15 ml were injected on to each filter, depending on the total bacteria count with DAPI. The aim was to inject an equivalent amount that produced 500 bacteria counts per slide with DAPI (see previous section). Fixation was performed in polystyrene Petri dishes (10 cm id) with the membrane filter placed on top of cellulose filter paper moistened with PBS (1xPBS at pH 7.2). Membrane filters were covered with 500 µl ice-cold paraformaldehyde (4% w/v) and incubated at 4°C for 60 min. Fixed samples were placed in centrifuge tubes and stored in -20°C until further analysis.

Probes were selected with the assistance of probeBase (Loy et al. 2007). Specificity was evaluated with probeCheck (Loy et al. 2008). \textit{Vibrio} was matched with G V (5' – AGGCCACAACCTCCAAAGTAG – 3') (Giuliano et al. 1999; Eilers et al. 2000) and total Bacteria with a mixture of EUB338 (5' – GCTGCCCTCCGTAGGAGT - 3') (Amann et al. 1990), EUB338 II (5' – GCAGCCACCCGTAGGTG - 3') (Daims et al. 1999) and EUB338 III (5' – GCTGCCACCGTAGGTG - 3') (Daims et al. 1999). G V was synthesized by ThermoFischer Scientific (Ulm, Germany) and dyed with Cy3 at the 5' end. EUB338-probes were synthesized by Invitrogen Ltd (Paisley, Great Britain) and dyed with Alexa 488 at the 5' end.

Filters were carefully washed with 3x7 ml PBS (1x) to remove paraformaldehyde. Each membrane was cut into 8 pieces, giving 16 sections per sample. Two sections were hybridized with EUB-mix + GV and two sections with only EUB-mix as control. Two sections were unhybridized, acting as control for autofluorescence. All sections were counterstained with DAPI.

Specific hybridization buffer was prepared with 225 µl hybridization stock solution (16 ml 5M NaCl, 1.78 ml 1M Tris, 2.22 ml MQ), X µl formamide to determine stringency (300 µl for G V + EUB-mix, 300 µl for EUB-mix), 1 µl SDS (10%) and X µl sterile MQ depending on probe (252 µl for G V + EUB-mix, 363 µl for EUB-mix). Specific hybridization solution was prepared with X µl of the corresponding specific hybridization buffer (480 µl for EUB-mix, 420 µl for G V + EUB-mix) and 60 µl of each probe working solution (25 ng µl\(^{-1}\) in MQ).

Hybridization chambers were constructed by soaking a piece of blotting paper in buffer solution (1.8 ml 5M NaCl, 0.2 ml 1M Tris, 8 ml MQ) and placing it in a 50 ml polyethylene tube. Chambers were heated to 46°C in water bath. Filter sections were placed on glass well slides (25 x 75 mm) and covered with 85 µl specific hybridization solution. Slides were carefully placed in chambers and incubated in oven at 46°C for 120 min.

Washing solutions were prepared with 1 ml 1M Tris, 1020 µl NaCl, MQ to 50 ml and 50 µl SDS (10%). Washing chambers were then preheated to 48°C in water bath. Hybridized filter sections were quickly rinsed with MQ and placed in washing chambers at 48°C for 15 min. Filter pieces were immersed in MQ and left to dry on blotting paper. Counterstaining was performed by placing filters on glass well slides (25 x 75 mm) and covering them with 50 µl DAPI (1 µg ml\(^{-1}\)). Filter sections were
incubated for 3 min in darkness, washed for 1 min in MQ, 1 min in ethanol (80%) and left to dry on blotting paper in darkness. Samples were then mounted back on well slides with a droplet of Citifluor and coverslip. Slides were stored in -20°C until microscopy.

Analysis of hybridized samples was made on a Zeiss Axiovert 200M epifluorescence microscope equipped with gfp, DAPI and texas red filters. Immersion oil was used and 10 x 100 magnification.

**Fatty acid profiles**

Upon arrival at the laboratory, the samples used for lipid analyses were prepared as follows. Fifty ml of each replicate was concentrated by centrifugation and removal of the supernatant. This was done in five steps on 10 000 rpm/5 min. The cells were then washed with PBS-buffer, resuspended and centrifuged followed by removal of the supernatant. This procedure was repeated six times on 10 000 rpm/5 min. The samples were frozen in -20°C before freeze-drying overnight. Freeze-dried samples were stored in -20°C for a maximum of three months.

A modified version of Bligh and Dyer (1959) was used to extract the lipids. The sample was weighed and transferred to a centrifuge tube followed by extraction with DCM:MeOH:Ammonium acetate-buffer (1:2:0.8 v/v). The solution was sonicated and centrifuged with the remaining supernatant transferred to a separation funnel. This procedure was repeated until the supernatant was colourless. 0.1 mg of internal standard C19:0 was added with DCM:MeOH:H2O to a final relation of 1:1:0.9 (v/v). The DCM phase was collected when separation had occurred and the remaining phase was washed twice with DCM. Extracted lipids were stored in -20°C for a maximum of 72 hours at this stage. The DCM was then evaporated under a stream of nitrogen. Lipids were weighed and stored for up to a month in -80°C.

The method used to derivate total lipids is described in Christie (1989). Lipids were dissolved in DCM after which 150 µl of sample was dried with nitrogen. 100 µl toluene and 200 µl sulfuric acid in methanol (1%) was injected. Samples were then kept at 50°C for 12 hours. 300 µl sodium chloride (5%) was added. Samples were extracted three times with n-hexane and collected in glass vials followed by evaporation. The extracted sample was dissolved in n-hexane and transferred to a glass inlet containing 100 µl injection standard. FAME were separated on a gas chromatograph (HP 3890) with a front inlet and a capillary column (DB-23, 60 m x 0.25 mm; 0.25 µm film thickness). Temperature of the column was programmed from 50 to 175°C at 10°C min⁻¹ and from 175 to 235°C at 4°C min⁻¹. Carrier gas was helium at 20 ml min⁻¹. FAME identification was made by comparing retention times with those from internal standards. C22:0 and C20:5 had overlapping retention times. Fatty acid peaks were manually integrated and analyzed using Enhanced Chemstation (MSD Chemstation D.02.00.275).
**Study 2: Identification of micro-contamination hot spots**

**The Hatchery**

The land-based oyster hatchery OSH has four major intertwined processes (fig. 3). The broodstock consists of adult oysters that are conditioned in order to spawn and release larvae. The nursery is where larvae are kept and reared until settlement. On growth is the third process where oyster spat are reared until they have reached the size when moved out to sea. The green house produces microalgae that are fed to adult oysters, larvae and spat. Microalgae are produced with three different methods. BioFences and BioReactors produce algae continuously, mainly for the broodstock. Batch cultures in polyethylene bags are mainly used for larvae.

All these processes require saltwater. The water intake is situated at 35 m depth in the Koster Fjord. Water is treated in different ways to prevent microbial growth and unwanted particles. The first step is filtration through four filters down to 25 µm. It is then treated with UV-radiation and collected in a cold water tank. From this tank, water is heated, degassed and transferred to another tank. The warm water tank provides water to broodstock, larvae and spat. For larvae and spat, water is filtered down to 5 µm and also UV-filtered. Large-scale cultures of microalgae get water from the cold water tank. This is filtered down to 0.2 µm and sterilized by pasteurization. Water to starter- and stock-cultures is sterilized by autoclave.

![Figure 3 Water treatment and sampling points at OSH](image)

**Sampling points**

Bacteria levels were measured between April – July 2011. Sampling points were chosen in agreement with OSH to identify possible micro-contamination hot spots and to evaluate the effects of water treatment. Sampling was focused on these key areas of the hatchery:

- Water intake before filtration
- Water intake after filtration
- Cold water tank
- Warm water tank
- Nursery water tank
- Bag cultures of microalgae
- Broodstock culture water
- Larval culture water
Bacteria measurements

Preparation of growth medium

Marine agar 2216 E was purchased in powder form (Difco). Powder was dissolved in distilled H₂O according to the manufacturer’s instructions. Agar solution was stirred magnetically and heated until boiling point. It was then sterilized by autoclave at 120°C for 15 min and left to cool to 50°C in a laminar flow cabinet. Bench area was sterilized with ethanol and bottleneck with a bunsen burner. Agar was poured into polystyrene Petri dishes (10 cm id) until bottom was covered. When not in use, the bottle was placed on a hot plate with low heat and gentle magnetic stirring. Plates were covered with lids and allowed to cool for 60 minutes in the laminar flow cabinet. Solidified agar plates were then inverted and left to dry overnight, or until no condensation was visible. Plates were stored in plastic bags at 4°C.

Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar is the classical medium to select for and measure *Vibrio* spp. bacteria. TCBS was purchased in powder form (Difco) and prepared as follows. Powder was dissolved in distilled H₂O according to the manufacturer’s instructions. Agar solution was stirred magnetically and brought to the boiling point three times on a hot plate. It was then left to cool down to 50°C in a laminar flow cabinet. TCBS agar plates were poured and processed in the same manner as described above for marine agar plates.

Sampling, dilution and plate spreading

From each sampling point, 10 ml was transferred to a sterile polypropylene centrifuge tube. Dilutions were made within 15 min of sampling. The number of dilutions made differed between samples, depending on the expected bacterial concentration at that point. Dilutions were made in a laminar flow cabinet sterilized with ethanol. Oceanic saltwater (approx. 32 ppt) from the hatchery was used. This was particle filtered down to 0.2 µm and autoclaved at 120°C for 15 min before dilutions were made. The sample was agitated gently but thoroughly before 1 ml was taken out with a sterile pipette. This was injected into 9 ml of sea water in a sterilized polypropylene centrifuge tube. Bottle neck and centrifuge tube neck was quickly heated with a Bunsen burner before injection. This procedure was repeated until the desired number of dilutions was reached.

Plate spreading was made in a laminar flow cabinet within 2 hours of dilution. The procedure was identical for TCBS- and marine agar plates. Centrifuge tubes were agitated before 0,1 ml was taken with a sterile pipette and injected onto an agar plate. A disposable polystyrene inoculatine loop was used to spread the liquid evenly onto the agar plate. The plate was then inverted and incubated at approximately 20°C for 48 hours. The number of plates differed between samples, depending on the expected growth from that point. Plates were not replicated.

Counting of colony forming units

Plates were evaluated and counted after approximately 48 hours of incubation. The plate with colony
forming units (CFU) in the range of 20-100 was counted and chosen to represent that sampling point and agar type. If no plate was within that range, the two neighboring plates were counted. Once counted, bacteria plates were destroyed in autoclave.

**Data analysis**

For statistical analyses and to obtain a more uniform distribution of values for linear regression, bacterial concentration values were logarithmically transformed with 10 as base, giving the formula: $\log_{10}(x+1)$. Addition of one was made to account for zero-values. Statistical tests were performed with IBM SPSS Statistics 21.

**Study 1**

**Bacteria**

Bacterial data from trial cultures were compared between diatom species as the percentage change from day 3 to day 7 in each replicate. A one-way ANOVA was used with the **percentage change** in logarithmically transformed bacteria concentrations as dependent and **algae specie** as factor. Tukey HSD was used for multiple comparisons. Homogeneity of variances was confirmed with Levene’s test and normal distribution with Kolmogorov-Smirnov. The bacteria sample from replicate no. 3 of *T. pseudonana* trials day 3 was discarded due to contamination (fig. 5).

Linear regression was used to evaluate the dependent variable **bacteria concentration** with predictors **time** and **algae concentration**. All observations were assumed to be independent in the data set.

Statistical analysis of **Vibrio** detection was not performed since no reliable data could be extracted.

**Fatty acids**

Statistical tests were performed on polyunsaturated FA (PUFA) known to be important nutritional constituents for the growth and survival of *O. edulis* larvae (See the introduction and fig. 7). CCAP diatoms were excluded from statistical tests since they were not replicated. The obtained data of individual fatty acids was in relative numbers to total FA (TFA). Normal distribution was confirmed with Kolmogorov-Smirnov. Homogeneity of variances could not be confirmed for all fatty acids with Levene’s test. Differences between species in selected PUFA were tested with Mann-Whitney U in a pairwise manner. Sample size was assumed to be adequate and therefore asymptotic significance was used.
Study 2

Larvae

Chi-square with Yates’ correction was used to test the hypothesis that larval survivability could be predicted by detection of *Vibrio* on TCBS agar. Yates’ correction was used to compensate for the scarce replicates of larval batches that reached settling. Bacteria samples from the first week after larval release were used. The data was trimmed to only use the most recent sample from first week if a batch was measured repeatedly. Data was grouped into “*Vibrio*” or “no *Vibrio*” and “reached settling” or “moribound”.

Microalgae

Mean bacteria concentrations in microalgal bag cultures were statistically compared between *C. muelleri*, *P. lutheri* and *Isochrysis* sp. (clone T-Iso). Normal distribution was confirmed with Kolmogorov-Smirnov. Homogeneity of variances could not be confirmed with Levene’s test. Mann-Whitney was used to compare differences. Sample size was assumed to be adequate and therefore asymptotic significance was used.

Watersystem

Effects of water treatment to bacteria concentrations were analyzed with paired t-tests. Intake water was paired with filtered intake water, filtered intake water with cold water tank, cold water tank with warm water tank and warm water tank with nursery water tank (fig. 3). Each sampling occasion was viewed as a replicate and as being separate from the next, since water flow in the system would have changed the present water between samplings. Mean differences were assumed to be normally distributed.
Results

Study 1: The Diatom evaluation

Algae counts

Trial cultures of *C. muelleri* demonstrated a positive growth while trial cultures of *C. calcitrans* and *T. pseudonana* presented negative growth (fig. 4). The same pattern was visible in the upscaling of culture volume from stock- to starter culture (fig. 13). No statistical analysis of algal growth rate was performed since trial cultures of *C. calcitrans* and *T. pseudonana* crashed.

Bacteria counts

Bacteria were detected in all stock cultures. The stock culture that contained the highest measured concentration was *C. muelleri* with 4.2 mill. bacteria ml$^{-1}$. *C. muelleri* also measured the highest bacteria level out of the starter cultures with 9.2 mill. bacteria ml$^{-1}$. Starter culture for *T. pseudonana* contained 1.1 mill. bacteria ml$^{-1}$ while *C. calcitrans* measured to 0.2 mill. bacteria ml$^{-1}$.

Bacteria concentrations in trial cultures at day 3 (fig. 5) demonstrated a similar cross species pattern to bacteria levels of the starter cultures.

Mean percent changes from day 3 to 7 in logarithmically transformed bacteria concentrations were not significantly separated between algae groups ANOVA, $p=0.054$. Post Hoc tests showed that percent changes were significantly higher in *C. calcitrans* compared to *C. muelleri*, Tukey HSD, $p=0.047$. Difference between *C. calcitrans* and *T. pseudonana* was not significant Tukey HSD, $p=0.408$. Neither was the difference between *C. muelleri* and *T. pseudonana*, Tukey HSD, $p=0.341$. 

\[\text{Figure 4 Boxplot of algae concentration in trial cultures at day 3 and 7}\]

\[\text{Figure 5 Boxplot of bacteria concentration in trial cultures at day 3 & 7}\]
Scatter plot of bacteria-against algae concentration (fig. 6) visualized the relationship between the two in trial cultures. Linear regression revealed no significant relations of bacteria concentrations with algae concentrations and time of sampling (table 1). Standardized coefficients indicated that time of sampling was a better predictor than algae concentration for all species: Beta 0,846 vs. –0,067; 2,825 vs. -2,196; 1,102 vs. 0,140 (table 2).

Table 1 ANOVA Linear regression. Bacteria no. as dependent, Algae no. and time as predictors

<table>
<thead>
<tr>
<th>Specie</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. calcitrans</em></td>
<td>Regression</td>
<td>2</td>
<td>1,03</td>
<td>7,24</td>
<td>0,07</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>3</td>
<td>0,14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. muelleri</em></td>
<td>Regression</td>
<td>2</td>
<td>0,05</td>
<td>4,71</td>
<td>0,12</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>3</td>
<td>0,01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. pseudonana</em></td>
<td>Regression</td>
<td>2</td>
<td>0,36</td>
<td>15,61</td>
<td>0,06</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>2</td>
<td>0,02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Coefficients Linear regression. Bacteria no. as dependent, Algae no. and time as predictors

<table>
<thead>
<tr>
<th>Specie</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. calcitrans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>6,05</td>
<td>10,09</td>
<td>0,60</td>
<td>0,59</td>
</tr>
<tr>
<td>Time</td>
<td>0,27</td>
<td>0,24</td>
<td>0,85</td>
<td>1,15</td>
</tr>
<tr>
<td>Algae conc.</td>
<td>-0,14</td>
<td>1,53</td>
<td>-0,07</td>
<td>-0,09</td>
</tr>
<tr>
<td><em>C. muelleri</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>18,99</td>
<td>6,72</td>
<td>2,83</td>
<td>0,07</td>
</tr>
<tr>
<td>Time</td>
<td>0,21</td>
<td>0,09</td>
<td>2,83</td>
<td>2,39</td>
</tr>
<tr>
<td>Algae conc.</td>
<td>-1,20</td>
<td>1,08</td>
<td>-2,20</td>
<td>-1,86</td>
</tr>
<tr>
<td><em>T. pseudonana</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Constant)</td>
<td>4,28</td>
<td>5,42</td>
<td>0,79</td>
<td>0,51</td>
</tr>
<tr>
<td>Time</td>
<td>0,22</td>
<td>0,12</td>
<td>1,10</td>
<td>1,89</td>
</tr>
<tr>
<td>Algae conc.</td>
<td>0,21</td>
<td>0,86</td>
<td>0,14</td>
<td>0,24</td>
</tr>
</tbody>
</table>

Figure 6 Bacteria concentration plotted against algae concentration in trial cultures
Detection of *Vibrio* with FISH

Bacteria samples from stock cultures and trial cultures at day 7 were hybridized. All were positively matched with the G V probe. Enumeration was however not practically viable. Bacteria were heavily clustered and fluorescent signals weak. This could have been due to a defaulted freezer which caused the samples to thaw, probes lacking in signal strength, poor quality of chemicals or repeated error when processing samples. No autofluorescence was observed with DAPI counterstained controls.

Fatty acid profiles

Table 3 Percentage composition of fatty acids in CCAP and CCMP strains of *Chaetoceros calcitrans* (CC), *Chaetoceros muelleri* (CM) and *Thalassiosira pseudonana* (TP). Data on CCMP diatoms from three replicate cultures per specie.

<table>
<thead>
<tr>
<th>FA</th>
<th>CC</th>
<th>CM</th>
<th>TP</th>
<th>CC1</th>
<th>CC2</th>
<th>CC3</th>
<th>CM1</th>
<th>CM2</th>
<th>CM3</th>
<th>TP1</th>
<th>TP2</th>
<th>TP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>0.1</td>
<td>0.2</td>
<td>-</td>
<td>0.0</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C13:0</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14:0</td>
<td>9.1</td>
<td>6.4</td>
<td>11.7</td>
<td>9.5</td>
<td>10.5</td>
<td>11.3</td>
<td>6.4</td>
<td>7.7</td>
<td>6.0</td>
<td>10.9</td>
<td>10.1</td>
<td>12.7</td>
</tr>
<tr>
<td>C14:1n-5</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>C15:0</td>
<td>2.2</td>
<td>1.5</td>
<td>2.1</td>
<td>2.2</td>
<td>2.3</td>
<td>2.3</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>2.1</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>16.5</td>
<td>18.0</td>
<td>13.9</td>
<td>15.2</td>
<td>15.1</td>
<td>14.5</td>
<td>11.8</td>
<td>14.3</td>
<td>14.9</td>
<td>16.6</td>
<td>16.0</td>
<td>13.8</td>
</tr>
<tr>
<td>C16:1n-7</td>
<td>62.4</td>
<td>54.8</td>
<td>48.6</td>
<td>54.0</td>
<td>54.6</td>
<td>52.5</td>
<td>52.7</td>
<td>51.2</td>
<td>49.8</td>
<td>57.1</td>
<td>54.6</td>
<td>53.0</td>
</tr>
<tr>
<td>C17:0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.4</td>
<td>4.3</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>-</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>C18:2n-9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.3</td>
<td>0.7</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>0.2</td>
<td>0.8</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>0.3</td>
<td>-</td>
<td>1.5</td>
<td>1.6</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>-</td>
<td>4.2</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>4.5</td>
<td>6.8</td>
<td>7.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:0</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.0</td>
<td>-</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:1n-9</td>
<td>0.2</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C21:0</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>0.0</td>
<td>0.1</td>
<td>-</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>C20:3n-6</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.5</td>
<td>6.9</td>
<td>7.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:0 + C20:5n-3</td>
<td>5.3</td>
<td>4.2</td>
<td>12.4</td>
<td>10.6</td>
<td>9.8</td>
<td>11.6</td>
<td>4.8</td>
<td>4.6</td>
<td>5.2</td>
<td>7.8</td>
<td>7.9</td>
<td>11.1</td>
</tr>
<tr>
<td>C24:0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>3.2</td>
<td>1.2</td>
<td>10.0</td>
<td>6.3</td>
<td>6.0</td>
<td>6.9</td>
<td>1.7</td>
<td>2.1</td>
<td>2.5</td>
<td>4.2</td>
<td>4.9</td>
<td>6.3</td>
</tr>
</tbody>
</table>

The fatty acid (FA) pool of all samples primarily consisted of the monounsaturated FA C16:1n-7, followed by the saturated FA C16:0 (Table 3). Selected PUFA that were statistically compared are visualized in fig. 7.

Percentage amounts of C18:3n-6 was highest in *C. muelleri* and not detected in *T. pseudonana*. Differences were significantly separated between all species (table 4). C18:3n-3 was detected in small amounts from *C. calcitrans* and from one replicate of *T. pseudonana*. Differences were significantly separated between *C. muelleri* and *C. calcitrans* (table 4). C20:4n-6 was only detected in *C. muelleri*.
and the amount was significantly separated from the other two diatoms (table 4). The pooled fatty acids C22:0 and C20:5n-3 were significantly higher in *C. calcitrans* and *T. pseudonana* compared to *C. muelleri* (table 4). One replicate of *T. pseudonana* resulted in high deviation in the species’ amount of C22:0 and C20:5n-3 (table 3). *C. muelleri* had significantly lower amounts of C22:6n-3 compared to the other species (table 4). Differences in C22:6n-3 between *C. calcitrans* and *T. pseudonana* were not significant *p*=0.127 (table 4).

![Figure 7](image) Levels of selected PUFA in relation to total fatty acids (TFA) of CCMP diatoms

**Table 4** Mann-Whitney tests of selected fatty acids. CC, *C. calcitrans*; CM, *C. muelleri*; TP; *T. pseudonana*.

<table>
<thead>
<tr>
<th>Group</th>
<th>C18:3n-6</th>
<th>C18:3n-3</th>
<th>C20:4n-6</th>
<th>C22:0 + C20:5n-3</th>
<th>C22:6n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CC - CM</strong></td>
<td>Mann-Whitney U</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Wilcoxon W</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>-1.20</td>
<td>-2.09</td>
<td>-2.09</td>
<td>-1.96</td>
</tr>
<tr>
<td></td>
<td>Asymp. Sig. (2-tailed)</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>CC - TP</strong></td>
<td>Mann-Whitney U</td>
<td>0</td>
<td>1</td>
<td>4.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Wilcoxon W</td>
<td>6</td>
<td>7</td>
<td>10.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>-2.12</td>
<td>-1.55</td>
<td>0</td>
<td>-1.09</td>
</tr>
<tr>
<td></td>
<td>Asymp. Sig. (2-tailed)</td>
<td>0.03</td>
<td>0.12</td>
<td>1</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>CM - TP</strong></td>
<td>Mann-Whitney U</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Wilcoxon W</td>
<td>6</td>
<td>9</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>-2.09</td>
<td>-1</td>
<td>-2.09</td>
<td>-1.96</td>
</tr>
<tr>
<td></td>
<td>Asymp. Sig. (2-tailed)</td>
<td>0.04</td>
<td>0.32</td>
<td>0.04</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Study 2: Identification of micro-contamination hot spots

Larvae and broodstock

Mean bacteria levels in larval tanks and broodstock tanks are visualized in fig. 8. Presumptive Vibrio bacteria were detected in every sample of broodstock culture water and more irregular in larval culture water. Bacteria concentrations were frequently higher in larval tanks compared to broodstock tanks (fig. 9).

![Figure 8](image_url)

**Figure 8** Mean bacteria concentrations (cfu ml⁻¹) in broodstock- and larval culture water at OSH.

Chi-square test to determine if larval outcome could be predicted by detection of Vibrio on TCBS agar (fig. 8) was not significant (Yates’ chi-square=0.943, p=0.332).

Microalgae

Bacteria were not detected on TCBS plates from P. lutheri and C. muelleri (fig. 10). Presumptive Vibrio counts were significantly separated between T-iso and both P. lutheri and C. muelleri (Mann-Whitney, IT-CM p=0.020; IT-PL p = 0.025). Bacteria counts from marine agar plates were significantly separated between C. muelleri and the other two algae but not between P. lutheri and T-iso (Mann-Whitney, CM-IT p=0.007; CM-PL p=0.000; PL-IT

![Figure 10](image_url)

**Figure 10** Measured bacteria concentrations (cfu ml⁻¹) in microalgal bag cultures at OSH. CM, Chaetoceros muelleri; PL, Pavlova lutheri; IT, Isochrysis sp. (clone T-iso).
p=0.080). Noticable is the variability between species in std. deviation of mean marine agar bacteria counts. Std. deviation was lowest in *P. lutheri* with 9 539, followed by T-iso with 217 229 and highest in *C. muelleri* with 2 084 126 (fig. 10).

**Water system**

Throughout the season, intake water contained on average $7.6 \times 10^4$ cfu ml$^{-1}$ less bacteria after filtration. UV-treated water in the cold tank contained on average $1.5 \times 10^3$ cfu ml$^{-1}$ fewer bacteria than filtered intake water. Heated water in the warm water tank had on average $1.0 \times 10^3$ cfu ml$^{-1}$ more bacteria than cold water. Nursery water tank contained more bacteria than warm water tank with $3.2 \times 10^3$ more cfu ml$^{-1}$ on average. Differences were significantly separated from 0 between filtered intake water and UV-treated cold water $p=0.001$ (table 5). Standard deviation was substantial in bacteria no. from unfiltered intake water ($1.8 \times 10^5$) and differences to filtered intake water were inconsistent (fig. 11).
Table 5 T-test of paired bacteria samples from water system. Cultured on marine agar. Logarithmically transformed concentrations. BF, before filtration; AF, after filtration.

<table>
<thead>
<tr>
<th>Paired Differences</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval of the Difference</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1 Intake BF - Intake AF</td>
<td>-1.18</td>
<td>1.11</td>
<td>.31</td>
<td>-.49</td>
<td>.85</td>
</tr>
<tr>
<td>Pair 2 Intake AF - Cold tank</td>
<td>1.58</td>
<td>1.21</td>
<td>.34</td>
<td>.85</td>
<td>2.31</td>
</tr>
</tbody>
</table>

Throughout the season, intake water contained on average $2.0 \times 10^2$ more cfu ml$^{-1}$ of presumptive *Vibrio* bacteria after filtration. Presumptive *Vibrio* bacteria were not detected on TCBS plates from cold water tank, warm water tank or from nursery water tank (fig. 11). Differences in presumptive *Vibrio* levels were significant between intake water and cold water tank throughout the season $p=0.001$ (table 6).

Table 6 T-test of paired bacteria samples from water system. Cultured on TCBS agar. Logarithmically transformed concentrations. BF, before filtration; AF, after filtration.

<table>
<thead>
<tr>
<th>Paired Differences</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval of the Difference</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1 Intake BF - Intake AF</td>
<td>-1.18</td>
<td>1.11</td>
<td>.31</td>
<td>-.49</td>
<td>.85</td>
</tr>
<tr>
<td>Pair 2 Intake AF - Cold tank</td>
<td>1.58</td>
<td>1.21</td>
<td>.34</td>
<td>.85</td>
<td>2.31</td>
</tr>
</tbody>
</table>

**Discussion**

The diatom evaluation experiment was hindered by crashes in trial cultures of *T. pseudonana* and *C. calcitrans*. Since new strains were brought in for the second trial run, bacterial data and nutritional data are discussed separately.

The present data could not find bacteria as causal agents of the microalgae crashes. Bacteria concentrations increased from day 3 to day 7 (fig. 5), but whether bacteria in these cultures were pathogenic or opportunistic remains to be seen. Linear regression of bacteria concentrations with algae concentrations and time did not demonstrate any clear relationship. Isolation of bacterial cells and challenging axenic diatoms with cultures of these bacteria may reveal more in future studies.

Bacteria levels in all trial cultures were substantial at day 7 (when normally harvested as larval feed) and well above the chosen reference value of $2.0 \times 10^6$ ml$^{-1}$. Post Hoc tests demonstrated a more
stable bacteria growth in trial cultures with *C. muelleri* compared to *C. calcitrans*. For a hatchery where bacterial spikes are undesirable, microalgae with a stable bacteria growth should be favored as larval feed. In that context, *C. muelleri* would be best suited as larval feed out of the CCAP diatoms. Noteworthy is that the measured bacteria concentrations from stock cultures at OSH were substantial (fig. 14) and it is conceivable that axenic stock cultures would have resulted in reduced bacteria levels in bag cultures. Bacterial samples for the detection of *Vibrio* with FISH were believed to be damaged and the results are therefore not viewed as reliable.

The FA C22:0 has in previous analyses not been detected in significant amounts from FA composition of *C. muelleri*, *C. calcitrans* or *T. pseudonana* (Thompson et al. 1990; Thompson et al. 1992). Present results of C22:0 + C20:5n-3 are therefore viewed as results of C20:5n-3 only. Table 7 demonstrates the variability in EPA and DHA proportion of examined diatoms. The compared values from Thompson et al. (1992) are from cultures at two different temperatures, which had a marked effect on FA composition. The results are not directly comparable with results of the present thesis since Thompson and colleagues used light intensities of 220 µmol. photons m⁻² s⁻¹ while OSH used app. 85 µmol. photons m⁻² s⁻¹. The comparison does however highlight what impacts the culture environment can have on FA composition. DHA levels in the examined diatoms were surprisingly high which could very well be explained by the specific culture environment at OSH.

Table 7 Comparison of EPA and DHA levels (% of TFA) in the present study (mean values) with redrawn levels from Thompson et al. (1992)

<table>
<thead>
<tr>
<th></th>
<th>Present study 17,5°C</th>
<th>Comparison</th>
<th>Present study 20°C</th>
<th>Comparison</th>
<th>Present study 17,5°C</th>
<th>Comparison</th>
<th>Present study 20°C</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA C20:5 n-3</td>
<td>10,6</td>
<td>15,7</td>
<td>10,5</td>
<td>4,9</td>
<td>11,6</td>
<td>5,8</td>
<td>8,9</td>
<td>13,7</td>
</tr>
<tr>
<td>DHA C22:6 n-3</td>
<td>6,4</td>
<td>1,5</td>
<td>0,7</td>
<td>2,1</td>
<td>1,5</td>
<td>0,5</td>
<td>5,1</td>
<td>3,2</td>
</tr>
</tbody>
</table>

In the present study, *C. muelleri* contained significantly lower relative amounts of the two fatty acids EPA and DHA, which have previously been identified as the most important FA for *O. edulis* larval development (Langdon and Waldock 1981; Webb and Chu 1983; Enright et al. 1986). On the other hand, *C. muelleri* contained higher relative amounts of ω-6 FA compared to *C. calcitrans* and *T. pseudonana* (table 3). Choosing one diatom out of these three as oyster larval feed based on nutritional value would consequently be a trade-off between ω-6 rich *C. muelleri* and abundance of EPA and DHA in *C. calcitrans* and *T. pseudonana*. Nutritional analyses of flagellate microalgae used at OSH would aid in the matching and electing of a diatom for the larval feeding mix. High levels of EPA and DHA with a ratio of ω-6 : ω-3 < 0.5 would be advisable according to the literature cited in the introduction. Another trade-off would be between nutritional value and the cultivability. CCAP as well as CCMP strains of *C. calcitrans* and *T. pseudonana* proved difficult to cultivate in large scale at OSH. That problem would have to be addressed if choosing one of these as larval feed.

Bacteria levels measured in the 2nd study of this thesis contained some interesting observations. Water treatment at OSH resulted in significantly reduced abundance of bacteria in the cold water tank compared to filtered intake water (table 5). Presumptive *Vibrio* abundance was also significantly
reduced in the cold water tank (table 6) and in this case to zero. Presumptive *Vibrio* spp. were neither found in the warm water tank or nursery tank (fig. 11). It seems likely that UV-treatment is effective in removing *Vibrio* bacteria from production water. Filtering alone did not significantly reduce bacteria levels, which is not surprising given that the minimum mesh size before UV-treatment is 25 µm. Overall the production water used at OSH seems to hold acceptable bacteria levels.

Sampled cultures of microalgae demonstrated that mean bacteria abundance in *C. muelleri* was significantly higher than in *P. lutheri* and T-iso. Standard deviation was remarkably low in *P. lutheri*, indicating a stable bacteria composition in the cultures of this specie. Presumptive *Vibrio* spp. were detected in cultures of T-iso on several occasions (fig. 10). Since no presumptive *Vibrio* was found in *C. muelleri, P. lutheri* or in the production water, it seems likely that the source of *Vibrio* found in T-iso was the stock culture of this algae. Regular sampling of microalgal stock cultures for *Vibrio* would be advisable.

*Vibrio* spp. appear to be inhibited by the UV-treatment of production water at OSH. Results point towards introduction of *Vibrio* from biological sources at OSH, i.e. the broodstock and cultures of T-iso (fig. 12). If the broodstock could be treated to prevent *Vibrio* spp. growth and if microalgal cultures could be kept *Vibrio*-free, then larvae would be less likely to be subjected to these bacteria according to the results.
Treatment of *Vibrio* infected cultures with metalloprotease inhibitors such as EDTA and Ovoglobulins have previously been successful at improving survival of *C. gigas* larvae (Takahashi et al. 2000; Hasegawa et al. 2008; Hasegawa et al. 2009). It is suggested that research in this area on *O. edulis* larvae might lead to fruitful results.

In conclusion the present data does not determine any cause–effect relations of the feed quality and bacteria levels to the survival of *O. edulis* larvae. The bacteria found at OSH could be pathogens or they could be opportunists. The data does however allow some interpretations to be made. *Vibrio* bacteria are present in larval cultures at OSH and they seem to emerge from the broodstock and microalgal stock cultures. Treatment of production water at OSH removes *Vibrio* spp. Feed quality of *C. calcitrans*, *C. muelleri* and *T. pseudonana* in terms of EPA could be enhanced. DHA levels were surprisingly high in these diatom cultures. Microalgal stock cultures were contaminated and it seems likely that an axenic state of the stock would have improved feed quality in terms of bacterial numbers.

**Acknowledgements**

Research for this thesis was conducted based on commercial production processes at the hatchery *Ostrea Sverige AB* in Stromstad, Sweden, where this study was part of a project to optimize feed quality and implement bacteriological monitoring (VINNOVA Dnr 2010-02193). Extensive research support was also received from EU Interreg IV A Kattegat-Skagerrak by the project Nord-Ostron.

I want to thank all those who have helped me finalize this thesis and particularly Susanne Lindegarth, Alyssa Joyce, Anders Karlsson, Kent Berntsson and everyone at Ostrea, Swantje Enge, Göran Nylund, Gunnar Cervin, Frank Persson, Robert Almstrand, Ann-Sofi Rehnstam-Holm, Malin Strand, Ricardo Pereyra and Elin Renborg.

**References**

Amann, R. I., et al. (1990), 'Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations', *Applied and environmental microbiology*, 56 (6), 1919-25.


Daims, H., et al. (1999), 'The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set', *Syst Appl Microbiol*, 22 (3), 434-44.


Garland, CD, et al. (1986), 'Effects of 0.2mm membrane-filtered seawater as a culture medium on fertilized eggs and larvae of the pacific oyster, Crassostrea gigas', *Marine and Freshwater Research*, 37 (6), 713-20.


Hasegawa, H., et al. (2008), 'The extracellular metalloprotease of Vibrio tubiashii is a major virulence factor for pacific oyster (Crassostrea gigas) larvae', *Appl Environ Microbiol*, 74 (13), 4101-10.


Jonsson, P. R., et al. (1999), 'Larval growth and settlement of the European oyster (Ostrea edulis) as a function of food duality measured as fatty acid composition', *Marine Biology*, 134 (3), 559-70.


Ley, J. E., et al. (1986), *Manual of hygiene for shellfish hatcheries* (Dept. of Agricultural Science, University of Tasmania, Hobart).

Ley, J. E., et al. (1988), 'The use of 0.2-μm membrane-filtered seawater for improved control of bacterial levels in microalgal cultures fed to larval Pacific oysters (Crassostrea gigas)', *Aquaculture*, 69 (3–4), 241-51.


Murchelano, R. A., Brown, C., and Bishop, J. (1975), 'Quantitative and Qualitative Studies of Bacteria Isolated from Sea Water Used in the Laboratory Culture of the American Oyster, Crassostrea virginica', *Journal of the Fisheries Research Board of Canada*, 32 (6), 739-45.


Paillard, Christine, Le Roux, Frédérique, and Borrego, Juan J. (2004), 'Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution', *Aquatic Living Resources*, 17 (04), 477-98.

Pernet, Fabrice, et al. (2003), 'Variation of lipid class and fatty acid composition of Chaetoceros muelleri and Isochrysis sp. grown in a semicontinuous system', *Aquaculture*, 221 (1–4), 393-406.


Romalde, J. L. and Barja, J. L. (2010), 'Bacteria in molluscs: good and bad guys', in A. Méndez-Vilas (ed.), *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* (1; Badajoz, Spain: Formatex), 136-47.


Walne, P. R. (1958), 'The Importance of Bacteria in Laboratory Experiments on Rearing the Larvae of Ostrea Edulis (L.)', *Journal of the Marine Biological Association of the United Kingdom*, 37 (02), 415-25.


**Appendix**

*Figure 13* Algae concentration in stock- and starter cultures from study 1

*Figure 14* Bacteria concentration in stock- and starter cultures from study 1