

Thesis for the degree of Doctor of Philosophy in Marine Ecology

Ecological aspects of marine *Vibrio* bacteria

Exploring relationships to other organisms and a
changing environment

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Cover photographs by Maria E. Asplund. Upper left: water sampling on the Indian coast; upper right: bubbles in the water-column; down left: sediment bottom in the Gullmar Fjord, Sweden; down right: blue mussels (*Mytilus edulus*) in the Gullmar Fjord, Sweden

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Abstract

Heterotrophic bacteria of the genus *Vibrio* are indigenous in the marine environment although environmental cues regulate their growth and distribution. The attention brought to this genus is due to its many species/strains that are pathogenic to humans and other organisms. *Vibrio* abundances are strongly coupled to water temperature and salinity but abundance dynamics occur even where these hydrographical parameters are stable. In this thesis, I have studied *Vibrio* dynamics in relation to other organisms such as phytoplankton (papers I, II and III) and a bivalve host-organism (paper IV) in a changing environment where increasing temperature (paper III) and ocean acidification (paper IV) may influence survival and proliferation of these bacteria. In particular paper I showed that in a tropical coastal area, where the water temperature and salinity were stable across seasons, abundances of *Vibrio* were tightly coupled to phytoplankton biomass and community composition. A diatom bloom during December seemed to support high numbers of vibrios in waters with otherwise low levels of dissolved organic carbon. Paper II further supports that some phytoplankton can favor *Vibrio* growth while others seem to have a negative influence on *Vibrio* abundances. For instance, *Skeletonema tropicum*, a common diatom in Indian coastal waters, easily eradicated *Vibrio parahaemolyticus* from sea water in our experiments. In temperate marine areas culturable *Vibrio* predominantly occurs in the water column during the warmer months. Sediments are suggested to be potential reservoirs when conditions in the water-column are harsh. Accordingly, in paper III we showed that cold-water sediments from geographically separate areas in a boreal region of Scandinavia all contained relative high abundances of total *Vibrio* spp. and that all sediments also included culturable *Vibrio*. In agreement with paper I, the fresh input of organic material from phytoplankton blooms, for which chlorophyll *a* was used as a proxy, seemed to positively influence *Vibrio* abundances also in the sediments (paper III). Therefore, the pelagic-benthic coupling which can supply the sediments with biomass from the primary production could influence the abundance of *Vibrio* spp. Increasing temperature had variable influence on sediment-associated *Vibrio* abundance, with a significant increase in abundances in sediments originating from one area when the temperature reached over 21°C and a generally negative influence of increasing temperature on abundances in sediments originating from another area (paper III). This suggests that the sediments contained different *Vibrio* communities with varying temperature tolerance traits. Rising levels of carbon dioxide in the atmosphere does not only lead to higher water temperature through the green house effect, but also to acidification of the oceans. Paper IV illustrated how a common bivalve pathogen, *Vibrio tubiashii*, can be favored in the interaction with a calcifying bivalve host, *Mytilus edulis*, when this host-pathogen combination was exposed to levels of ocean acidification projected to occur by the end of the 21st century. Thus, global environmental changes may enhance the probability of *Vibrio* infections in higher organisms.

Keywords: *Vibrio* spp. / Phytoplankton / Organic material / Sediments / Increasing temperatures / Ocean acidification / Bivalves / Host-pathogen interactions.

Populärvetenskaplig sammanfattning

Vibrio är ett släkte av bakterier som förekommer naturligt i den marina miljön. De kan antingen simma fritt i vattenmassan, befinna sig i sedimentet, sätta sig på någon yta eller leva anknutna till någon annan organism. De allra flesta av dessa bakterier är ofarliga för andra organismer och bidrar främst till nedbrytningen av organiskt material i havet men det finns också inom detta släkte en rad arter/stammar som är skadliga (patogena) för andra marina organismer och även för människor. I Indien orsakar *Vibrio* ca 25% av alla diarrésjukdomar och de är allra vanligast i kustområden. Den mest ökända bland *Vibrio*-stammarna är de som orsakar diarrésjukdomen kolera (*Vibrio cholerae*) som har härjat i många delar av världen och orsakat minst 7 världsomspännande pandemier. I Sverige finns historiskt sett spår av kolerans framfart i form av kolera-kyrkogårdar från återkommande epidemier under 1800-talet. Kolera-bakterien förekommer främst i brackvattensområden medan andra mänskliga patogener så som *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus* är marina arter som människan kan få i sig via fisk och skaldjur eller genom att bada i vatten med höga koncentrationer av dessa bakterier. De kan orsaka blodförgiftningar, sårinfektioner, öroninfektioner och magåkommor som alla kan ha dödlig utgång framförallt hos personer med nedsatt immunförsvar. Under sommaren 2006 rapporterades från sydöstra Sverige flera fall av *Vibrio*-infektioner hos personer som hade exponerats för Östersjövatten. Andra arter/stammar är kända ostron- och musselpatogener (t.ex. *Vibrio tubiashii* och *Vibrio tapetis*), några är kända fiskpatogener (t.ex. *Vibrio anguillarum*) och några kan bidra till blekning av korallrev (t.ex. *Vibrio corallinolyticus* och *Vibrio shiloi*). På grund av att släktet *Vibrio* rymmer alla dessa patogener är det av särskilt intresse att studera vad som styr hur många de är, vilka arter som förekommer och hur skadliga dessa är i sin naturliga miljö. Det övergripande syftet med den här avhandlingen har varit att studera relationen till andra vanligt förekommande organismer och om dessa påverkar förekomsten av *Vibrio* i havet samt hur förändringar i miljön skulle kunna påverka hur framgångsrika dessa bakterier är. Avhandlingens olika delstudier utgör tillsammans en liten pusselbit för att bättre förstå de komplexa sambanden kring vad som styr förekomsten och mängden av marina *Vibrio* i deras naturliga miljö.

De flesta *Vibrio* trivs bäst i vattentemperaturer över 17°C, och därför är de vanliga i tropiska områden. I tempererade havsområden är *Vibrio* vanligast förekommande i vattenmassorna under sommarmånaderna. Sedan tidigare vet

man att förekomsten av de flesta *Vibrio* ökar när vattentemperaturen ökar, men det är också många andra faktorer som också kan påverka deras förekomst. Till exempel vet vi att *Vibrio* växelverkar med andra organismer i havet. Bland annat kan de använda sig av de kolföreningar som växtplankton producerar och *Vibrio* producerar också enzym som kan bryta ner djurplanktons yttre skelett för att utnyttja kolet däri för sin energiförsörjning. I delstudie I visade vi att i ett tropiskt kustnära havsområde, där temperaturen och salthalten var stabila under olika säsonger, kunde antalet *Vibrio* länkas till växtplanktonsamhällets sammansättning. Vidare visade vi experimentellt i delstudie II att vissa arter av de växtplankton som återfanns i detta område kan gynna tillväxten av *Vibrio* medan andra arter verkade vara giftiga för *Vibrio*. I vårt försök eliminerade de bakterierna helt i vattnet. Om man bättre kan förstå sambanden mellan *Vibrio* och resten av planktonsamhället och inverkan av andra miljöfaktorer skulle man kunna utnyttja den etablerade planktonövervakningen som redan idag är vanlig i många länder för att förutspå när det föreligger risk för högre tätheter av *Vibrio* i vattenmassan. Sådan information skulle kunna användas för att utveckla varningssystem för när det är potentiellt ökad infektionrisk vid bad eller vid intag av fisk och skaldjur.

Tempererade havsområden utgör en relativt sträng miljö för *Vibrio* under den kallare delen av året och det är då särskilt svårt att återfinna odlingsbara *Vibrio* i vattenmassan. Under sådana perioder då den omgivande miljön är ogynnsam för *Vibrio* så kan de övergå i ett stadie där de fortfarande är levande (viabla) men minskar sin aktivitet. Då är de inte odlingsbara på de odlingsmedium som finns tillgängliga på marknaden idag. Däremot kan de fortfarande övergå till väldigt potenta patogener om omgivningen i ett senare skede förändras och blir mer gynnsam. Vibrios kan också ta sin tillflykt till reservoarer, t ex olika typer av värdorganismer eller sediment. I delstudie III visade vi att sediment, insamlade från geografiskt vitt skilda områden längs den skandinaviska västkusten då vattentemperaturen var under 10°C, alla innehöll relativt höga halter av *Vibrio* som också visade sig vara odlingsbara. Dessa sediment innehöll varierande mängder organiskt material. *Vibrio* är beroende av organiskt material för sin energiförsörjning. Det visade sig dock i delstudie III att också kvalitén av organiskt material var av betydelse för mängden *Vibrio* i sedimentet. I likhet med delstudie I som fokuserade på vattenmassan så kunde vi här se att *Vibrio* gynnas av algiomassan även i sedimenten, här grovt mätt som klorofyll *a*. Däremot verkar det inte som att den totala mängden organiskt material eller phaeopigment som är en nedbrytningsprodukt av klorofyll *a* har samma betydelse. Således drog vi slutsatsen att fräscht tillskott av organiskt material

från algbloomingar kan vara av stor betydelse för den totala mängden *Vibrio* både i vattenmassorna (delstudie I) och i sedimenten (delstudie III). Vidare visade vi i delstudie III att en ökad temperatur i sediment inte var odelat positivt för den totala mängden *Vibrio* i sedimenten. Troligen beror detta på att olika sediment innehåller olika *Vibrio*-samhällen varav vissa eventuellt endast kan tillväxa vid lägre temperaturer. Sammansättningen av de odlingsbara bakterierna i olika sediment i delstudie III stödde också hypotesen att *Vibrio*-samhället skiljer sig mellan områden.

Den ökande mängden koldioxid i atmosfären som beror på människans användning av fossila bränslen har oönskade effekter på vårt klimat. Detta gör inte enbart att vattentemperaturen ökar utan också att en större mängd koldioxid löser sig i havet, vilket i sin tur leder till havsförurning. Vi kan redan idag se att pH-värdet i havet har sjunkit med 0.1 enheter i jämförelse med nivån före den industriella revolutionen och man räknar med att pH kommer att sjunka med ytterligare 0.4 enheter fram till nästa sekelskifte (2100). Detta motsvarar en ökning på 150% i antalet vätejoner i havet. Havsförurningen kommer att påverka hela den kemiska strukturen i havsvattnet, vilket i sin tur kommer att påverka många av de organismer som lever där. Det finns vissa indikationer på att växtplankton eventuellt kan gynnas av den ökande mängden löst koldioxid medan andra organismer som är beroende av kalk för uppbyggnad av skal- och skelettdelar kan komma att missgynnas av förurningen eftersom mängden löst karbonat minskar i vattnet. Det är ännu oklart hur bakterier i havet påverkas av havsförurningen men det finns tecken på att vissa av dessa patogener kan vara mer skadliga när de befinner sig i en surare miljö. Det finns också forskning som visat på att de bakterier i havet som måste utnyttja organiska kolföreningar för sin energiförsörjning generellt producerar mera enzymer som kan bryta ner proteiner och sockerföreningar vid lägre pH. Dessa enzymer kan också vara kopplade till hur skadliga bakterierna är för andra organismer. I delstudie IV har vi studerat hur samspelet mellan en musselpatogen (*V. tubiashii*) och en kalkbildande värdorganism, den vanliga blåmusslan (*Mytilus edulis*), påverkas av havsförurning. Vuxna blåmusslor har ett utvecklat immunförsvar mot patogena bakterier som *V. tubiashii* och det är framförallt larv- och unga stadier som blir infekterade. Delstudie IV visade i enlighet med detta att det krävs mycket höga halter av *V. tubiashii* för att infektera vuxna musslor då de utsätts för den medelnivå av löst koldioxid och pH som finns i havet idag. Dock visade det sig att förmågan hos *V. tubiashii* att infektera blåmusslorna ökade när de blev exponerad för de koldioxid-/pH-nivåer som är beräknade för slutet av detta århundrade. Detta trots att varken *V. tubiashii*:s överlevnad, tillväxt och/eller

patogenicitet eller blåmusslornas immunförsvar blev mätbart påverkade av havsförsurningen när de blev studerade var för sig. Om kalkbildande organismer blir mer infektionsbenägna på grund av ett surare hav så kan det få stora konsekvenser för många organismer som utgör livsutrymme och/eller födobas för en rad andra organismer, inklusive människan. Globala klimatförändringar som ger upphov till varmare och surare hav kan således potentiellt även öka risken för *Vibrio*-infektioner hos djur och människor.

Till Martin, familj och vänner

List of papers

- I. **Asplund ME**, Rehnstam-Holm A-S, Atnur V, Raghunath P, Saravanan V, Härnström K, Collin B, Karunasagar I, Godhe A (2011) Water column dynamics of *Vibrio* in relation to phytoplankton community composition and environmental conditions in a tropical coastal area. *Environmental Microbiology* 13:2738–2751*
- II. Olofsson M, **Asplund ME**, Karunasagar I, Godhe A. *Prorocentrum micans* promote and *Skeletonema tropicum* disfavours persistence of the pathogenic bacteria *Vibrio parahaemolyticus* (accepted for publication in *Indian Journal of Marine Sciences*)
- III. **Asplund ME**, Engström P, Granberg M, Norling K, Hernroth B. Are sediments reservoirs for viable *Vibrio* bacteria in cold water marine environments? (manuscript)
- IV. **Asplund ME**, Baden S, Russ S, Ellis RP, Gong N, Hernroth B. Ocean acidification and host-parasite interactions: blue mussels, *Mytilus edulis*, encountering *Vibrio tubiashii* (submitted manuscript)

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Related articles not included in thesis:

Godhe A, **Asplund ME**, Härnström K, Saravanan V, Tyagi A, Karunasagar I (2008) Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Applied and Environmental Microbiology* 74:7174–7182

Peterson O, **Asplund M**, Karunasagar I, Godhe A (2010) Phytoplankton community composition and diversity effects on the growth of marine *Vibrio* bacteria. ICHA14 Conference Proceedings, Crete



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Photo to the left: Fishing
boat sailing into
Mangalore harbor, on the
south-west coast of India
(Photo: Maria E. Asplund)

Introduction

Vibrios are heterotrophic bacteria that naturally occur in marine environments which provide the bacteria with an enormous number of habitats. *Vibrio* can be found in sediment (e.g. Kaneko & Colwell 1973, Vezzulli et al. 2009), produce biofilms on surfaces (Hood & Winter 1997, Grau et al. 2005), be free-swimming in the water-column (McCarter 1999) or be attached to or live associated within other organisms (e.g. Huq et al. 1983, Colwell & Huq 1994, Hood & Winter 1997, Lipp et al. 2002, Pruzzo et al. 2005a). In the marine environment, heterotrophic bacteria, such as members of *Vibrio* spp., are of great importance for the remineralization of organic matter in the sea (Fukami et al. 1985). However, the foremost attention brought to this genus is related to its many pathogenic strains.

The *Vibrio* genus includes more than sixty defined species (Thompson et al. 2005a), whereof at least 12 are human pathogens (Chakraborty et al. 1997) and a number of species that are known to infect marine animals, such as corals, shellfish, shrimp and fish (reviewed by Pruzzo et al. 2005b). The most renowned human pathogen species is *Vibrio cholerae* with over 200 existing serotypes but only two (O1 and O139) are associated with the worldwide epidemics of cholera (Kaper et al. 1995). Since 2005 statistical trends show that the number of cholera cases is steadily increasing. In 2011 there were almost 600 000 reported cases worldwide (WHO 2012); however, true numbers including all unreported cases, most likely exceed these figures by far (Zuckerman et al. 2007). Some of the non-O1/O139 *V. cholerae* serotypes can cause vibriosis and milder forms of gastroenteritis (e.g. Morris 1990, Kaper et al. 1995). In the 1970s, it was still thought that the *V. cholerae* was unable to survive long periods outside its human host but during the 1980s it was concluded that this bacterium was naturally occurring in aquatic environments (reviewed by Colwell 1996). In Asia there are often recurring seasonal outbreaks of cholera. These can originate in several places simultaneously, which indicates that environmental factors trigger these epidemical outbreaks (Kaper et al. 1995).

Within the *Vibrio* genus there are several species that have a high tolerance for different salinity levels (Wright et al. 1996). *Vibrio cholerae* is mainly found in brackish- or limnic waters, while other human pathogens such as *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* are found in coastal marine areas (Urakawa & Rivera 2006). *Vibrio vulnificus* can in some areas commonly be

found in seafood, can cause primary septicemia and wound infections (Oliver & Kaper 2001), which may be lethal to persons with compromised immune systems or liver disorders (Blake et al. 1979). Indeed, *V. vulnificus* is proposed to be responsible for most deaths related to food-associated bacterial infections (Todd 1989). *Vibrio parahaemolyticus* is recognized as one of the most important agents for seafood-associated gastroenteritis and stands for 10% of the Indian outbreaks (Deepanjali et al. 2005). *Vibrio alginolyticus*, *V. vulnificus* and *V. cholerae* are also common causes for otitis and wound infection in persons handling shellfish or being in contact with seawater (Pezzlo et al. 1979, Morris & Black 1985). Many of the human pathogens can also cause infections in marine organisms. For instance, *V. vulnificus* is associated with disease outbreaks in eel farms (Hoi et al. 1998) and *V. alginolyticus* is known to infect fish and shrimp (Lee 1995, Liu & Chen 2004). Other examples of animal pathogens of major importance are those that cause infections in fish, shellfish and shrimp such as *V. anguillarum*, *V. harveyi*, *V. splendidus*, *V. tapetis* and *V. tubiashii* (e.g. Hada et al. 1984, Alvarez et al. 1998, Gomez-Leon et al. 2005, Larsen et al. 2006, Paillard et al. 2006), and therefore pose particular threats to marine aquacultures where animal densities are high. However, most of these *Vibrio* species are not exclusive to one host and may also cause infections in humans. Further, *V. coralliilyticus* and *V. shiloi* can be involved as the causing agents of coral bleaching (Kushmaro et al. 2001, Rosenberg & Falkovitz 2004), which may in turn severely impact reef-building organisms.

The most commonly observed infective pathway for bacteria to humans are through seafood (Thompson et al. 2005b). Especially bivalves and other filter-feeding marine animals can concentrate high numbers of bacteria in their tissues (Murphree & Tamplin 1995, Hernroth et al. 2000, Canesi et al. 2002). During warm periods in temperate waters almost 100% of oysters can carry *V. vulnificus* and/or *V. parahameolyticus*, and an annual study on the southwest coast of India showed that 57% of all oysters contained toxic vibrios (Parvathi et al. 2004). *Vibrio* species have also been reported from shrimp farms (Gopal et al. 2005) and in crayfish, which thus can be potential vectors for transmission of *Vibrio* diseases (Bean et al. 1998). Increasing abundances and distributions of pathogenic *Vibrio* in the marine environment consequently pose a real threat not only to local fisheries and seafood industries but also to the people living in coastal areas and depend on seafood as a vital contribution of proteins to their diet.

In India, *Vibrio* are suggested to give rise to as much as 25% of all diarrheal illnesses (Deepanjali et al. 2005). Despite this fact, surveillance programs of marine vibrios are few. In US *Vibrio*-associated illnesses are estimated to about 8000 cases yearly (Mead et al. 1999). Vibrios also occur in Swedish coastal waters and a study conducted along the Swedish coastline (Eiler et al. 2006) found the highest abundance of *Vibrio* within the Skagerrak area, on the west coast. Cases of *Vibrio* infections with domestic origin in Sweden have been registered during the last 9 years by the Swedish institute for communicable disease control (Fig. 1). The highest numbers, in 2006, coincide with unusually high summer surface water temperatures. From this period Andersson and Ekdahl (2006) also reported wound infections caused by non-O1/non-O139 *V. cholera* in people who had been in contact with seawater outside Blekinge, in southern Sweden. *Vibrios* have further been isolated from mussels and water samples collected in southern Sweden during the summers of 2006, 2008, 2009 and 2010, with highest recorded abundances in 2010 (Collin & Rehnstam-Holm 2011, Rehnstam-Holm & Collin 2011) coinciding with the second peak in domestic *Vibrio* infections (Fig 1).

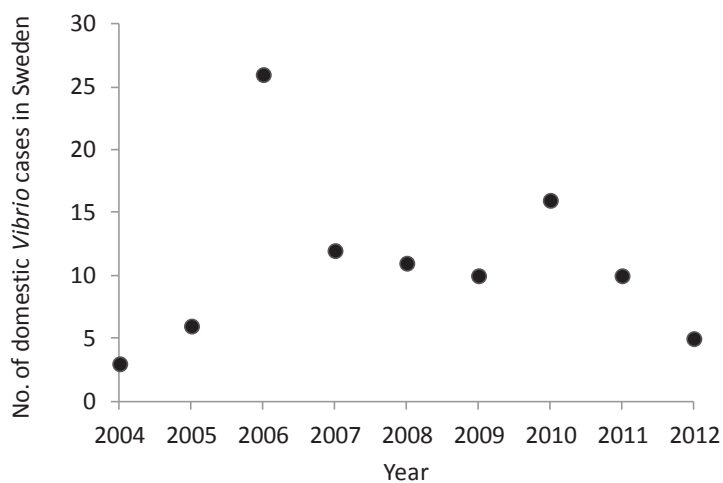


Figure 1. Number of cases of *Vibrio* infections with domestic origin reported in Sweden during 2004 to 2012, excluding cholera-infections (data were extracted from the Swedish institute for communicable disease control, www.smittskyddsinstitutet.se, 2012)

Vibrios are geographically spread all over the world but are more commonly occurring in warmer waters (Wright et al. 1996). In temperate regions abundances of many vibrios can be strongly coupled to water temperature and hence more frequently occurring during the warmer seasons (Oliver et al. 1995, Blackwell & Oliver 2008). Global climate change inducing water temperature increases is suggested to promote proliferation of *Vibrio*, particularly in temperate aquatic regions, and in its footsteps *Vibrio*-associated diseases (Harvell et al. 2002, Baker-Austin et al. 2012, Vezzulli et al. 2012). However, during a large part of the year the water-column is a harsh and too cold environment for many *Vibrio* strains to proliferate in temperate areas, and thus vibrios have to find refuge in suitable reservoirs to survive. Important properties for the survival and propagation of *Vibrio* in harsh environments are their extensive adaptive capabilities. In response to environmental cues, they can for instance alter from free-swimming cells to “swarmer cells” that prosper in more viscous environments such as biofilms (McCarter 1999). During extreme conditions vibrios can switch from an active stage to a dormant, viable but not culturable (VBNC) stage; yet they may still be very potent opportunists if favorable conditions recur (Colwell et al. 1985, Roszak & Colwell 1987, McDougald & Kjelleberg 2006). Although the capability to cause fatal infections is reduced in vibrios in the VBNC stage, these may still account for a small numbers of infections during colder periods in temperate regions (Oliver & Bockian 1995).

In tropical areas, the water temperatures can remain stable over different seasons and therefore be of minor importance for variations in *Vibrio* abundances (e.g. Parvathi et al. 2004, Deepanjali et al. 2005). This suggests that any seasonal patterns in *Vibrio* abundances in such tropical areas must be controlled by other factors. The sources of the organic material that *Vibrio* uses as energy supply may be the factors determining *Vibrio* dynamics. Vibrios display broad metabolic ranges and enzyme activities that enable them to use a wide variety of carbon sources (Thompson & Polz 2006). Other organisms, serving as sources for energy could therefore be important determinants for *Vibrio* outbreaks when large-scale hydrographical parameters fail to explain variability in *Vibrio* abundances. In subtropical areas, such as in Mozambique, the numbers of culturable *Vibrio* coincide with the warmer season which in turn overlap with heavy rains bringing a high load of land run-off into the coastal areas (Collin et al. 2013). It can therefore not be excluded that energy sources supporting vibrios may be of terrestrial origin.

Since the 1980s there is increasing awareness of pathogen *Vibrio* in the marine environment with a growing number of peer-reviewed articles each year focusing on the ecology of these heterotrophic bacteria in their natural habitat (Fig. 2). Knowledge of which biotic factors that promotes proliferation of vibrios in the sea is still fragmented. Particular attention has been devoted to *Vibrio* attachment to and growth in association with zooplankton (e.g. Kaneko & Colwell 1973, Huq et al. 1983, Tamplin et al. 1990, Lobitz et al. 2000, Lipp et al. 2003). *Vibrio* is known to produce chitinases which enable them to use the chitin layer of copepods as energy source but also as protection against predators and the surrounding environment (reviewed by Pruzzo et al. 2008).

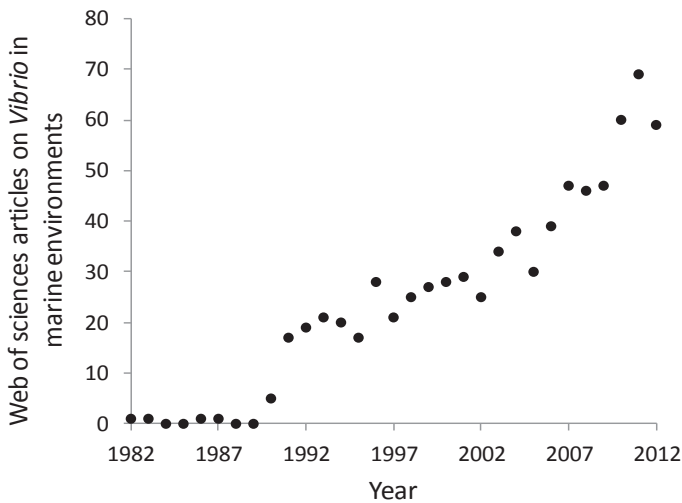


Figure 2. Number of peer-reviewed articles with search words *Vibrio* and marine environments. Data were extracted from the database *Web of sciences expanded* in January 2013).

Phytoplankton blooms may be another important biotic factor to fuelling the proliferation of *Vibrio* in marine environments (Hsieh et al. 2007). Phytoplankton blooms can serve as substrates by providing attachment surfaces and can also sustain bacterial communities by providing dissolved and particulate organic matter through a variety of processes such as excretions, exudation and cell death (Karl 2007). It is suggested that bacteria can sense the release of these organic molecules through chemical gradients and thus be attracted to their origin (Azam & Long 2001). This type of chemotaxis can lead

to the establishment of a microbial community around phytoplankton which Bell and Mitchell (1972) renamed as the “phycosphere”, as an allegory for the terrestrial rhizosphere (microflora surrounding root systems). The phycosphere constitutes an organic-rich microhabitat where heterotrophic bacteria like *Vibrio* could proliferate. During the development of a phytoplankton bloom organic material will undergo significant changes that can support a succession of different bacterial populations (Smith et al 1995). Accordingly, earlier studies have shown that the amount of bacteria increases especially in the decline phase of phytoplankton blooms (Rehnstam et al. 1993, Mourino-Perez et al. 2003). A mesocosm study following the propagation of an algal community and subsequent development of *Vibrio* populations found that the vibrios were clearly associated with and possibly favored by the algal biomass (Rehnstam-Holm et al. 2010). However, Rehnstam-Holm et al. (2010) also discovered that the *Vibrio* growth seemed to be favored by certain phytoplankton diatom species, e.g. *Cosinodiscus* sp., whereas others like *Chaetoceros* sp. appeared to inhibit growth. This indicates that associations between phytoplankton and *Vibrio* can be highly complex and depend on the composition of both communities as well as on interactions with other organisms of different trophic levels that are included in the concurrent plankton-bacteria bloom.

Phytoplankton blooms may also trigger large net downward fluxes of organic material through formation of aggregates, so called “marine snow”, consisting of a variety of polymers and clustered phytoplankton (Azam & Long 2001). As the marine snow sinks through the water column it leaves a plume of dissolved organic material that can be a “hot-spot” for attached and free-swimming bacteria (Azam & Long 2001, Kiorboe & Jackson 2001). A large portion of the bacteria will be consumed in the water column (Jürgens & Massana 2008). Thus, depending on the plankton community structure, the time the bacteria spend on sinking and the turnover rate in the water column some attached bacteria may reach the seafloor. The fate of some of the bacteria that reach the seafloor is to end up in the sediment where they may constitute potent seeding populations. Yet others may be ingested by filter-feeding organisms.

Bivalves, such as mussels, oysters and clams can accumulate high numbers of bacteria and are therefore hosts for many of these bacteria, including potent pathogens (e.g. Hernroth et al. 2002). However, adult specimens of bivalves rarely get infected (Nottage & Birkbeck 1990, Venier et al. 2011) due to well established defense systems against invasive microorganisms. The defense systems of bivalves are composed by several different components. The first line

of defense to fend off outside invaders is the physicochemical barrier of the shell (Canesi et al. 2002, Girón-Pérez 2010), which they can close when sensing virulent bacteria (Collin et al. 2012). Some vibrios seem to inhibit mussel filtration which can be interpreted as that the mussel is selective in its uptake of bacteria (Birkbeck et al. 1987, Hernroth et al. 2000). Further, bivalves have a cellular defense through their phagocytotic hemocytes (Canesi et al. 2002, Girón-Pérez 2010) which are able to kill bacteria in the phagolysosomes with reactive oxygen radicals and degrading enzymes such as lysosyme and antimicrobial peptides (Pipe 1990, Hernroth 2003, Wootton et al. 2003). These peptides can as well be excreted and act in the gill mucus (Mitta et al. 2000). On the other hand, pathogens have an enormous evolutionary potential to avoid the immune defense through frequent mutations, faster generation times and horizontal gene transfer between bacteria. So the balance between host and pathogen in the marine environment is based on the so called arms-race between the two organisms where the host usually is well adapted to their surrounding local micro-flora (Roth et al. 2012). However, host immune defense is energetically costly (Sheldon & Verhulst 1996), and if host and pathogens are exposed to external stressors due to changes in their surroundings, it might shift the balance between these organisms.

Changes in the environment can occur on different spatial and temporal scales. Because of the heterogeneity of sources of organic material in the sea microbes can experience dramatic changes that occur on the 1-mm³ scale and within minutes (Azam & Malfatti 2007). In shallow coastal ecosystems, organisms are subjected to natural variability of numerous factors, such as temperature, salinity, pH, tides, waves, and nutrient availability. Therefore, organisms living in such environments are used to cope with these different changes. However, more long-term exposure to unfavorable conditions may be the tipping-point that some organisms cannot endure. For microorganisms definition of long-term may be a matter of days while for more long-lived organisms this could be seasons or years. Global climate change induced by anthropogenic emissions of carbon dioxide into the atmosphere increases mean temperatures in the oceans, which could have drastic consequences by e.g. favoring proliferation of pathogens (Harvell et al. 2002). Further, the increasing levels of carbon dioxide are bound by the oceans and cause decline in seawater pH (Sabine et al. 2004, Zeebe et al. 2008). During the last 200 years the average pH in the oceans has decreased by approximately 0.1 units (The Royal Society, 2005) and it is predicted to decrease with approximately 0.4 units by the end of the 21th century (Caldeira & Wickett 2003). Ocean acidification, OA, drastically changes the

seawater chemistry shifting the equilibrium towards more dissolved carbon dioxide, hydrogen ions, carbonic acid and bicarbonate, and with a decreasing amount of carbonates due to buffering (Feely et al. 2004, Doney et al. 2009). Recent research has revealed mostly negative effects of future OA on marine biota, although there are inter-specific differences (Kroeker et al. 2010), which may have severe consequences for marine biodiversity (Widdicombe & Spicer 2008). Calcifying organisms, like bivalves, are suggested to be particularly vulnerable (e.g. Orr et al. 2005, Gazeau et al. 2007), while there is so far few data on impacts of OA on heterotrophic bacteria, despite the fact that bacterial physiological processes are influenced by changes in their surrounding, such as changes in external pH levels (Liu et al. 2010). Recent research shows that OA has immune suppressive effects on diverse marine biota (Bibby et al. 2008, Hernroth et al. 2011, Hernroth et al. 2012) and therefore it is essential to study these organisms' interaction with pathogens to more comprehensively understand the consequence of future global changes in the marine environment.

Picture next page: Traditional fishing with Chinese fishnets, Cochin, India (Photo by Maria E. Asplund)



Scope of the thesis

Problem statement and overall aim

Many *vibrios*, which were previously thought to be exclusively communicable between humans or from contaminated water supplies, are since the 1980s known to originate from and therefore also be able to survive and proliferate in aquatic environments. Therefore, there has been an increasing research effort directed towards understanding the ecology of this bacterial genus in its natural environment. However, many knowledge gaps still remain such as where to find the bacteria and what factors that favor their growth and their pathogenicity. Attention has recently been brought to how the effects of global climate change, in terms of increasing sea water temperatures and ocean acidification, affect *Vibrio* in the marine environment and in turn what consequences these potential effects will have for other marine life as well as for humans. This research topic, especially concerning ocean acidification, is still in its infancy. In my thesis, I have in particular focused on the relationship between *Vibrio* and a couple of other organism groups, which can generate essential organic material, i.e. phytoplankton (papers I, II and III), or can be potential hosts or vectors for disease transmission for vibrios, i.e. phytoplankton and bivalves (papers I, II, and IV). Although there are a few studies on *Vibrio*–phytoplankton interactions, this relationship has been generally overlooked in comparison to *Vibrio*–zooplankton interactions. Depending on the level of turnover rate in the water column some of the organic material and associated heterotrophic bacteria may through sinking processes end up in the sediments. In the boreal region of the western Scandinavian coast, facing Skagerrak and the North Sea, there are many enclosed sediment bottoms that receive high degree of organic loading due to net downward fluxes of material from the water column. These bottoms may serve as potential reservoirs for *Vibrio* when the conditions in the water column are harsh. Even though there are regional studies regarding specific *Vibrio* species, no previous investigation in this area has focused specifically on total *Vibrio* abundances in sediments.

Vibrio coupling to bivalves is a well established research area and we know that bivalves through filtering particles from the water can accumulate high numbers of bacterial pathogens without getting infected. However, little is known about how the interaction between host and pathogen, such as bivalves

and *Vibrio*, will be affected in nature by the stress posed on the organisms inflicted by global climate change.

The overall aim of this thesis is to increase the knowledge on how the occurrence, abundances and potential pathogenicity of marine *Vibrio* relate to other organisms and a changing environment. Particular emphasis has been placed on exploring how *Vibrio* abundances relate to phytoplankton community composition and a common invertebrate filter-feeding host-organism under changing environmental conditions. Investigations have been carried out through *in situ* studies and experimental manipulations. Knowledge on how *Vibrio* relate to other organisms from different trophic levels under changing conditions can be useful in predictive modeling and risk assessment of bacterial- and plankton blooms, accumulation in host organisms and associated disease outbreaks in marine animals and in humans. Compiling such information from research about *Vibrio* relationships to other organisms can be used to develop monitoring programs to create early warning systems for disease outbreaks and to increase awareness of anthropogenic activity that might lead to increased numbers of virulent bacteria in the sea.

The objectives of this thesis were to:

1. Explore how the occurrence and abundances of *Vibrio* spp. could be related to the community composition of phytoplankton and a variety of environmental factors when temperature and salinity are stable.
2. Explore survival and proliferation of *Vibrio* when encountering selected phytoplankton species.
3. Explore the occurrence and abundances of *Vibrio* in boreal sediments and examine how increasing temperature will affect *Vibrio* communities from different sediments.
4. Explore a host-pathogen interaction between adult specimens of a calcifying bivalve host and a common bivalve pathogen, *Vibrio tubiashii*, when exposed to a future scenario of ocean acidification.

Research frame

The research covered in paper I in particular aimed to investigate *Vibrio* dynamics in relation to the phytoplankton community composition. The study was performed in a tropical marine area during two distinct periods, when the water column was stable in terms of temperature and salinity. These hydrographical parameters are otherwise known to strongly influence *Vibrio*

abundances. The periods were chosen due to the expectation of differences in phytoplankton composition between the periods based on earlier phytoplankton records. Further, *Vibrio* abundances were also related to a range of other environmental variables as well as potential *Vibrio* predators. The intention was to explore, using uni- and multivariate statistical modeling techniques, whether the biomass of phytoplankton, certain composition of plankton communities and/or particular environmental variables influence *Vibrio* abundances and occurrences of certain species in a seasonal manner.

In the microcosm experimental study, covered in paper II, we aimed to explore if blooms of certain phytoplankton species commonly occurring in the Arabian Sea could affect persistence and potential growth of a pathogenic *Vibrio* strain using simulated conditions from this tropical area. In specific, we used exponential phase cultures of the mixotrophic dinoflagellate *Prorocentrum micans* and the diatom *Skeletonema tropicum*, assuring phytoplankton viability, to which cultured *V. parahaemolyticus* was added.

The study described in paper III aimed to investigate if boreal marine sediments in Scandinavian waters may serve as potential reservoirs for *Vibrio* spp. when water temperatures are low. To achieve high variability, we examined occurrences and abundances of *Vibrio* in sediments from a range of marine environments in this region, with different depth, organic loading and seasons represented. In laboratory experimental manipulations, we further aimed to investigate the influence of increasing temperature on the growth of *Vibrio* in sediments which were collected at two sites affected by different water bodies and therefore expected to contain different bacterial communities. Particular focus in the experiment was placed on presence of culturable *Vibrio* which may be redistributed to the water column if conditions are suitable. Total *Vibrio* abundances, from both *in situ* samples and the temperature manipulation, were explored by uni- and multivariate statistical modeling techniques. The models tested potential relationships with a few relevant environmental variables such as temperature, salinity and organic content in the sediment where loss on ignition, chlorophyll *a*, and phaeopigment were used as proxies for different qualities of organic material. Chlorophyll *a* was also used as a rough measure for the biomass of primary producers.

In the experimental study covered by paper IV, we aimed to illustrate a host-pathogen interaction between adult specimens of a calcifying bivalve host, and a common bivalve pathogen, *Vibrio tubiashii*, when exposed to a future scenario of ocean acidification (OA) projected to occur by the end of the 21th century.

These organisms are especially attractive as models since they naturally occur in shallow coastal areas and therefore are expected to have developed adaptations for encountering each other and for natural fluctuations in seawater carbon dioxide levels and pH. In specific, we explored the effects of OA on: (1) pathogen growth, viability and virulence, (2) host growth, immune defense and stress response, and (3) the interaction between the two organisms.



Above: Malpe harbor fishing fleet, north of Mangalore, Karnataka, India

Below: Sampling in the Hardanger Fjord, Norway (Photos by Maria E. Asplund)

Study areas

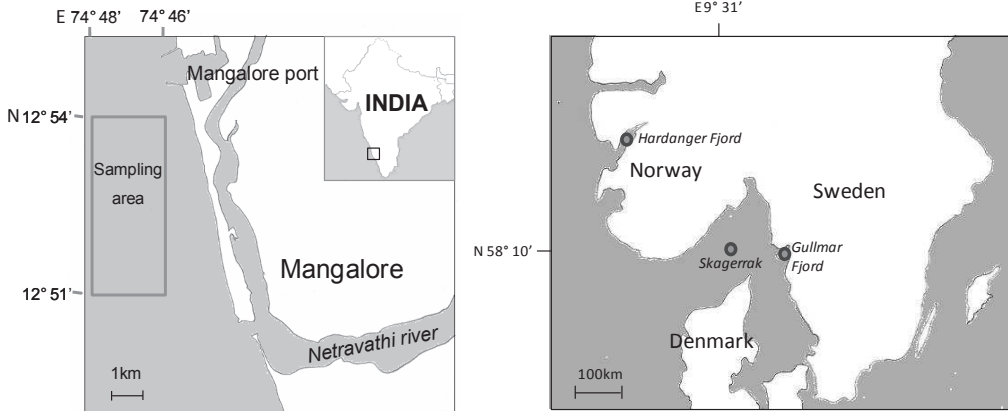


Figure 3. Maps showing study areas: (a) A tropical area off the west coast of India (paper I), and (b) A boreal region on the Scandinavian west coast (paper III).

The research in the thesis was geographically set in one tropical coastal area in the Arabian Sea on the west coast of India and in one boreal region at the Scandinavian west coast. Tropical settings have naturally higher abundances of *Vibrio*, and in India there are numerous *Vibrio*-related infections reported each year (Deepanjali et al. 2005). The field study covered in paper I was conducted approximately 1-2 km off the Karnatak coast outside Mangalore, India (Fig. 3a). Seasonal variation in the hydrographical conditions arises from semi-annual reversal of the monsoonal winds (Banse 1959, Wyrтки 1973, Shankar et al. 2002, Roy et al. 2006). During the strong south-westerly (SW) summer monsoon coastal upwelling with cold nutrient-rich water occurs (Banse 1959) which fuels primary production. The northeast winter monsoon (NE), in which the study covered in paper I was conducted, extends from November through February-March. This period is characterized by a milder, dry climate with stable water temperatures and salinities, and with low nutrient levels (Matondkar et al. 2007). However, since evaporation exceeds precipitation it can enhance convection, which may in turn mix up nutrients to maintain primary production (Banse 1968, Luis & Kawamura 2004). The field study was carried out in two distinct periods during the NE monsoon, one in December when the sea is normally

dominated by diatoms and one in February-March, when the community is usually dominated by dinoflagellates and cyanobacteria (Subrahmanyam 1958). In the microcosm experiment described in paper II we used phytoplankton and *Vibrio* specimens that are common in this tropical area and manipulated the conditions according to those found in the area.

In temperate regions culturable *Vibrio* spp. primarily occurs in the water-column during the warmer season. During the colder part of the year concentrations of most *Vibrio* are low in the water column and therefore may take refuge in reservoirs. Sediments are one such potential reservoir. The sediments described in paper III were collected from three geographically separated areas, i.e. the Hardanger Fjord in Norway, the Gullmar Fjord in Sweden and the Skagerrak deep trench between Norway and Sweden, on the Scandinavian west coast (Fig. 3b). The sampling sites within these areas were chosen to represent gradients of deposits, which provide the sediments with variable amounts of organic material. The supply of organic material to these sediments is generated by net downward fluxes of particulate organic material mainly originating from extensive phytoplankton blooms, organic excess from fish cultures or high land run off. Exudates from diatom blooms constitute the main carbon supply to the vertical flux in the Gullmar fjord (Waite et al. 2005). This material may have been produced locally or transported from other areas through horizontal water movements. The main origin of organic material in the Skagerrak is from the North Sea (Aure & Dahl 1994). The Skagerrak sediments were collected within a depth gradient lasting from the deep trench at 668 m up the slope to 176 m depth. In the fjord systems different water bodies with different density are often generated due to the origin of the water. The Gullmar Fjord sediments were collected from depths encompassing three such different water bodies; the deepest part with highly saline waters originating from the North Sea, the mid part with surface water from Skagerrak and the top part with water from the Baltic current and local run-offs. In the Hardanger Fjord activities from fish farms were expected to accumulate different degree of organic loading depending on the bottom current regime at different distances from two such farms. Deep-water exchange of oxygen-rich water occurs in the fjords when dense enough water appears in sufficient quantity outside the sill (Stigebrandt & Aure 1989), which mostly occur during the winter-spring period (Bjork & Nordberg 2003). Water movements such as during periodical water exchange or by convection may cause resuspension of organic material and potentially associated bacteria (Wainright 1990).

Methodological considerations

Numerous methods have been used within the framework of this thesis. Since most of these methods are exclusive to one study and not of general interest for discussion, details are referred to and are further explained in the different papers. Some considerations are nevertheless highlighted here regarding the core work of *Vibrio* detection and quantification, as well as the overall discussion on phytoplankton biomass and of organic content measurements.

Detection and quantification of Vibrio

To detect *Vibrio* in samples containing natural bacterial communities, methods have to be used to distinguish vibrios from the rest of the bacteria. Within this thesis I have used both culture-dependent (papers III and IV) and culture-independent techniques (papers I, II and III) to describe occurrence and abundances. Occurrence has been observed on Thiosulfate citrate bile salts sucrose (TCBS) agar plates in combination with PCR (paper III) and directly with PCR (paper I). Abundances have been determined by counting colony forming units (CFU) on TCBS agar (paper IV) and molecular methods (paper I, II and III). In paper III the number of yellow and green CFUs was only used as a relative measure of the composition of the bacterial community that is able to grow on TCBS. That this community included (if not solely was constituted of) *Vibrio* was verified by PCR. Culture-dependent techniques have within the frame of this thesis been based on culturing on TCBS-agar (Kobayashi et al. 1963), which selects for *Vibrio*-like bacteria. The plates have limitations since some *Vibrio* strains may fail or grow poorly on the plates and some other bacteria like members of *Aeromonas*, *Pseudomonas* and *Proteus* may also grow on the plates. However, these usually produce smaller colonies than vibrios. There is also other agar media on the market, e.g. HiChrome-agar that has seems to work excellent for isolation of *V. parahaemolyticus* (Rehnstam-Holm pers. com.). Generally all agar types will select for the species that grow most optimally on the chosen medium. To ensure that colonies are formed by *Vibrio* the culturing has to be followed up by, for instance, biochemical testing or molecular techniques. Biochemical testing by commercially supplied phenotypic tests such as API20E (Biomérieux, Durham, US) have given variable results for our isolates with sometimes no reactions. For identification of for instance fish

pathogens, from boreal marine regions, manufacturer protocols have to be altered using lower temperatures, longer incubation times and higher salinities in the diluting media (Popovic et al. 2007) than for human pathogen. Further, before culturing of bacteria it has to be determined which *Vibrio* to select for, since different kinds of *Vibrio* have different temperature optima. Human pathogens will of course readily be cultured at 35°C, while other vibrios with lower temperature optimum may even be killed at such high temperature level (e.g. *V. tubiashii*). Therefore, depending on the culturing temperature there will most likely be dominance of species that have growth optima closest to the selected temperature. Isolation of *Vibrio* on agar plates is, except time-consuming, limited to detection of culturable *Vibrio* and thereby leaving out viable but not culturable (VBNC) cells. This method may therefore underestimate the total number of *Vibrio* cells in the community. However, the advantage using culture-dependent techniques is that live bacteria can be isolated and further investigated for biochemical, enzymatic and growth characteristics, and thus further used in experimental studies as was the case in Paper II.

To estimate the relative abundances of the total *Vibrio* abundance from environmental samples, quantitative real-time PCR with genus-specific primers were used, which in contrast to culture-dependent techniques will also include VBNC-stages of *Vibrio* spp. This technique may however also overestimate the *Vibrio* community through detection of dead cells with still intact DNA. The PCR-step provides a more rapid tool for quantification of *Vibrio*. In estimation of vibrio abundances it can be favorable to use gene target sequence that occurs in several copies in the genome to, such as 16S rRNA, which enhances detection levels. This however is followed by the disadvantage that the number of copies can differ between species. Therefore for total *Vibrio* abundances estimated from real-time PCR were based on *V. parahaemolyticus* (containing 11 copies of 16S rRNA) equivalents, which were used as standard in each run. Furthermore, to estimate *Vibrio* abundance in environmental samples, which often is comprised of complex matrices such as phytoplankton communities, sediments or mussel tissue, effective DNA extractions that yield highly pure DNA have to be used. High purity of the DNA is essential to avoid inhibition of the PCR amplification. The DNA extraction adds to the work load for this method. Therefore, different DNA extraction methods have been applied depending on the matrix of the environmental sample. For the filtered (0.20µm membrane filters) water samples in paper I and II phenol-chloroform extraction (Godhe et al. 2008) was used, which is a very environmental and human hazardous method

but effective in breaking the cell walls of the phytoplankton. For the sediments in paper III a commercial kit, Fast DNA Spin Kit for Soil (MP Biomedicals, Solon US) and FastPrep instrument (BIO 101, SAVANT Instruments Inc. Holbrook, NY) were used. This method is based on bead-beating and showed to give high yields of highly pure DNA. Effectiveness of the DNA extraction methods have been tested by replication followed by spectrophotometric determination of the DNA-concentration, where the level of purity has also been observed. Hence, all quantitative data must be evaluated as relative data to compare samples that have been treated the same way and not absolute values. Further, to determine occurrence of certain species of *Vibrio* conventional PCR using species-specific primers followed by agarose gel-electrophoresis have been used.

Phytoplankton biomass and organic content

In paper I diatoms dominated the phytoplankton biomass. Biomass can be recalculated from the phytoplankton biovolumes (Eppley et al. 1970, Cornet-Barthaux et al. 2007). To determine phytoplankton biovolumes by hand takes skilled taxonomic knowledge and tedious microscopy work. Therefore in paper I we used an indirect scheme to decide the numbers of SSU rDNA copies using diatom-specific reversed primers and SSU rDNA universal primers according to method developed prior to this study (Godhe et al. 2008). Diatom biovolumes were derived from the linear relationship with the concentration of SSU rDNA copies and thereafter used as a relative measure (between samples) of diatom biomass. In addition, in papers I and III, Chlorofyll *a* (Chl *a*) was estimated as a rough approximation of the biomass of primary producers. Chlorophyll *a* is widely used for estimations of the oceans phytoplankton biomasses since it is easily observed with satellite remote sensing (Behrenfeld & Falkowski 1997) and easy, quick and affordable to measure in laboratory. However, the results must be viewed as very rough relative estimations of phytoplankton biomass since the relationship is not linear (Wang et al. 2013). Likewise with Chl *a*, loss on ignition (LOI%), used in paper III, is a relatively quick, inexpensive and commonly used measure of organic matter content. In paper III, LOI% was used as a proxy for the organic loading in the sediments. Also here the results must be viewed as rough relative estimates since during the procedure inorganic carbonates can to some extent be lost during the procedure. Furthermore, some of the organic material can be un-combusted (Luczak et al. 1997).

Synthesis of main results and discussion

Understanding spatiotemporal patterns of variability and processes that structure heterotrophic bacterial communities in the marine environment is a challenge for ecologists worldwide. Nevertheless, this type of knowledge is essential for assessment of the occurrence and proliferation of potential pathogens included in these bacterial communities. The marine environment provides an extremely heterogeneous environment for bacteria, which can be compared to range from wandering in vast sterile deserts to feasting in hot-spots with unlimited food resources. Other organisms can provide potential attachment surfaces and the high concentration of organic material needed by the bacteria in an otherwise oligotrophic environment. Yet, others can be hosts and vectors for transmission of the bacteria to reservoirs or other organisms including humans. These relationships may be symbiotic or antagonistic. For some organisms the bacteria can constitute the essential food supply, which might in turn be vital to limit bacterial distribution.

In this thesis I have highlighted a few ecological aspects of relationships of bacteria versus other organisms and the changing environment within which they prevail. In particular the thesis emphasizes the significance of phytoplankton blooms as determinant for *Vibrio* occurrence and abundance (papers I, II and III). Papers I and II also indicate that the composition of phytoplankton communities is of importance for *Vibrio* survival, growth and abundance. Papers I and III show that phytoplankton biomass is of importance for *Vibrio* occurrence and abundance both in the pelagic environment and in marine sediments indicating that there could be a distinct pelagic–benthic coupling. Further, paper I indicates that *Vibrio* may periodically be top-down controlled by bacterioplanktivores such as ciliates. In the marine environment organisms are subjected to altered conditions at different temporal and spatial scales that may in turn change their abilities for survival and proliferation. In paper I vertical mixing shifted the prerequisites for plankton blooms from a pronounced established fluorescence maximum to a completely mixed water column, which may subsequently have diluted the occurring blooms. An increasing water temperature and ocean acidification due to global climate change may also affect *Vibrio* communities in different ways. Paper III shows a variable effect of increasing temperatures on *Vibrio* abundances in sediments.

Paper IV describes how a bivalve pathogen, *V. tubiashii* successfully can infect a common bivalve host, *M. edulis*, when exposed to ocean acidification.

In specific, paper I shows that temporal variation in *Vibrio* abundance can occur in an oligotrophic tropical area despite stable water temperature and salinity. *Vibrio* abundances varied with up to two orders of magnitude between the first sampling period (December) and the latter one (February-March). Phytoplankton biomass, in terms of the dominating diatom bloom biomass, was the single most important factor coinciding with *Vibrio* abundance. A study by Simidu et al (1971) showed higher proportions of *Vibrio* in the bacterial assemblages when samples were isolated from phytoplankton in comparison when samples were isolated from water samples. In addition, Smith et al. (1995) showed that bacteria living attached to diatoms grew more rapidly than those that are free-living and that they consumed 40-60% of the total primary production. Our model presented in paper I suggests that over the whole period under the current conditions, the resources provided by the primary production were of greater importance than top-down regulation by *Vibrio* predators such as ciliates and heterotrophic nanoflagellates (HNFs). Bacterial mortalities generally are due to viral attacks (e.g Murray & Eldridge 1994) engulfment by phagotrophic protists, which consists of e.g. HNFs, mixotrophic and heterotrophic planktons (Jeong et al. 2010) and to lesser extent ciliates (reviewed by Pernthaler 2005). Prevailing theories suggests that HNFs can easily suppress *Vibrio* abundances (Beardsley et al. 2003, Pernthaler 2005). Our model showed weak links between HNFs and *Vibrio*, and even when a lag phase was included in the analysis. However, periodically ciliates seemed to negatively influence *Vibrio* abundances. In aquatic ecosystems rapid change can occur between bottom-up and top-down control of bacterioplankton (Psenner & Sommaruga 1992) depending on the structure of the plankton community. Bacteria in the sea is suggested to be mostly top-down controlled by predators especially in oligotrophic areas where resources are scarce but can switch to bottom-up control when resources are more generally available (Sanders et al. 1992). *Vibrio* abundances were more tightly linked to the phytoplankton community during the first period when the bloom was more intense, despite that total Chl *a* was relatively low during both periods. Therefore our results suggest that at least periodically phytoplankton blooms can sustain high proliferation of *Vibrio*, but the structure of predator components and viral phages will greatly influence this relationship.

Zooplanktons (e.g. copepods) are suggested to be important as hosts where *Vibrio* can attach and proliferate (Kaneko & Colwell 1973). They can also be vectors for transmission of vibrios to other marine organisms or if they occur in drinking water to humans (e.g. Huq et al. 1996). The numbers of copepods in the waters were relatively high (i.e. mean 55 ind. L⁻¹ in December and 36 ind. L⁻¹ in December – March). Paper I showed that copepods were important for the model but did not have an explanatory power comparable to that of the phytoplankton biomass. A possible explanation is that during periods when phytoplankton bloom support *Vibrio* growth, the attachment to copepods is less important for the bacteria. However, during other periods within the same area, zooplankton has been shown to be tightly linked to at least culturable *Vibrio* abundances (Peterson et al. 2010). Vibrios are suggested to attach to copepods to avoid predation (Matz et al. 2005) or for the resources that the copepod chitin (reviewed by Pruzzo et al. 2008) or sloppy feeding, exudation and excretion can provide (Olsen et al. 2007). Therefore, *Vibrio* attachment to copepods may be driven by high abundances of predators or scarcity of other resources.

This thesis also highlight that the composition of a phytoplankton bloom can influence *Vibrio* abundance, survival and growth (papers I and II). A diatom bloom with high diversity of species supported high *Vibrio* abundances in the oligotrophic tropical study area during the first sampling period in December, as described in paper I. The importance of higher diversity of phytoplankton for *Vibrio* abundance in this area was further supported by Peterson et al (2010), showing that natural net samples with higher number of phytoplankton species can coincide with high *Vibrio* abundances. However, some phytoplankton species can have negative impacts on *Vibrio*, which can be explained by that they may produce antimicrobial compounds, that they may actually feed on bacteria or that they may compete for the same resources as *Vibrio*. The findings show that the abundance of cyanobacterium *Trichodesmium* spp. was negatively correlated with *Vibrio* abundances. Nevertheless these results indicate that the relationship between phytoplankton and *Vibrio* is not general; rather our results suggest that the composition of phytoplankton blooms is essential and may determine the composition of *Vibrio* bacteria. This suggestion was further supported in paper II, which clearly indicated that the dinoflagellate *Prorocentrum micans* can provide resources for *Vibrio* to be able to grow, while the diatom *Skeletonema tropicum* completely eradicated the bacteria in our experiment. Since *P. micans* is mixotrophic it could be hypothesized that they instead also could feed on the bacteria and thereby surpress *Vibrio* numbers. However, since *Vibrio* clearly increased in numbers by almost two orders of

magnitude when encountering the dinoflagellate, we could conclude that this was not the case in this study. The two phytoplankton species used in the study are commonly found in the Arabian Sea. Trials to use Cell Tracking probe labeled active *Vibrio* cells to follow potential association to certain phytoplankton groups when added to natural net samples of plankton from the Arabian Sea, in epi-fluorescent microscopy, further indicated higher degree of clustering around dinoflagellates (Eliasson 2009). However, the method had some constraints since only a part of the bacteria were labeled and that diatom auto-fluorescence generally was weaker than that of dinoflagellates. Association to phytoplankton blooms, with higher proportion of dinoflagellates, has earlier been shown in boreal waters of Sweden (Eiler et al. 2006). However, in boreal waters dinoflagellates are more dominant when sea surface temperatures (SSTs) increases (Hare et al. 2007), and may therefore co-occur with *Vibrio* that also thrive in warmer waters. Contrastingly, if *Vibrio* abundances truly are positively influenced by dinoflagellates, phenological shifts in the plankton community, due to increased SST, towards earlier dinoflagellate blooms (suggested by Edwards & Richardson 2004) could also increase *Vibrio* numbers in the Sea. If there is a true relationship, positive or negative, with dinoflagellates and if this kind of association is of general importance have to be further elucidated.

When resources are scarce or conditions harsh in the water column vibrios may seek refuge in reservoirs such as host organisms or in sediments. Sediments constitute a realm often with high proportions of organic material. In paper III we found that boreal sediment from geographically separated areas on the Scandinavian west coast all contained relatively high *Vibrio* abundances, despite the overall cold temperatures (<10°C). All the sediments also contained culturable *Vibrio*. The sediments contained variable degree of organic matter content that was expected to have different origins. In coherence with the results in paper I, the biomass of primary producers, here determined by the Chl *a* content, was the single most important variable tested for explaining variability in *Vibrio* abundances in these boreal sediments. Interestingly, although the sediments were quite variable in terms of sampling depth, organic content and expected origin of the sediment material, Chl *a* showed an explanatory power in the model. Thus vibrios seem to be favored by fresh input of microalgae biomass. The origin of the Chl *a* has not been encompassed by this study. Also sediments at depths below the euphotic zone, where irradiance is less than 1% of that at the surface, benthic diatoms have shown to contribute substantially to Chl *a* levels (Wulff et al. 2005). However, at the deeper sites it is not likely that Chl

a originates from benthic microalgae. Deposition bottoms are common along these coasts and especially in the fjord systems. In deposition bottoms large fractions of the organic material can originate from extensive phytoplankton blooms (Davies & Payne 1984, Waite et al. 2005), which can aggregate and sink. These aggregates and plumes of dissolved organic material following the sinking algal biomass can be “hot-spots” for attached or free-living heterotrophic bacteria (Azam & Long 2001, Kiorboe & Jackson 2001). Most likely, depending on the activity and temperature in the water column, most of the Chl *a* will be degraded by microorganisms before reaching the seafloor. Still, the small fraction of Chl *a* in the sediments seems to be of more general importance for *Vibrio* abundance than phaeopigments that occurred in higher proportions. Therefore, we speculate that the pelagic-benthic coupling may be of importance for the numbers of *Vibrio* species/strains in these boreal sediments. The explanatory level of the model indicates that there are other factors of importance for *Vibrio* dynamics in these boreal sediments, however not encompassed by this study. For instance, meiofaunal predators may control bacterial abundance (Pascal et al. 2008) or macroscopic invertebrates that rework the sediments may create micro-niches where bacteria can thrive (Gamble & Lovell 2011).

Rising sea surface temperatures during the last decades have increased the dominance of marine *Vibrio* bacteria in northern latitudes (Vezzulli et al. 2012) and this coincides with increased risks of *Vibrio* infections (Baker-Austin et al. 2012). The findings from paper III show that increasing temperature had variable effects on *Vibrio* abundances in two boreal sediments with high degree of organic content collected from two sites influence by distinct water bodies within the Gullmar Fjord. *Vibrio* abundances increased in numbers in sediments from the deepest part of the fjord only when temperature reached 21°C. In sediments from the mid-water body, close to the sill, the increasing temperatures negatively influenced *Vibrio* numbers in the sediments. This suggests that the *Vibrio* community composition differs between the two sediment sites, a result which was also confirmed by the composition of culturable bacteria. A possible explanation is that the *Vibrio* communities include species with variable temperature tolerances. However, if this negative trend is due to true overall negative impact of increased temperature on these vibrios or if they turn to be free-swimming and leave the sediment is not clarified by the study in paper III. Contemporary environmental conditions in conjunction with how the assemblage of seeding *Vibrio* strains is structured will certainly strongly affect

how *Vibrio* bacteria can proliferate in the water column, if they are resuspended from the sediment. To understand if sediment reservoirs of *Vibrio* spp. could pose a potential threat of emerging disease risk it has to be better understood whether *Vibrio* communities include virulent strains.

Ocean acidification (OA) alters the entire chemical structure of the seawater, which in turn will affect many of the organisms that live there. Study IV, illustrates how an interaction between a mussel pathogen (*V. tubiashii*) and a filtrating calcifying host organism, the common blue mussel (*Mytilus edulis*) is affected by ocean acidification. Since bivalves filter large volumes of water they are normally exposed to high numbers of pathogenic bacteria occurring in the water column. However, they seem to be adapted to cope with microbes and more resistant to high doses of pathogens compared to other marine invertebrates such as Norway lobsters and sea stars (Oweson & Hernroth 2009). In accordance, study IV showed that in ambient average CO₂/pH-levels occurring in the sea today, very high concentrations of *V. tubiashii* was needed to infect adult mussels. This can be explained by the mussels' powerful immune defense that they have adapted to battle invasive pathogens (Canesi et al. 2002, Girón-Pérez 2010). However, it was shown that *V. tubiashii* ability to infect the mussels increased when both host and pathogen were exposed to the CO₂/pH-levels projected to occur in the sea at the end of the 21th century, despite no evident effect on survival, growth and pathogenicity of *V. tubiashii* or immune response of the mussels, when studied separately from each other. Therefore, this study highlighted the necessity to explore impacts of OA on host-pathogen interactions or on other interacting groups of organisms, not only on separate species. The ability of microbial pathogens to faster adapt in a changing environment provides them significant advantage in comparison to their hosts. If the increasing rate of OA progress according to predictions, we may face a future with emerging host-infections by marine pathogens. The emergence of microbial pathogens that proliferate at faster rates in the marine environment due to higher temperatures but also due to their ability for fast adaptation to OA conditions could therefore have major implications for many calcifying host organisms.

Furthermore, if calcifying organisms, due to OA, becomes more prone to *Vibrio* infections, it can pose serious threats to many key species in the marine environment that constitute habitat and food supply for a variety of other organisms and thereby also human food security. Recent studies of edible

shellfish indicates that the negative influence of OA on host immunity (Bibby et al. 2008, Hernroth et al. 2012) could as well increase the risk for transmission of pathogens to humans consuming them. Global climate change resulting in warmer and more acidic oceans may thus increase the risk of bacterial infections in both animals and humans.

Concluding remarks

The main findings covered within the frame of this thesis showed that:

- Temporal variation of *Vibrio* abundance can be strong in oligotrophic tropical waters, despite stable hydrographical conditions in terms of temperature and salinity
- Temperatures is not necessarily the driving main force for *Vibrio* growth, neither in tropic waters nor in boreal sediments
- The biomass of primary producers can be an important determinant of *Vibrio* spp. abundance, both in pelagic and benthic marine environments
- Phytoplankton blooms can periodically be of greater importance for *Vibrio* abundance compared to top-down control by predators and/or associations to zooplankton in oligotrophic tropical waters
- Phytoplankton composition and diversity seems to influence *Vibrio* survival, persistence and proliferation, as certain species of phytoplankton may favoring *Vibrio* growth, while others species or compositions inhibit the growth of *Vibrio*
- In boreal sediments, the fresh input of phytoplankton biomass, in terms of Chl *a*, seems to be more significant for *Vibrio* abundance than more degraded organic material, in terms of phaeopigments and total organic content.
- The composition of vibrio species seems to differ between sediments with different organic contents as judged by of their temperature tolerance.
- Adult host specimens can be well adapted to pathogens occurring in the sea and thereby resist high numbers of the pathogen under pH/CO₂ conditions on average occurring in the sea today
- *Vibrio tubiashii* can successfully infect adult blue mussels when both host and pathogen are subjected to levels of ocean acidification projected to occur by the end of the 21th century despite no evident effects on either pathogen growth, viability and pathogenicity or on host immune defense and stress respons when studied under these conditions separately
- This indicates a threat for a sustainable biomass of mussels and other marine species and constitutes as well an increased threat for transmission to humans consuming them.

Future research challenges

A major challenge for the future studies of *Vibrio* in the marine environment, from my perspective, is to find rapid, easily accessible and affordable tools to distinguish the composition of *Vibrio* communities as well as other marine pathogens and to allure occurrence of pathogenic strains. So far most studies, like in this thesis, concerns total *Vibrio* abundances or detection of some certain species or strains most often human pathogens. A number of tools like multiplex real-Time PCR (Kim et al. 2012), quantitative PCR combined with denaturant capillary electrophoresis (Thompson et al. 2004) and denaturant gradient gel electrophoresis (Eiler & Bertilsson 2006), PCR combined with restriction length polymorphisms (Maeda et al. 2003) and PCR combined with DNA sequencing (Ki et al. 2009) are highly useful to determine diversity in the *Vibrio* community. Some of these tools are, however, very time-consuming and cannot give the rapid answer that is needed when considering the fast turn-over rates in the marine environment. Most methods also results in very inconclusive answers which rather distinguish populations of genetically similar vibrios than exclusive strains. Yet others, like next-generation sequencing, are so far economically untenable but are developed to lower the costs through parallel sequencing of millions of sequences at once. However, they still require largely developed databases and bioinformatic computerizing tracking systems, which are not yet accessible for most laboratories, especially not in developing countries where this information may be of major importance. Therefore, before such advanced tools are generally available for rapid determination of diversity of pathogens in the seawater and to be able to alert warnings in advance we must rely on knowledge on how these pathogens are coupled to environmental conditions. We need to more translucently define the factors which promote proliferation and pathogenicity of *Vibrio* in the marine environment. Sea surface temperature and salinity can to some extent be used to predict large-scale patterns of *Vibrio* abundances in areas where these hydrographical parameters show seasonal and/or spatial variation (Lobitz et al. 2000, Baker-Austin et al. 2012). However, as shown by this thesis other events like phytoplankton blooms can be tightly coupled to development of the *Vibrio* abundances. Therefore one challenge is to improve our knowledge on which compositions of phytoplankton blooms that may sustain high *Vibrio* abundances and in turn also which strains of *Vibrio* that are promoted by such blooms. The information provided by these programs

could be used to understand the coupling of *Vibrio* and phytoplankton blooms. Such knowledge can in turn be used as an indirect estimate for predicting when there is a higher risk of being infected by *Vibrio* from eating seafood or swimming in waters with high number of pathogens. Further, if it could be elucidated that the bloom-forming phytoplankton that support high *Vibrio* abundances, also have discrete pigment signals, potentially satellite imaging may potentially be used for indirect predictions of *Vibrio* occurrences in marine areas.

To comprehend the emerging risks of bacterial infections in a marine environment under global climate change and ocean acidification we need to study the interactions between pathogens and their host-organisms under the influence of these parameters combined. This thesis showed that it is difficult to distinguish significant disturbances of mussel immunity induced by ocean acidification. However, when subsequently encountering *V. tubiashii* exposed to the same pCO₂/pH-conditions the mussels do show increased susceptibility for infection, indicating that mussels are affected by ocean acidification in the way that they are less fit to fight an additional stressor. This further stresses those studies on host-parasite interactions are sensitive tools to explore the consequences of increasing temperature and levels of ocean acidification. Ideally, in order to better understand the dynamics of pathogen development and toxicity we should strive to include as many trophic levels of organisms possible in our studies, and expose these systems in settings which simulate as natural conditions as possible.

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