Characterization and persistence of potential human pathogenic vibrios in aquatic environments

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Vibrio spp., natural inhabitants of aquatic environments, are one of the most common causes of bacterial gastroenteritis in the world, being spread to humans via the ingestion of seafood, contaminated drinking water or exposure to seawater. The majority of *Vibrio* spp. are avirulent, but certain strains may sporadically be human pathogenic. *Vibrio cholerae* may cause cholera and fatal wound infections, *Vibrio parahaemolyticus* may cause gastroenteritis and *Vibrio vulnificus* may cause wound infections and sepsis. To expand current knowledge of the occurrence, ecological niche and persistence of potential human pathogenic *Vibrio* spp. in aquatic environments, occurrence and laboratory studies were performed.

The seasonal variation of *Vibrio* spp. in clams and mussels from Mozambique and Sweden were studied, with isolated strains characterized and compared with those isolated from water samples collected in India. Results showed that the numbers of *Vibrio* spp. in Mozambican clams peaked during the warmer rainy season and that the dominating species was *V. parahaemolyticus.* Biochemical fingerprinting and virulence screened by PCR revealed a high similarity among strains from the different aquatic environments. However, isolate functional hemolytic analyses and antibiotic resistance patterns differed between strains; Swedish and Indian strains were less sensitive to the tested antibiotics and had a lower hemolytic capacity than those from Mozambique. Molecular analysis of bacterial DNA from Swedish mussels showed the presence of the three *Vibrio* spp. most commonly linked with human illness, as well as their associated virulence genes. The strains isolated from marine and clinical environments were equally and highly harmful to the tested eukaryotic cells.

The persistence of clinical *V. cholerae* in aquatic environments was investigated *in vivo.* Strains were exposed to mussels, with bacterial uptake and elimination then examined. The mussels were able to avoid the most potent strain by complete closure of shells. The less potent strain was accumulated in mussel tissue in low levels and one marine control strain to a higher degree. Mussels eliminated the pathogenic strain less efficiently than they did the marine strain. One clinical and one marine strain were then exposed to 4°C for 21 days, with the temperature then increased to 20°C. The clinical strain was more prone to lose culturability than the marine strain at 4°C, the former performed significantly better in regaining culturability after the temperature up-shift. Subsequently, the persistence of the clinical strain in natural bottom sediment, incubating as above, was studied and results showed a similar decrease in culturable numbers in the sediment as in the water. As the clinical *V. cholerae* strains did not carry any of the standard set of virulence genes, the ability to change from non-culturable to culturable may be of great importance to strain pathogenicity. The results also show that natural bottom sediment may be a potential reservoir of human pathogenic *Vibrio* spp.

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ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numeral (I-IV)

I   **B. Collin**, A.-S. Rehnstam-Holm, S.-M. Ehn Börjesson, A. Mussagy and B. Hernroth
     Characteristics of potentially pathogenic vibrios in sub tropic Mozambique compared to isolates from tropic India and boreal Sweden
     Submitted

II   **B. Collin** & A.-S. Rehnstam-Holm
     Occurrence and potential pathogenesis of *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus* on the South Coast of Sweden.
     FEMS Microbiology Ecology. 2011; **78**: 306-313.

III **B. Collin**, A.-S. Rehnstam-Holm, B. Lindmark, A. Pal, S. N. Wai and B. Hernroth
     The origin of *Vibrio cholerae* influences uptake and persistence in the blue mussels *Mytlius edulis*
     Journal of Shellfish Research, 2012, **31**: 87-92

IV   **B. Collin**, B. Hernroth, and A.-S. Rehnstam-Holm
     The importance of marine sediments as a reservoir for human pathogenic *Vibrio cholerae* in cold water conditions
     Submitted

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ADDITIONAL PAPERS

The author has also contributed to the following studies not included in this thesis:

B. Collin, A.-S. Rehnstam-Holm and B. Hernroth
Faecal Contaminants in Edible Bivalves from Maputo Bay, Mozambique: Seasonal
Distribution, Pathogenesis and Antibiotic Resistance
Open Nutrition Journal 2008; 86-93.

M. E. Asplund, A.-S. Rehnstam-Holm, V. Atnur, P. Raghunath, V. Saravanan, K. Härnström,
B. Collin, I. Karunasagar, A. Godhe
Water column dynamics of *Vibrio* in relation to phytoplankton community composition
and environmental conditions in a tropical coastal area
Environmental Microbiology, 2011**; 13**, 2738–2751

A.-S. Rehnstam-Holm, A. Godhe, K. Härnström, P. Raghunath, V. Saravanan, **B. Collin**, I.
Karunasagar, I. Karunasagar
Association between phytoplankton and *Vibrio* spp. along the southwest coast of India:
a mesocosm experiment.
Aquatic Microbiology Ecology 2010; **58**: 127-139.

Rehnstam-Holm A.-S. & **Collin B**
Vibrio-arter i sydsvenska vatten orsakade badsårsfeber. Ökande frekvens av
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caused bath-wound fever. Increased bacteria frequency according to studies on clams.
Läkartidningen 2009; **106**: 435-438.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vibriobakterier är naturligt förekommande i akvatiska miljöer och påträffas ofta i sjöar, brack- och havsvatten. Arterna som ingår i vibriosläktet är oftast ofarliga för människor, men vissa kan ge upphov till sjukdom, t o m dödlig sådan. *Vibrio cholerae* kan orsaka, om än väldigt sällan, diarrésjukdomen kolera och är en av de mest fruktade bakterierna - uttrycket "pest eller kolera" berättar om dess betydelse för människan genom historien. Spridningen av sjukdomen är framför allt kopplad till bristande tillgång på rent dricksvatten och drabbar därför ofta länder med otillräcklig rening av avloppsvatten. Koleraepidemier uppkommer ofta i samband med naturkatastrofer då de lätt kan få fäste i tältläger där människor lever tätt och hygienen är dålig, som t.ex. efter översvämningen som drabbade Moçambiques huvudstad Maputo 2009 och efter jordbävningen på Haiti 2010. Kolera skördar varje år många människoliv (120 000- 100 000 enligt WHO, 2012). Ett annat sätt att drabbas av vibrioinfektioner är via skaldjur. Musslor filtrerar vatten för att syresätta sina vävnader och få i sig föda och med vattnet följer mikroorganismer som de kan ackumulera i sin vävnad. Detta leder till att musslor ibland bär på bakterier som är potentiellt sjukdomsframkallande, patogena, för människan. Eftersom skaldjur ofta äts råa eller lättkokta hinner inte temperaturen döda alla organismer i musslans vävnad. Skaldjur räknas som den viktigaste spridningsvektorn av bakteriell maginfektion i världen och arten *Vibrio parahaemolyticus* är en av de vanligaste orsakerna. För att få djupare kunskap om vibrios förekomst och uthållighet i akvatiska miljöer samt dess virulens har vi utfört laborativa och fältstudier.

Moçambique är ett land på den sydöstra kusten av den afrikanska kontinenten med en 2400 km lång kuststräcka mot Indiska oceanen. Reningen av avloppsvatten från den växande huvudstaden Maputo är bristfällig och en stor del av detta vatten mynnar ut i Maputo Bay, där invånare samlar musslor för sin dagliga föda. För att studera förekomst och säsongsvariation av vibriobakterier i musslor och vatten från Maputo Bay köpte vi musslor av plockare och provtog vatten vid fyra provtagningstillfällen under ett års tid; november, mars, maj och augusti som representerar tidig och sen regn- (varmare) och torrperiod (svalare). Medeltemperaturen på vattnet sjönk inte under 22°C vid något av tillfällena och *Vibrio* isolerades vid samtliga provtagningar. Vi fann att antalet *Vibrio* i musslor var högt under hela året och följde vattentemperaturen med högst värde (ungefär 5.5 miljoner bakterier/100g mussla) när det var som varmast (mars) och lägst (ungefär 60 000 bakterier/100g mussla) då det var som kallast (augusti). Infektionsdosen för *Vibrio* är vanligen hög, det krävs ca 1 miljoner patogena bakterier för att insjukna. Antalet överskrids lätt i dessa fall, framför allt om musslorna inte värms tillräckligt eller inte äts med det samma och bakterierna tillåts tillväxa. Vi kunde dock se att inte alla isolerade bakterierna verkade kapabla att orsaka sjukdom.

V. parahaemolyticus var den vanligaste *Vibrio* arten som isolerades från proverna och när vi studerade deras virulens såg vi att endast en av de 109 stammarna bar på virulensgenen *tdh* (thermostable hemolysin gene), som gör att bakterien kan producera ett protein som orsakar infektion genom att förstöra tarmcellernas membran. Vi studerade även om bakterierna kunde påverka cellmembran genom att låta dem växa på agar som innehöll blodceller. Resultaten jämfördes sedan med studier på *V. parahaemolyticus* som vi isolerat från svenska och indiska vatten. Resultaten visar att nästan 70% av de moçambikanska bakterierna var kapabla att bryta ned blodcellerna medan siffran för de svenska och indiska stammarna endast var ca 40%. Vi kan därför

inte utesluta att de är virulenta även om *tdh*-genen inte kunde detekteras. Vi undersökte även deras eventuella resistens mot antibiotika och kunde se att resistensen var mest utbredd bland de svenska stammarna, något mindre bland de indiska och minst bland de moçambikanska stammarna. Vi utvärderade även odlingsmediet och såg att procentandelen av de bakterier som växte på TCBS-agar (selektivt för *Vibrio* spp.) som verkligen var *Vibrio* spp. skiljde sig mycket mellan säsongerna, vilket är av stort intresse när man utför övervakningsstudier och identifieringar.

Sverige har länge ansetts förskonad från sjukdomar orsakade av vibriobakterier, men eftersom det under senare år ofta har anmälts inhemska sjukdomsfall till smittskyddsinstitutet har det blivit klart för oss att så inte är fallet. Det är inte kolera eller maginfektioner som drabbar svenskar utan olika typer av sårinfektioner, bl.a. den s.k. "badsårsfebern" orsakad av *V. cholerae*. Under den varma sommaren 2006 insjuknade flera svenskar i denna sjukdom, varav två avled till följd av infektionen. Patienterna berättade att de hade varit i kontakt med östersjövatten dagarna innan infektionens utbrott, och eftersom den är vattenburen kan man anta att bakterien härstammade därifrån. Under perioden juni till september 2006 utförde vi en kvalitativ studie av förekomst av vibriobakterier i Öresund. Vi kunde se att vibrios förekom vid alla provtagningar då vattentemperaturen översteg 17°C och 86% av de positiva proverna var även positiva för testade virulensgener, vilket alltså visar att bakterierna kan ha förmågan att orsaka sjukdom hos människan. Vi gjorde även laborativa tester på de isolerade bakterierna och såg att många av dem var väldigt farliga för en typ av eukaryota celler, dvs. den typen av celler som bl.a. människor består av. De bakterier som orsakade de allvarliga sårinfektionerna har vi sedan studerat mer ingående.

När *Vibrio* som infekterat människan hamnar i havsvatten via avloppsvatten möter de en ny och annorlunda miljö som de snabbt måste anpassa sig till. För att få djupare kunskap om hur uthålliga *V. cholerae* från patientprover är i vattenmiljöer studerade vi dem laborativt. Vi jämförde först dess uppsättning av virulensgener med en *V. cholerae* stam som isolerats från Öresund och såg att patientstammen inte skiljde sig från den akvatiska stammen. Därefter utsatte vi dem för blåmusslor och resultaten visade att musslor stänger sina skal och slutar filtrera om bakterierna de träffar på är högpatogena. Om bakterierna är något mindre farliga ackumulerade musslorna dessa till låg grad i sin vävnad, men när bakterierna väl fanns i musslan var det svårt för dem att göra sig av med dem. De minst patogena bakterierna kunde musslorna både äta och bryta ned väldigt effektivt. Alltså, musslorna kunde avgöra redan vid filtreringen om bakterien var skadlig för dem eller ej och om de ackumulerat bakterier som var patogena var dessa svåra för musslorna att bryta ned.

Därefter studerade vi hur uthålliga bakterierna var vid låg vattentemperatur (4°C) i tre veckor och därefter en snabb temperaturhöjning till 20°C. Vi kunde vi se att patientstammen ströp sin ämnesomsättning under den kalla perioden, men mycket snabbt slog om till hög ämnesomsättning när omgivningsfaktorerna blir bättre. Denna reaktion jämförde vi med en bakterie som isolerats från Öresund. Den var inte var alls lika uthållig och kunde inte återuppta sin ämnesomsättning efter temperaturhöjningen, inte ens efter en vecka i 20°C. Vi undersökte även uthålligheten hos patientstammen i naturligt bottensediment vid 4°C och efterföljande temperaturhöjning och kunde då se att bakterien överlevde bättre i sedimentet än i vattnet trots att sedimentet innehöll mikroorganismer som både konkurrerar med och äter *V. cholerae.*

SLUTSATSER

- Potentiellt patogena vibriobakterier fanns i musslor från Maputo Bay, Moçambique oberoende av säsong, med högst antal odlingsbara vibriobakterier när vattentemperaturen i vattnet var högst (ca 30°C).

- *V. parahaemolyticus* var den vanligaste vibrioarten i Maputo Bay, men endast en av 109 stammar bar på virulensgenen *tdh*. 70% kunde dock bryta ned röda blodkroppar, vilket endast ca 40% av de svenska och indiska stammarna kunde.

- Antibiotikaresistensen hos moçambikanska *V. parahaemolyticus* var mycket lägre än hos bakterier isolerade från Sverige och Indien. De svenska bakterierna var mest resistenta.

- Potentiellt patogena vibriobakterier fanns i Öresund under sommaren 2006 när vattentemperaturen steg över 17°C. Bakterierna isolerade från vattnet var lika skadliga för eukaryota celler som de bakterier som orsakat allvarlig sjukdom hos människor.

- Musslor är filtrerande organismer, men om de utsätts för högpatogena bakterier stänger de av sin filtrering. Musslor kan dock ta upp mindre patogena vibriobakterier och ackumulera dem i sin vävnad. Ju mer patogena bakterier musslan tagit upp i sina vävnader desto svårare är de för immunförsvaret att bryta ned. Alltså kan musslan utgöra en viktig vektor och överföra bakterier från vatten till människa. Musslan kan också utgöra ett bra skydd för vissa patogena *V. cholerae* i en vattenmiljö som annars kan vara tuff.

- Den kliniska *V. cholerae* stammen var mer uthållig vid låg vattentemperatur än den stam som isolerats från vatten. Efter temperaturhöjningen ökade patientstammens ämnesomsättning väldigt snabbt medan stammen från Öresund aldrig kom tillbaka. Att snabbt kunna anpassa sig till nya förutsättningar i miljön kan vara en viktig egenskap för bakteriers överlevnad och patientstammar kan vara bättre anpassade för föränderlig miljö, vilket kan vara ett viktigare karaktärsdrag än att bära på virulensgener.

- Naturligt bottensediment visade sig kunna utgöra en reservoar för kliniska *V. cholerae* när vattentemperaturen är låg.

ABBREVATIONS

TCBS	Thiosulfate Citrate Bile Sugar
APW	Alkaline Peptone Water
PBS	Peptone Buffer Sulfate
BHI	Brain Heart Infusion
VBNC	Viable But Non-Culturable
spp.	species
toxR	toxin regulation gene (*V. cholerae*)
ctx	cholera toxin gene (*V. cholerae*)
tlh	thermolabile hemolysin gene (*V. parahaemolyticus*)
tdh	thermostable direct hemolysin gene (*V. parahaemolyticus*)
trh	TDH-related hemolysin gene (*V. parahaemolyticus*)
vvh	hemolysin gene (*V. vulnificus*)
viuB	iron acquisition gene (*V. vulnificus*)
CFU	Colony forming units
PCR	Polymerase Chain Reaction
qPCR	quantitative PCR
WHO	World Health Organization
PhP	Phene Plate system
API 20NE	Biochemically based identification method
CHO-cells	Chinese Hamster Ovary cells
PSU	Practical Salinity Units
16S rRNA	Gene coding for component of prokaryotic ribosome

INTRODUCTION

Bacteria are present almost everywhere. Some may be found in cold environments, while others thrive at high temperatures. Many bacterial species are found in the soil, metabolizing dead plants and making the nutrients available to other living organisms. Bacteria are also exploited for their abilities on an industrial scale, such as in the production of antibiotics and vitamins or in the treatment of sewage and wastewater. Many are important for human health since they form part of normal gut flora, which is essential to deal with pathogens. The majority of bacteria are harmless to humans and necessary for our wellbeing. However, some may cause illness and as such have been feared throughout history, including *Yersinia pestis* (causing the Black Death), *Mycobacterium tuberculosis* and *Vibrio cholerae*. The latter is one of the bacteria I will focus on in this thesis. This bacterium belongs to the family *Vibrionaceae,* which includes several potential human pathogenic species.

Historically, vibrios (*Vibrio* spp.) were the first bacteria to be isolated and identified from the environment. In 1854, *Vibrio* were described by the Italian medical student Pacini (Bentivoglio & Pacini, 1995) and became an important argument in the contemporary debate of germ theory vs. miasma theory – i.e. identifying the causative agent of disease as an organism or as polluted vapor in the air. However, a few years earlier, John Snow had isolated the bacterium *V. cholerae* after a cholera outbreak tracked to a contaminated drinking water well in London. Robert Koch, originator of Koch's postulates, isolated *V. cholerae* during an outbreak in Egypt/India in 1883 and suggested that the bacterium was the causative agent of pandemic cholera, the most feared disease at the time. John Snow declared that cholera could not be tracked back further than 1769, but this may be due to the fact that epidemics in Asia were not documented in Europe (Snow, 1855). Seven cholera pandemics have been noted, with the first identified in 1817 and the seventh still ongoing (Blake, 1994, Colwell, 1996). Statistics presented by the WHO illustrate that the estimated annual number of cholera cases is still very high, with 3-5 million patients and 100 000-120 000 deaths each year (2011), while the African continent in particular is frequently hit by epidemics (Mintz & Guerrant, 2009).

V. cholerae is only one of several potential human pathogenic *Vibrio* species (Table 1). *Vibrio parahaemolyticus* is the major bacterial cause of gastroenteritis associated with seafood in the world (Joseph*, et al.*, 1982, Janda*, et al.*, 1988, Honda & Iida, 1993), while *Vibrio vulnificus* is a highly lethal bacterium most often linked to wound infection and sepsis (Torres*, et al.*, 2002, Hsueh*, et al.*, 2004, Kuhnt-Lenz*, et al.*, 2004, Ruppert*, et al.*, 2004, Oliver, 2005a).

Taxonomy - Vibrionaceae
The bacterial genus *Vibrio* is, according to Bergey's Manual of Systematic Bacteriology (Garrity, 2005), classified as belonging to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Vibrionales* and family *Vibrionaceae.* Other bacterial orders in this class include *Aeromondales* and *Enterobacteriales.* The taxonomy is widely debated however, as the gene sequencing of the 16S rRNA, normally used as accurate genetic identification, is unreliable, since several *Vibrio* spp. have nearly identical 16S rRNA. To date the genus includes over 60 species (Thompson*, et al.*, 2004). The *Vibrio* spp. under focus in this thesis - *V. parahaemolyticus, V. cholerae* and *V. vulnificus* - consist of both human pathogenic and non-pathogenic strains, which inhabit the same environmental niche.

Characteristics
Vibrio spp. are gram-negative bacteria, straight or rod-shaped and motile, with one or more flagella. They are facultative anaerobes, i.e. with a respiratory or fermentative metabolism, chemo-organitrophs, oxidase positive, Na$^+$ stimulates their growth and they may be luminescent. The LPS - lipid A, core polysaccharide and O polysaccharide side chain - determines serological specificity. *V. cholerae* is the most extensively studied *Vibrio* sp. and includes over 200 serogroups, with O1 and O139 the two identified as causative agents of pandemic cholera and which seem to be very similar in composition. *V. parahaemolyticus* is also grouped according to antigens and by 2005, 75 combinations of the O and K antigens had been identified, of which 11 belong to the pandemic clone (Iida*, et al.*, 2001, Ansaruzzaman*, et al.*, 2005).

Several factors may be stressful for the bacteria, such as starvation and a decrease in temperature and salinity, and these may provoke them into adopting one of a number of different survival strategies. One is to produce biofilm, which has been shown to protect the bacteria from starvation, predation and UV-radiation (Elasri & Miller, 1999, Yildiz & Schoolnik, 1999, Matz*, et al.*, 2005). Another strategy is that of the non-culturable state (VBNC), which is said to represent a response to low nutrient levels or low temperatures (Colwell, 2000, Wong & Wang, 2004, Oliver, 2005b). These tactics may lead to difficulties when trying to isolate vibrios from aquatic environments.

Reservoirs
Vibrio spp*.* are found both in their natural habitat (aquatic environments) and accidentally in humans after ingestion or contact with contaminated seafood/water. The bacteria are frequently isolated from fresh, brackish and seawater, and are often found in association with other marine organisms, such as planktonic copepods and protists (Kaneko & Colwell, 1975, Sochard*, et al.*, 1979, Huq*, et al.*, 1983). The species focused on in this thesis have been shown to prefer a water temperature exceeding 17°C, a salinity of 5 to 30 PSU, and may be favored by a high plankton density in the water (Kaneko & Colwell, 1973, Motes*, et al.*, 1998, Bauer*, et al.*, 2006, Collin & Rehnstam-Holm, 2011). Indeed, this positive correlation between plankton and vibrios seems in some cases more important than actual water temperature (Chowdhury*, et al.*, 1990). However, *V. parahaemolyticus* has been grown in the laboratory at salinities as high as 80 PSU (Joseph*, et al.*, 1982, Garrity, 2005) and at 40 PSU in the field (Gonzalez-Acosta*, et al.*, 2006).

Virulence and antibiotic resistance
The majority of vibrios are harmless to humans, but strains of several species are able to cause disease (Table 1). Those most commonly isolated from patients are *V. parahaemolyticus, V. cholerae* and *V. vulnificus,* while numerous case reports and reviews of these and other human pathogenic vibrios have been published (Rubin & Tilton, 1975, Schmidt*, et al.*, 1979, Shandera*, et al.*, 1983, Colwell, 1996, Shinoda*, et al.*, 2004). The virulence genes of *V. cholerae* (*ctx, tcp* and *toxR*), *V. parahaemolyticus* (*tdh* and *trh*) and *V. vulnificus* (*vvh* and *viuB*) - are found integrated in a chromosome. Antibiotic resistance is on the other hand more often found on mobile genetic elements, which may be circulating within the aquatic environment (Heidelberg*, et al.*, 2000, Chen*, et al.*, 2003, Chen*, et al.*, 2011). These elements are easily transferred between hosts by integrating conjugative elements (ICEs), and may be present in both aquatic and clinical

strains. A summary of the species-specific illnesses and virulence patterns of the most common human pathogenic species is presented in Table 1.

Generally, antibiotic treatment is not used on patients with diarrheal diseases, with liquid and electrolyte compensation considered sufficient, but the most severe cases are treated with e.g. tetracycline and ciprofloxacin. Wound infections and septicemia are normally treated with antibiotics such as tetracycline, cephalosporin and ciprofloxacin (Pitrak & Gindorf, 1989, Liu, *et al.*, 2006, Bross, *et al.*, 2007). The antibiotic sensitivity pattern of *Vibrio* spp. isolated from different parts of the world, both from clinical and aquatic environments, has been investigated previously. Studies carried out at the U.S. governmental Centers for Disease Control and Prevention (CDC), published in the Bergey's Manual of Systematic Bacteriology (Garrity, 2005), show a lower sensitivity in *V. parahaemolyticus* compared to *V. cholerae* and *V. vulnificus.* Bhattacharya *et al.* (2000), Das *et al.* (2008) and Taviani *et al.* (2008) have presented the resistance patterns of strains from India and Mozambique, but as yet no study of strains isolated from Sweden has been published.

Vibrio cholerae
V. cholerae isolated from aquatic environments are most commonly non-pathogenic to humans. The few pathogenic strains include cholera toxin-producing strains which cause pandemic cholera; serogroups O1 (biotype Classical and El Tor) and O139 according to the LPS antigen (Banwell, *et al.*, 1970, Holmgren, 1981). Here the bacterium uses the flagellum to reach the small intestine and attaches to mucosa cells by the pili (coded by *tcp* genes; the toxin co-regulated pili). Consisting of two subunits (coded by the genes *ctxA* and *ctxB*), the cholera toxin is then engulfed by the mucosa cells which increases cell cAMP levels. This in turn leads to a stimulation of intestinal secretion-inducing neurotransmitters within the cells, followed by an increase in Cl⁻ secretion. The ion channels normally normalizing the ion balance are then blocked and large amounts of water flow into the lumen from the mucosa cells, causing massive diarrhea. Some strains belonging to additional serogroups other than O1 and O139 may also be cholera toxin-producing (Tobin-D'Angelo, *et al.*, 2008).

As well as those producing the cholera toxin, other strains of *V. cholerae* may also be pathogenic to humans, with the bacterium potentially responsible for otitis, ulcus cruris, septicemia and fatal wound infections (Dalsgaard, *et al.*, 2000). The HlyA protein represents one plausible cause of these non-cholera diseases, since it may permeabilize eukaryotic cells such as HeLa and Vero cells (Purdy, *et al.*, 2005). However, no specific genes have been definitively linked with the illness, as it may reflect a synergy between different abilities. Interestingly, Simpson, *et al.* showed in (1987) that 7 out of 12 nonO1/O139 strains inoculated in mice were lethal, compared to only one of the 12 injected O1/O139 strains.

Vibrio parahaemolyticus
V. parahaemolyticus is the most common bacterial cause of food-borne gastroenteritis and the infection itself is often associated with the ingestion of shellfish or contaminated drinking water (Joseph, *et al.*, 1982, Honda & Iida, 1993). The main symptoms of the illness are diarrhea and abdominal pain, fever, vomiting, nausea and fatigue. The primary virulence trait of *V. parahaemolyticus* is the production of thermostable direct hemolysin (TDH), although TDH-related hemolysin (TRH) may also be present. Both the TDH and TRH proteins are linked to different biological activities

(Honda & Iida, 1993), such as lysis of erythrocytes, cytotoxicity to eukaryotic cells and as a cause of diarrhea (Raimondi*, et al.*, 2000).

Only a few of the strains previously isolated from aquatic environments have carried known human pathogenic virulence genes, with 1-2% of isolated *V. parahaemolyticus* strains testing positive for *tdh* and/or *trh* (Nishibuchi & Kaper, 1995). Similar results have also been shown in this thesis. Molecular screening of bacterial genes in mussel tissue has revealed that virulence genes can be present, but that the culturability of virulence-carrying strains may be lower and that they are less competitive than those that do not carry virulence genes (Pace*, et al.*, 1997). However, strains carrying human pathogenic virulence genes have been isolated, most commonly in studies involving the screening of clinical samples and shellfish for potential causative agents of gastroenteritis outbreaks (DePaola*, et al.*, 2003a, Vongxay*, et al.*, 2008, Mahoney*, et al.*, 2010).

In 1996, the number of infections caused by *V. parahaemolyticus* dramatically increased worldwide and its first pandemic clone was recorded. Since then, the pandemic clone O3:K6 has been isolated in many parts of the world, including India, Bangladesh, Mozambique, Italy and Brazil (Okuda*, et al.*, 1997, Ansaruzzaman*, et al.*, 2005, Ottaviani*, et al.*, 2008, Ansede-Bermejo*, et al.*, 2010).

Variation in the antibiotic resistance patterns of different *V. parahaemolyticus* strains has also been reported. Commonly, isolated strains - both clinical and aquatic - have been found to be susceptible to the tested antibiotics (Okuda*, et al.*, 1997, Nair*, et al.*, 2007), but other studies have discovered increasing bacterial resistance to antibiotics such as ampicillin (Wong*, et al.*, 2000, Baker-Austin*, et al.*, 2008, Chao*, et al.*, 2009).

Vibrio vulnificus

Three different biotypes of *V. vulnificus* have been identified. Number 1 is the human pathogenic biotype, which causes gastroenteritis, primary septicemia and wound infections, and is the most lethal of all *Vibrio* species (Torres*, et al.*, 2002, Oliver, 2005a). Cases of gastroenteritis are the least severe of the three and may include diarrhea and abdominal pain; no fatalities have been reported (Hlady*, et al.*, 1993, Mead*, et al.*, 1999). In contrast, primary septicemia linked to consumption of oysters and clams is often severe, with a high hospitalization rate. This illness is the number one cause of seafood-linked death in the US (Mead*, et al.*, 1999), with a fatality rate of 50-60% largely via immunity deficiency, heart and liver failure. Infections may also occur within an existing wound (which may be as small as an ant bite) after exposure to seawater (Oliver & Kaper, 2001), with fatality rates in this instance reported to be 20-25% (Oliver, 1989). The identified virulence genes include the hemolysin genes (*vvhA*) and (*viuB*) (Panicker*, et al.*, 2004a)*.*

Other potential human pathogenic Vibrio *spp.*

Species that may cause infection in humans are presented in Table 1, which includes many species other than those focused on in this thesis. A number of species also cause illness in aquatic organisms, such as *Vibrio coralliilyticus* (coral disease) (Ben-Haim*, et al.*, 2003), *Vibrio anguillarum* (Kitao*, et al.,* 1983), *Vibrio salmonicida* (Egidius*, et al.,* 1986) (vibriosis among cultured and wild fish) and *Vibrio splendidus* (molluscs, fish and shrimps) (Vandenberghe*, et al.*, 1998, Gatesoupe*, et al.*, 1999, Lacoste*, et al.*, 2001).

Table 1. Summary of *Vibrio* spp. and human infections

Species	Human infection		
	Gastroenteritis /diarrhea	Wound/Ear	Septicemia
V. cholerae O1/O139	Yes	Yes	No
Non O1/O139	Yes	Yes	Rare
V. parahaemolyticus	Yes	Yes	Rare
V. vulnificus	Yes	Yes	Yes
V. mimicus	Yes	Rare	Rare
V. hollisae	Yes	Rare	Rare
V. fluvialis	Yes	Rare	Rare
V. alginolyticus	Yes	Yes	Yes
Photobacterium damsela	No	Yes	Yes
V. metschnikovii	Rare	Rare	Rare
V. cincinnatiensis	Rare	No	Rare
V. harveyi	No	Rare	No
V. furnissii	Rare	No	No

Bivalvia

Phylum Mollusca, class Bivalvia are aquatic organisms with soft bodies enclosed between hard $CaCO_3$ shells. They are very efficient filter-feeders (Hernroth, *et al.*, 2002, Forster & Zettler, 2004) – one kilo of the blue mussel *Mytilus edulis* filters approximately 90 liters of water per hour (Haamer, 1996) - and can create a localized water current with their lateral cilia. In this way bivalves may accumulate microorganisms from the surrounding water in their tissue (Hernroth, *et al.*, 2002). Mussels are able to control filtration and when looking at a mussel bed one can easily see both filtrating and non-filtrating individuals simultaneously.

Clams stay burrowed in the sediment and have enlarged gills that are used both for respiration and filter feeding. Tube-like mantle formations called siphons are employed in order to prevent sediment from entering the exhalant openings. In contrast, mussels such as the common blue mussel *Mytilus edulis* live in the water column, attached to hard surfaces by byssus threads. As bivalves are frequently exposed to microorganisms, their cellular immune system needs to be very efficient. Usually they are able to rapidly clear tissue of microorganisms, but some pathogens can prove more resistant to their immune defense. The innate immune response of bivalves consists of both cellular defense, which includes phagocytosis, and degradation by lytic enzymes antimicrobial peptide activity, and humoral defense involving lysosome, agglutinins and antimicrobial peptides. Characterization of hemocytes has shown that they consist of different cell types, including hyalinocytes and basophilic and eosinophilic granular cells (Pipe, 1990, Pipe, *et al.*, 1997, Canesi, *et al.*, 2002, Hernroth, 2003a, Hernroth, 2003b, Ottaviani, 2006).

Study areas

Mozambique and Sweden differ in many respects, including in terms of their climate, socio-economy, infrastructure and diet. The two countries have also been affected rather differently by vibrios. Several epidemics of both cholera and infections caused by *V. parahaemolyticus* have struck Mozambique in the last decade. According to the WHO

(2009), cholera has been endemic in Mozambique since at least 1973 and cases have been reported almost weekly since October 2007, with most occurring during the rainy warmer season from December to March. The latest epidemic was recorded in January 2009, with approximately 13 000 cases of which around 120 proved fatal. Screening of water and sediment revealed that *V. cholerae* was present during the epidemic, with the pandemic serotypes O1 and O139 isolated in the Beira area of Mozambique, situated north of the capital Maputo. In May 2004, infections caused by *V. parahaemolyticus* were reported from the same area (Ansaruzzaman*, et al.*, 2005). 81% of strains were identified as the pandemic serovar (O3:K6 and O4:K68) and all strains were *tdh+*.

Although no vibrio epidemic has been recorded in Sweden since the beginning of the 19th century, as people today are frequent travellers, Swedes may be exposed to gastroenteritis caused by vibrios at tourist destinations, with the bacteria then brought back to Sweden either with returning tourists or in contaminated shellfish sold at the local grocery store. However, human pathogenic vibrios are in fact present in Swedish waters; severe and even fatal wound infections caused by *V. cholerae* have been recorded in the country, primarily in patients who had been in contact with the Baltic Sea. Case reports from countries along the Baltic coast has been reported by a number of authors (Bock*, et al.*, 1994, Melhus*, et al.*, 1995, Dalsgaard*, et al.*, 2000, Ruppert*, et al.*, 2004, Lukinmaa*, et al.*, 2006, Shönning*, et al.*, 2008). However, none have focused on the presence of potentially human pathogenic vibrios in the aquatic environment.

AIMS OF THE STUDIES

The primary objective of this thesis was to study the occurrence and characteristics of vibrios in aquatic environments, as well as the persistence of human pathogenic strains when encountering an aquatic environment that is clearly different from their human hosts. This was achieved through both field study in Mozambique and Sweden and laboratory-based microcosm studies. Bivalvia were used as the host organisms for vibrios.

The specific aims were to:

- Investigate the seasonal distribution of vibrios in clams and water samples from Maputo Bay, Mozambique.
- Characterize the Mozambican strains in terms of their antibiotic resistance, virulence and biochemical diversity, and to compare these properties with those of strains from tropical (Indian) and boreal (Swedish) waters.
- Investigate the presence of potential human pathogenic vibrios in the Sound between Sweden and Denmark (Öresund) and the eukaryotic cell toxicity of isolated strains.
- Study the uptake and persistence of marine and clinical (isolated from a wound infection) *V. cholerae* strains when exposed to the common blue mussel *M. edulis.*
- Study, in laboratory experiments, the persistence of clinical *V. cholerae* strains when exposed to low water temperatures and natural sediment.

The main questions raised for the experiments were:

- Are human virulent strains favored by higher water temperatures?
- Is a low water temperature always unfavorable for vibrio strains?
- Can strains isolated from aquatic environments be harmful to eukaryotic cells?
- Is the virulence and antibiotic resistance pattern different in strains of the same species from different areas of the world?
- Do the blue mussel accumulate and eliminate bacteria independently of the latter's level of pathogenicity?
- Are human clinical strains persistent in aquatic environments?

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis will be discussed in the following section. A more detailed description of these methods is included in papers I-IV.

Study area description
Mozambique is a sub-Saharan country situated on the southeast coast of the African continent, with its 2400 km of coastline facing the Indian Ocean. Maputo, the capital, is situated by the Maputo Bay, in the southern part of country. Two rivers discharge into the Bay; the Maputo River and the N'komati River. The study area Costa do Sol is found in the northern part of the city, 25°54'52"S, 32°38'55"E (Fig. 1).
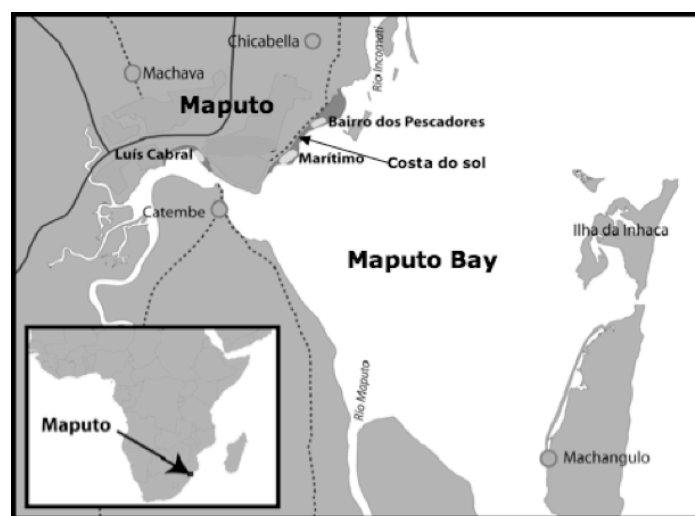

Fig. 1. Map of Maputo Bay showing the sampling site at Costa do Sol (paper I). Originator: Lars-Ove Loo

In tropical and sub-tropical developing countries, a high percentage of wastewater is discharged untreated into the ocean where seafood is gathered, which constitutes a major health hazard. In Maputo, most waste drains are situated in residential areas and approximately 30% of buildings are connected to the sewer system. However, the Maputo storm water system is supplied with the overflow of the Maputo river and some buildings dump their wastewater into this system (CERA, 2012). In any case, although the local treatment plant is well maintained, it lacks chemical treatment facilities. Groundwater contamination from pit latrines and storm water effluent currently pollutes Maputo Bay to the extent that swimming is inadvisable in many areas. The Ministry of Health tests fecal coliform levels regularly, with a general ban on consumption of shellfish from the bay enforced in 2001. However, people living close to the shoreline are highly dependent on fishing, while the collection of clams for either family consumption or for sale at the local market is common practice among women in Maputo (De Boer, *et al.*, 2002). The gathering is simple and no advanced equipment is needed, which contributes to the locally widespread consumption of molluscs. Earlier studies have shown a high volume of fecal contaminants to be present in the clams, and the area would be categorized as non-usable for human consumption according to EU-standards (Collin, *et al.*, 2008). According to the WHO, the country suffers from a 14.2% mortality rate for children under the age of 5, of which 11% is a direct result of

diarrheal disease (2009). In 2010, 7430 cases of cholerae were recorded in Mozambique (WHO, 2010).

Sweden is a country in northern Europe with surface coastal waters ranging in salinity from 35 PSU on the northwest Skagerrak coast, to 0.1 PSU in the northern Bay of Bothnia. In the Sound, saline water mixes with brackish water. The surface water consists of a north flowing, low density, brackish water from the Baltic Sea. At deeper layers (normally a depth of 10 to 12 meters), a south going current with more dense water from the Kategatt and the Atlantic Ocean supplies the Baltic Sea with salty water. Mussels were collected from collection sites at Domsten 56°06'58''N 12°36'12''E and Råå 55°59'31''N 12°44'30''E (Paper II), water and sediment collected in Lomma Bay 55°40'37''N 13°03'24''E (Paper IV) (Fig. 2).
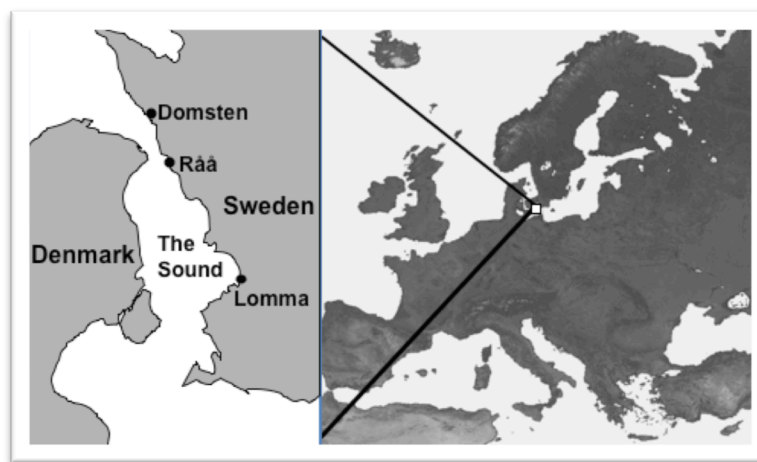

Fig. 2. Map showing the sampling sites in the Sound (paper II). Originator: Betty Collin

Sweden has not been hit by diarrheal cholera since the early nineteenth century. However, imported crayfish contaminated with *V. parahaemolyticus* have been known to cause diarrheal cases and at one given outbreak, 350 instances were reported, which was an exceptionally high number. Cases originating outside the country constitute approximately half of those recorded each year (Table 2), with the countries of origin representing the most common Swedish vacation destinations, e.g. Thailand, Spain and Greece (SICDC, 2012). Among the clinical cases of Swedish origin, the majority have been identified as *V. cholerae* non-O1/O139; the statistics show that these bacteria cause external otitis more commonly among younger patients (up to 30 years old) and gastroenteritis more commonly among older patients (50 years and older). It has also been demonstrated that men are more often infected by vibrios than women. As *V. cholerae* was previously only associated with the feared cholera, hospital wards were surprised when they isolated this species from otitis and wound infections.

Table 2. Number of *Vibrio* infections recorded in Sweden (source: Swedish Institute for Communicable Disease Control).

Year	2004	2005	2006	2007	2008	2009	2010	2011
Domestic cases	3	6	26	12	11	10	16	10
Total number	8	25	41	22	24	20	30	20

The statistics also reveal *V. cholerae* infections to be more common among patients infected on the east coast, where the salinity is lower, while *V. parahaemolyticus* and *Vibrio alginolyticus* are more common on the more saline west coast. Just a single clinical case of *V. vulnificus* has been recorded in Sweden since 2006. This bacterium is known to be responsible for several lethal infections in people who had been in contact with the Sound, mostly on the Danish coast. The frequency of recorded *Vibrio* infections is shown in Table 2.

COLLECTION AND PREPARATION OF WATER SAMPLES AND CLAMS

Molluscs, water and sediment
Both clams and mussels were studied in this thesis. In paper I, edible clams, *Meretrix meretrix, Eumarcia paupercula* and *Scincilla bologna*, (Fig. 3) collected from Maputo Bay (Costa do sol) were screened for seasonal variation of vibrios. The common blue mussel *Mytilus edulis* was chosen for both occurrence screening in the Sound (paper II) and for the *in vivo* test (paper III), as it is the most abundant edible species in Swedish coastal waters.

Fig. 3. Clams bought from collectors in Maputo Bay. Photo: Betty Collin

Water samples and clams were collected in Maputo Bay (Fig. 1) at low tide on four different occasions during a single year: early rainy (November); late rainy (March); early dry (May); and late rainy season (August) (paper I). Twelve separate sampling days were chosen in each season according to the availability of gatherers, i.e. when the tide was low. Two separate batches of clams were bought from collectors (most commonly women and children) gathering clams at a popular harvest site in the northern part of Maputo (Fig. 4). In parallel, two separate batches of water were collected in clean 1.5L bottles.

Mussels from two different sampling sites in the Sound (Fig. 2), Domsten and Råå, were collected from June through September 2006 on twelve separate sampling days (paper II).

Clams and mussels were prepared in similar fashion, with each individual scrubbed and rinsed with distilled water before being opened with a sterilized shucking knife. All tissue, including liquid, was subsequently collected in a sterilized blender and sequentially homogenized for two minutes at maximum speed. The resulting homogenate was spread onto agar plates to produce colony forming units (CFU).

Fig. 4. Collectors at the gathering site of Costa do Sol in Maputo Bay. Photo: Stina-Mina Ehn-Börjesson

Water samples were filtered through a 0.22 μm filter, with the filters then cut in half along the grids on the vacuum pump with a pair of sterilized scissors and tweezers. After placing the cut filters onto agar and incubating at 37°C overnight, CFU were subsequently counted. Culture from the pre-enrichment stage was also spread onto agar plates and incubated at 37°C for approximately 24h. Colonies from both plates were picked and transported in Liquid Media Transport Swabs tubes (COPAN Italia S.p.a., Brescia, Italy) to Sweden for identification, together with extracted DNA. The colonies grown in Sweden were directly identified as described below.

Water and sediment for the microcosms in paper IV were collected in Lomma Bay in sterile flasks.

COUNTING, ISOLATING AND IDENTIFYING VIBRIOS

CFU and pre-enrichment

A few standard methods have been presented as preferable when isolating *Vibrio* spp.: Marine, Thiosulfate Citrate Bile Sucrose (TCBS) and colistin-polymyxin B-cellobiose (especially for *V. vulnificus*) agar. TCBS is generally considered an ideal selective medium when isolating vibrios from aquatic environments. However, in order to isolate vibrios from an environmental sample, pre-enrichment may be required. According to most protocols, pre-enrichment should preferably be performed in Alkaline Peptone Water (APW) with a pH of 8.0-8.5 and 2% NaCl. In order to isolate different *Vibrio* spp. it has been suggested that samples should be extracted from a pre-enrichment stage and recultivated on TCBS agar after different time intervals, thus achieving earlier isolation of *V. cholerae* (after 6h) with respect to *V. parahaemolyticus* (after 18-24h) (Farmer, *et al.*, 2003, DePaola & Kaysner, 2004), (Analytical methods, Detection isolation (NMKL No 156, FDA, ISO/TS 21872-1)). This method of isolating different *Vibrio* spp. was performed in papers I and II.

Biochemical identification

Isolated strains were identified via use of the biochemical API 20NE (bioMérieux Inc., Hazelwood, USA). In paper I the PhenePlate™ System (PhPlate AB, Stockholm, Sweden), which works by evaluating the kinetics of biochemical reactions, was used in order to

verify the API 20NE identification results, as well as to produce a dendrogram of the isolates of different origin. Strain identification was then confirmed molecularly.

Total cell count, identification and virulence screening
PCR: The presence of vibrios in aquatic environments and total count of vibrios in microcosms was investigated on a molecular basis in papers II and IV (performed but not presented in paper I). DNA was extracted from samples via different methods depending on sample type: from mussel/clam samples in paper II (and in paper I) using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), from water samples in paper IV (and I) using the Blood & Tissue kit (Qiagen, Valencia, USA) and from sediment samples in paper IV using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, USA). PCR targeting of genes (presented in Table 3) was used in order to detect and verify the identification of vibrios in environmental samples and microcosms. In paper II, conventional PCR was used qualitatively to determine whether genes were present in mussel samples. In paper IV, real-time qPCR (7500 Real Time PCR System (Applied Biosystems)) was employed in order to quantify vibrios in samples. Standard curves were developed using serial dilutions of samples with known DNA concentration, with the concentration of vibrio cells then calculated. The target genes and PCR references are presented in Table 3.

Table 3. Primers and probes used in papers I, II and IV

Vibrio spp.	Gene	Sequence	Reference	Paper
Vibrio spp. primers	16S rRNA (total count)	F' – GGC GTA AAG CGC ATG CAG GT R' – GAA ATT CTA CCC CCC TCT ACA G	Thompson *et al.* (2004)	IV
Vibrio spp. probe	16S rRNA (total count)	5' – XGG CGT AAA GCG CAT GCA GGT	paper I	I
V. cholerae	*toxR* (regulatory protein)	CCT TCG ATC CCC TAA GCA ATA C AGG GTT AGC AAC GAT GCG TAA G	Panicker *et al.* (2004b)	II
primers	*ctx* (cholera toxin)	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCGGTATTACG	Brasher *et al.* (1998)	II
V. parahaemolyticus	*tlh* (thermolabile hemolysin)	AAA GCG GAT TAT GCA GAA GCA CTG GCT ACT TTC TAG CAT TTT CTC TGC	Panicker *et al.* (2004b)	II
primers	*tdh* (thermostable hemolysin)	GTA AAG GTC TCT GAC TTT TGG AC TGG AAT AGA ACC TTC ATC TTC ACC	Panicker *et al.* (2004b)	II
	trh (TDH- related hemolysin)	TTG GCT TCG ATA TTT TCA GTA TCT CAT AAC AAA CAT ATG CCC ATT CCC G	Panicker *et al.* (2004b)	II
V. parahaemolyticus	*tlh* (thermolabile hemolysin)	5'-XAA AGC GGA TTA TGC AGA AGC ACTG-3'	McCarthy *et al.* (1999)	I
probes	*tdh* (thermostable hemolysin)	5'- XGG TTC TAT TCC AAG TAA AAT GTA TTT G -3'	McCarthy *et al.* (2000)	I
	trh (TDH-related hemolysin)	5'- XCA TAT GCC CAT TTC CGC TCT CAT ATG C -3'	Raghunath *et al.* (2007)	I
V. vulnificus	*vvh* (hemolysin)	TTC CAA CTT CAA ACC GAA CTA TGA C ATT CCA GTC GAT GCG AAT ACG TTG	Panicker *et al.* (2004b)	II
primers	*viuB* (iron acquisition)	GGT TGG GCA CTA AAG GCA GAT ATA CGG CAG TGG ACT AAT ACG CAG C	Panicker *et al.* (2004b)	II

Sybr®Green was used during qPCR, while visual judgment of the terminating melting plot was employed to verify accuracy in amplicons.

Probe hybridization
Probe hybridization was carried out in order to confirm the biochemical identification of Mozambican *V. parahaemolyticus* strains in paper I, according to the FDA Bacteriological Analytic Manual. As no specific DNA-extraction is needed, this method is useful when screening numerous isolates. Bacteria were lysed on filters and the chosen probe hybridized onto the filter, as shown in the photos in Fig.5.

Fig. 5. Left: The lysed filters, ready to be transported to Sweden when dried. Right: A hybridized filter. Bluish colonies are counted as positive and white/brownish as negative. The total number of colonies was first counted and the percentage of positive colonies calculated after hybridization. Photos: Betty Collin

LABORATORY EXPERIMENTS

To study the persistence of the bacteria when exposed to variation in a single environmental factor, laboratory-based research is often performed. In this thesis, a number of microcosm experiments were carried out in order to examine the effect of temperature, as well as to evaluate the effectiveness of natural sediment as a potential reservoir for pathogenic *V. cholerae* in cold water conditions. For the investigation of cell toxicity to Chinese Ovary cells (CHO-cells) (paper II) and the killing index of vibrios when exposed to mussel hemocytes (paper III) *in vitro*, microplate systems were used.

Mussels
In paper III, bacterial uptake and elimination by *M. edulis* was investigated. The experiment was performed at the Sven Lovén Centre for Marine Sciences at Kristineberg, which is located on the northwest coast of Sweden. Mussels were collected near the Centre and immediately placed in acclimatization buckets for two days prior to the experiment start. The animals were then transferred to buckets containing 2.5 l natural seawater, each of which were inoculated with one of the different *V. cholerae* strains to a final concentration of 5.0×10^6 bacteria/ml. The mussels were analyzed on four occasions after the start of the experiment to measure the extent of bacterial uptake and elimination.

Water and sediment microcosms
In paper IV, the effect of low temperatures on *V. cholerae* was investigated. Seawater was collected from Lomma Bay. The experiment was broadly prepared as described above, although here 40 mL water was added to 50mL cell culture in suspension flasks (NUNC) and the microcosms incubated at 4°C. After 21 days, the temperature was increased to 20°C and the microcosms incubated for an additional 7 days. All microcosms were protected from light for the entire duration of the experiment. The CFU (i.e. the number of culturable cells) was compared with the total cell number (as calculated by qPCR).

In paper IV, the persistence of a clinical *V. cholerae* strain in natural bottom sediment at 4°C (experiment performed in February) was also investigated. The chosen sandy surface sediment was collected in Lomma Bay at a water depth of 0.5 m on the

same day as the start of the experiment, with the sediment visually inspected before use. The experimental design was identical to that involving the water microcosm, except for the addition of 20g sediment and 20 ml sterile filtered seawater to 50ml flasks. On each sampling occasion, 1 ml was extracted from the sediment surface and analyzed for CFU, while DNA was extracted from 200 µl for total *Vibrio* cell count.

Fig. 6. A sediment microcosm. Photo: Betty Collin

In vitro *cell toxicity test*
Cell toxicity on Chinese Hamster Ovary cells (CHO-cells) of the vibrios isolated from the Sound was investigated *in vitro* (paper II). CHO-cells were exposed to the different vibrio strains for 1 hour, with the former's survival then measured via the colorimetric method described in paper II. Briefly, a tetrazolium dye, which is converted to a formazan product by dehydrogenase activity only produced by living cells, was added as a substrate. The formazan produced is proportional to enzyme activity and could thus be measured in a microplate reader. The absorbance of wells containing only CHO-cells or only bacteria was compared with that of wells in which CHO-cells and bacteria had been mixed, with the results reported in terms of a percentage killing index (KI%). The survival of bacterial cells after exposure to mussel hemocytes was tested *in vitro*, as described in paper III. Formazan was again measured and the survival index of bacteria presented as a percentage (survival index, SI%).

Antibiotic resistance pattern
The sensitivity of the isolated *V. parahaemolyticus* strains to antibiotics was tested via the disc diffusion method and was performed in clinical microbiology laboratories at either the Central Hospital of Kristianstad or Lund University Hospital. The antibiotics tested were ampicillin, cefadroxil, tetracycline, trimethoprim-sulphamethoxazole, ciprofloxacin, nalidixic acid, cefuroxime, gentamycin and chloramphenicol. These drugs are standard for vibrio treatment in Sweden and, according to registration protocols kindly provided by the Health Ministry in Maputo, also include the most commonly used antibiotic in Mozambique (nalidixic acid). Briefly, bacterial colonies were diluted in 0.85% NaCl and spread onto Müller Hinton agar. Filter paper discs containing different antibiotics were placed on the inoculated agar plates which then were incubated overnight. The resultant inhibition zones were measured and the resistance pattern calculated according to the Swedish Reference Group for Antibiotics (www.SRGA.org).

Evaluation of cultivation and identification methods used in the screening of Vibrio *spp.*

Enumeration: TCBS agar was consistently used for cultivation in all studies included in this thesis. It is possible that the results would have differed slightly if other media had been chosen. For example, earlier investigations have shown that other bacterial species grow on these plates, such as *Aeromonas* spp. and *Shewanella* spp., and that different brands of TCBS vary in the recovery of spiked samples (Nicholls*, et al.*, 1976). Here, the same brand of TCBS (Merck, Darmstadt, Germany) was used in each experiment, which allowed all results to be validly compared. Recently, HiCrome agar has been suggested as a suitable alternative to TCBS. In a recent unpublished pilot study carried out in our laboratory, it was shown that TCBS and HiCrome agar favor different *Vibrio* spp., with the growth of one *V. cholerae* strain and one *V. parahaemolyticus* strain tested on each. The results showed fairly low recovery rates occurring after pre-cultivation in Brain Heart Infusion (BHI), and that TCBS agar favors *V. cholerae* while HiCrome agar favors *V. parahaemolyticus.* This difference may influence study results, especially considering *Vibrio* populations alter throughout the seasons, as was the case in our analysis of clams from Mozambique. However, if this study was significantly biased by the choice of agar, CFU numbers should have been the highest when the *V. parahaemolyticus* percentage was lowest. In the present study the opposite pattern was observed; *V. parahaemolyticus* was the dominant isolated species on all sampling occasions with the exception of the early rainy season, with the lowest number of *Vibrio* recorded during the late dry season when the percentage of *V. parahaemolyticus* was at its highest.
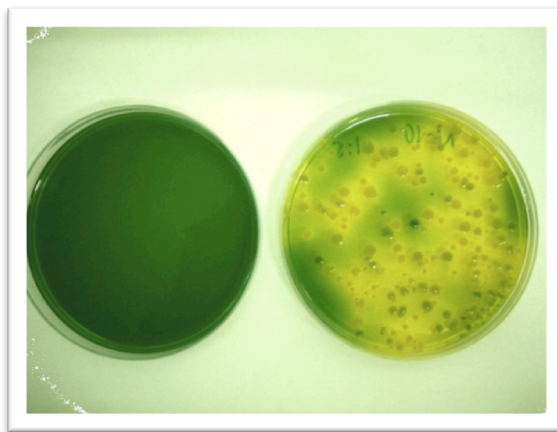

Fig. 7. *Vibrio* spp. growing on TCBS agar. Photo: Betty Collin

Pre-enrichment: Pre-enrichment of *Bivalvia* homogenate was performed in papers I and II following the manuals presented in the material and method section. However, the results showed that after isolating *Vibrio* spp. according to this method, the majority of species identified after 6h were *V. alginolyticus,* not *V. cholerae*. This result thus strengthens the idea that samples contained a very small number of *V. cholerae*. The occurrence of *Vibrio* spp. in clams and mussels in papers I and II, and in laboratory experiments in papers III and IV, was therefore measured in terms of CFU on TCBS agar without pre-enrichment.

Identification: The strains isolated from water samples and mollusc homogenate collected from Maputo Bay (paper I) and the Sound (paper II) were identified using API 20NE, a biochemically-based identification method. However, when comparing this data with the identification results derived from the PhP we could see that several strains were identified differently. When confirming identity on the 16S rRNA level, it was possible to reach several conclusions: the selectivity of the TCBS agar is quite low, as shown by the percentage of strains identified as vibrios during the sampling year (96-40%); additional strains were then excluded after the PCR based species-specific gene tests. This resulted in quite a low percentage of *Vibrio* spp. identification, which may be due to different factors: *i:* the API 20NE system is optimized for clinical isolates and thus will not ensure 100% identification of species that are to date classified as non-pathogenic, *ii:* the bacterial species in question are molecularly quite similar, with strains of the *Vibrionaceae* genus in particular having almost identical 16S rRNA sequences and therefore not easily differentiated.

Comparison of strains: When tested biochemically, the *V. parahaemolyticus* strains from Mozambique, Sweden and India were very similar, while a large number of the tested strains from Mozambique and India were identified as being of the same phenotypical clone. This pattern has been shown earlier, in *V. parahaemolyticus* strains isolated from different Asian countries (Rahman*, et al.*, 2006) and on the Adriatic coast (Barbieri*, et al.*, 1999). However, it was interesting to see an almost identical relationship between strains from India and Mozambique, as it has been suggested that the pandemic *V. parahaemolyticus* clone from Asia has travelled from India to Mozambique, causing illness among inhabitants. Virulence analyses and antibiotic resistance patterns are evenly spread throughout the dendrogram, which indicates that biochemical fingerprinting is not suitable for further characterization of the strains. However, this method may be useful for the identification of a large number of strains during an epidemic, especially as both the medium and equipment are relatively inexpensive.

Molecular analyses of extracted DNA and accuracy of chosen primers

DNA extraction and analysis: In the studies presented in papers I, II and IV, DNA was analyzed molecularly (not reported in paper I) in order to investigate the occurrence and total cell count of vibrios in samples. For the screening of molluscs, water and sediment, different DNA extraction kits were used, as described earlier. The DNA concentration of samples was measured in a BioPhotometer (Eppendorf). The DNA extracted from mussels, water and sediment at the Swedish laboratory was found to be of high quality when analyzed. In contrast, the DNA content in samples from Mozambican clams differed greatly between samples. Double-stranded DNA was then measured using the picogreen method, which showed that DNA was totally degraded and thus further analysis was excluded. The actual reason for this remains unclear, since the extraction kit used was chosen due to the high recovery rates observed in earlier investigations (Lothigius, 2009). However, the temperature in the Mozambican laboratory may have exceeded the upper limit for kit storage due to occasionally electricity failure that may have affected the functioning of the air-conditioning system. These conditions may not only have damaged the buffers, proteinase K or tubes, but may also have affected the extracted DNA which was kept refrigerated during the sampling period and thus may also have been subjected to temperature variation. The exact temperatures experienced during the 24 h transport to Sweden are unclear, but

upon arrival, samples were transferred to -20°C and kept frozen until analysis. In contrast, DNA extracted from Mozambican water samples was intact and ready to be analyzed, which indicates that the use of the extraction kit caused the degradation in clam-derived DNA and not its storage once extracted.

PCR and real-time qPCR primers: In both the occurrence study presented in paper II and the experimental studies in paper IV, the presence of vibrios was investigated molecularly. This proved to be a useful method as recovery of vibrios from aquatic environments on TCBS-agar plates was shown to be low (paper IV, Table 4), while strains carrying virulence genes are also low in number in aquatic environments and would therefore be even harder to find.

As analysis of DNA obtained from Swedish mussels in paper II was qualitative – i.e. no cell count was performed – we used a semi-nested approach. A second round of PCR was carried out which involved the addition of the PCR product instead of DNA in the second amplification run. This approach was taken for two reasons: *i:* the detection limit of PCR is higher than that of qPCR and *ii:* the samples required dilution as their DNA concentration was too high and possible PCR inhibitors may have been present in the mussel homogenate. In order to exclude primer dimer effects, the same procedure was performed on the negative control. Primers targeting species-specific genes coding for virulence factors or housekeeping genes were chosen (Table 1).

Table 4. Culturability of the *V. cholerae* strains exposed to low temperature, calculated as CFU number divided by the total *Vibrio* spp. count according to qPCR (paper IV).

	Marine strain in seawater	Clinical strain in seawater	Clinical strain in sediment
Days	**CFU (%)**	**CFU (%)**	**CFU (%)**
0,2	0,14	<0,00	93,1
1	0,07	0,01	0,28
4	0,06	<0,00	0,49
8	0,09	0,01	1,62
14	0,12	<0,00	0,17
21	0,05	<0,00	0,03
22	<0,00	<0,00	0,20
28	<0,00	0,02	0,26

Temperature up-shift from 4°C to 20°C (between days 21 and 22)

In the experimental studies presented in paper IV, microcosm total cell count was analyzed via real-time PCR. 16S rRNA was targeted with the primers shown in Table 3. As the samples were spiked, only one primer pair was used. Standard curves were included in each run, as described in paper IV.

Occurrence of Vibrio *spp. in Mozambican clams*

The presence of vibrios in aquatic environments has been frequently studied, with strains isolated from both the water column and shellfish (Colwell*, et al.*, 1977, Hervio-Heath*, et al.*, 2002, DePaola*, et al.*, 2003b, Gil*, et al.*, 2004, Ottaviani*, et al.*, 2005, Lhafi &

Kuhne, 2007, Du Preez, *et al.*, 2010, Collin & Rehnstam-Holm, 2011). However, in some areas the presence of vibrios has not yet been investigated. In Maputo Bay, earlier research has examined the occurrence of enteric viruses and bacteria, with the results indicating a high incidence of Hepatitis A and *Salmonella* spp. in the area (Nenonen, *et al.*, 2006, Collin, *et al.*, 2008). Case reports from Maputo and environmental studies from Beira, located north of Maputo (Cliff, *et al.*, 1986, Mandomando, *et al.*, 2007b, Du Preez, *et al.*, 2010), have also been published. In our study the CFU content of clam homogenate showed higher numbers occurring when water temperatures were high.

Mussels may be used both in screening studies as indicators of bacterial occurrence in the water body and evaluated as a food resource. However, there are certain precautions that should be taken when interpreting data. Firstly, highly pathogenic strains may not accumulate in mussels, as shown in paper III, which can give false negative results. Secondly, it has also been shown that some pathogenic strains may need additional bile to grow on agar plates, which again may result in a deceptively low number of pathogenic strains being identified (Pace, *et al.*, 1997). This is in contrast to the results presented by Matté et al. (1994), who assumed, after comparing the numbers of CFU found in water and mussels, that the abundance of fecal bacteria in the two was comparable. However, if studied molecularly their results may have been different – especially regarding human pathogenic strains. Additionally, in the Matté study, mussel tissue was pre-enriched prior to the CFU count, which may have given a false negative result. Pre-enrichment prior to quantification is now a commonly used method, with many papers presenting CFU counts after enrichment for *Vibrio* numbers both in environmental samples and in laboratory studies (Croci, *et al.*, 2002, Bauer, *et al.*, 2006, Das, *et al.*, 2008). However, this was shown in paper I to be misleading. Moreover, as the percentage of vibrios of total bacteria on TCBS agar differs between seasons, exact counts can be rather inaccurate, while other authors have shown that there are differences in the number of *Vibrio* grown on TCBS agar of different brands (Nicholls, *et al.*, 1976), as mentioned earlier.

In addition to the CFU study, DNA was extracted from the homogenized clam tissue and water samples. Unfortunately, the DNA obtained from the clams was degraded and could not be analyzed as discussed earlier. However, that extracted from seawater was found to be intact and was able to be analyzed for total *Vibrio* number, targeting genus specific 16S rRNA (Table 3). This analysis revealed a significant difference between CFU numbers in clam tissue and the water body, as can be seen in Figure 8 (not discussed in paper I).

The results show that the total number of vibrios in the water peaked during the late dry season, a period in which not only were temperatures low (22°C) and salinity high (40 PSU), but also in which CFU numbers were at their lowest. This pattern may have many different explanations: *i*: when water temperatures are high, UV-radiation is also high and thus a higher number of bacteria take refuge in sediment; *ii*: the clams may have been stressed at these higher temperatures and therefore did not eliminate the bacteria as efficiently, resulting in higher bacterial numbers in mussel tissue than in the water; *iii*: as the *Vibrio* population differed between seasons, so the species may also grow differently well on the agar.
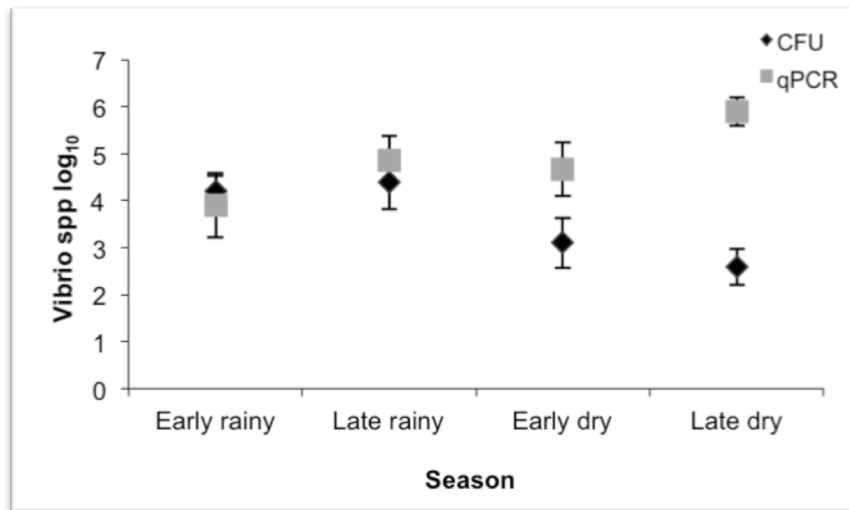
Fig. 8. The occurrence of *Vibrio* spp. in water (qPCR) and clams (CFU) from Maputo Bay.

Occurrence of Vibrio *spp. in Swedish mussels*

In Sweden, no screening of potential human pathogenic *Vibrio* spp., similar to that presented in paper II, had been performed at the time of study, despite the number of recorded infections caused by vibrios at Swedish hospitals (Collin & Rehnstam-Holm, 2011). Research that has been carried out has largely been ecologically-centered, focusing on population densities along the Swedish coast (Eiler, *et al.*, 2006), while screening studies of vibrio occurrence in mussel cultivating areas have been presented from neighboring Norway (Bauer, *et al.*, 2006). However, analysis of cultivated mussels may lead to false negative results, as it has been shown that only a very small percentage of strains in aquatic environments carry virulence genes and that they are easily missed amongst the vast majority of non-virulent strains. The occurrence of *V. vulnificus* was investigated in the Danish part of the Sound in 1994 (Høi, *et al.*, 1997), but no human virulence traits were reported, while incidences of the disease in humans and screening findings have been reported from the German Baltic coast (Frank, *et al.*, 2006) and from Finland (Lukinmaa, *et al.*, 2006). However, publications examining the presence of vibrios in the environment are few and none have focused on the actual occurrence of potential human pathogenic *Vibrio* spp. in the Baltic Sea.

The results of the molecular analysis of mussel samples from the Sound presented in paper II show that during a summer with relatively high water temperatures, virulence genes in *V. parahaemolyticus* and *V. vulnificus* were present on 13 out of 15 dates, i.e. on 87% of occasions. No vibrios were detected in either of the two remaining samplings, the dates of which coincided with water temperatures of below 17°C (in June and September). The screening of *V. cholerae* virulence genes was restricted to *ctx*, which is not associated with the occurrence of wound infections recorded among Swedish patients, and none of the samples were *ctx* positive. However, as shown in paper III, one clinical and one marine strain were screened for an expanded set of virulence genes, with the results showing them to be both *hly* A and *rtx* positive. This wider gene set would likely be of interest in future screening studies, as they were present in the wound infection-causing strains. In any case, the *ctx* gene is always of interest and earlier studies have shown that environmental strains may carry this gene

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(Rivera*, et al.*, 2001, Blackstone*, et al.*, 2007). Regarding *V. vulnificus,* the strain isolated in our study was carrying the *vvh* gene, which codes for a hemolysin. However, when the strains isolated (four *V. cholerae,* three *V. parahaemolyticus* and one *V. vulnificus* strain) were tested for pathogenicity to CHO-cells, the results revealed that the CHO-cells were very sensitive to the bacteria, with all tested vibrio strains having a high killing index, i.e. killing >90% of the eukaryotic cells. Even if the latter are not the normal target cells for the bacteria, our findings show that the bacteria express cell toxic products to a significantly higher level than the tested *Escherichia coli* strain (KI 33%). The effect of environmental vibrios on human intestinal epithelial cells would surely be of future interest.

These two occurrence studies are the first to demonstrate the presence of potential human pathogenic vibrios in these particular study regions. As both adjoin shellfish-culture sites and recreational areas, tests for vibrios should be carried out regularly. According to the EU, fecal contamination is currently still the only microbial control test performed, which may encourage false negative results due to the fact that vibrios are natural inhabitants of aquatic environments while human pathogenic strains may be very persistent in shellfish. We could also expect that the number of human pathogenic strains in clam/mussel tissue may be higher than reported, due to many virulence genes being not yet identified.

Is antibiotic resistance evenly spread among strains of different origin?

As shown in paper I, the antibiotic resistance pattern varied between strains of different origin, with the Mozambican *V. parahaemolyticus* strains less resistant to the tested antibiotics than those from India and Sweden. The Swedish strains in particular showed the most pronounced pattern, having a high resistance to both cefuroxime and chloramphenicol. Cefuroxime, a β-lactam antibiotic, inhibits the synthesis of cell walls and is not normally used to treat infections caused by *Vibrio* spp., but chloramphenicol is a broad-spectrum antibiotic which inhibits protein synthesis and may be efficient against cholera. Some of the Indian strains were resistant to tetracycline, which inhibits protein synthesis and is normally the first choice treatment of cholera.
Both tetracycline and chloramphenicol are widely used in many countries due to their low cost. Additionally, all strains were non-sensitive to ampicillin and cefadroxil, which are both broad-spectrum antibiotics and may be used in *Vibrio* treatment. During cholera outbreaks in Mozambique, strains have shown resistance to several antibiotics, including ampicillin, chloramphenicol and tetracycline (Cliff*, et al.*, 1986, Dalsgaard*, et al.*, 2001a, Mandomando*, et al.*, 2007a). Despite this, all isolates were sensitive to ciprofloxacin, another recommended antibiotic for *Vibrio* treatment. However, recent studies have shown a reduced sensitivity to this antibiotic (Quilici*, et al.*, 2010, Islam*, et al.*, 2011, Nelson*, et al.*, 2011).

Does Mytilus edulis *react differently to* V. cholerae *strains of varying origin?*

As described earlier, mussels and clams are filter-feeding organisms able to accumulate large amounts of bacteria from the surrounding environment. Bivalves may control filtering by closing their shells, which is a normal reaction to danger or events of high particle concentrations in the water (Hernroth*, et al.*, 2000). In paper III, we investigated whether mussels reacted differently to *V. cholerae* strains of varying origin,

using the same final bacterial concentration in the water. One clinical strain, isolated from a wound infection, was compared with one marine and one highly pathogenic reference strain (O1 biotype El Tor). The results revealed that the mussels accumulated the marine strain to a much higher level than they did the others, and when exposed to the El Tor strain in particular, they closed their shells even after extension of exposure time. However, the mussels also filtered continually throughout exposure to the clinical strain, which indicates that they either let the bacteria quickly pass through the digestive glands to be eliminated in feces, or rejected them at the gills as pseudo feces. This is a reaction often exhibited by mussels when the concentration of food is too high. Since bacterial concentrations were equal in all three experimental groups, it seems more likely that the mussels could recognize - and therefore reject - certain bacteria at the gills. Such behavior has previously been observed in mussels encountering different virulent strains of *Salmonella enterica* with modified cell surface properties (Hernroth, 2003a)*. Another possibility is that filtration activity was inhibited by the highly virulent strain, although such toxicity has to my knowledge not been reported. We could also see that mussel elimination of the clinical strain was much less efficient than that of the marine strain, which indicates that mussels do not have the same ability to utilize the former as a food resource as they do the latter (paper III). In the future, it would be very interesting to test more clinical strains and investigate whether different bacterial concentrations affect mussels differently. It would also be of interest to extend the elimination time in order to observe whether the bacterial elimination rate eventually reaches 100%. It should of course be taken into consideration that mussels are individuals; the accuracy of future investigations could perhaps be improved by increasing the number of animals analyzed, expanding the elimination time and by including incubation at different temperatures. When water temperature is below or exceeds that to which bivalves are adapted, they may experience stress and thus their immune response may not be optimal (Monari*, et al.*, 2007, Wang*, et al.*, 2011).

It has been suggested that bacteria that are natural inhabitants of the water column may be less easy to depurate than those introduced by humans, e.g. via fecal contamination (Croci*, et al.*, 2002), although this requires further investigation (Jackson*, et al.*, 1999). The depuration process itself has been studied experimentally; Marino et al. (2005) showed *V. cholerae* nonO1 to be more resistant in blue mussels than *E. coli*, while Power and Collins (1989) studied the depuration of Poliovirus, a coliphage and *E. coli.* As clam species differed between seasons in the Mozambican study, further investigation into the immune system of mussels may also be of interest.

In the study presented in paper III, we also investigated the persistence of the bacteria after exposure to mussel hemocytes. The results showed that survival rates were >90% for all tested strains, independent of origin, i.e. clinical or aquatic. Resistance to mussel hemocytes is rare, and as shown earlier, other enterobacteria may not be as resistant to mussel hemocytes as the strains tested in paper III (Hernroth*, et al.*, 2009).
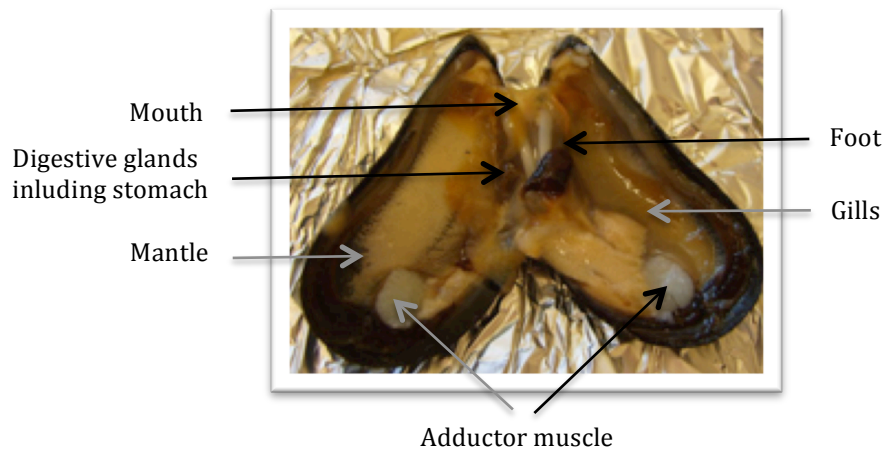
Fig. 9. Blue mussel *Mytilus edulis.* Photo: Rose-Marie Nilsson

Is there a difference in persistence to environmental factors between a clinical and a marine V. cholerae *strain?*

In paper II we showed that vibrios are undetectable in the water column when temperatures are below 17°C. In an unpublished study, we tried unsuccessfully to isolate vibrios from winter sediment using a variety of cultivation methods. However, it was shown in paper IV via qPCR and cultivation that a small number of vibrios were present in the sediment (although the particular strain isolated from the sediment was not identified). If identified as a *Vibrio* this proves that vibrios may hibernate in the sediment, but does not reveal to what degree the bacteria are capable of regaining culturability when the water temperature increases. In order to investigate this phenomenon, we initiated a microcosm persistence study of *V. cholerae* of different origins at low temperature and through a temperature up-shift. As presented in paper IV, the clinical strain responded to low temperatures by becoming less culturable very quickly, with culturability close to the detection limit after only three weeks. However, after temperature up-shift the strain regained culturability and reached peak CFU numbers for the whole experiment after just one week at 20°C. In contrast, the marine strain was not as inactive at the lower temperature and was also not able to regain culturability after temperatures were increased. As shown in Table 4, while the culturability of both strains was quite low directly after inoculation in the microcosms, the marine strain did not lose culturability completely until after the temperature up-shift.

The results also reveal the culturability of the clinical strain to be significantly higher in natural sediment than in sterile seawater, despite the presence of bacteriovores, while the strain's ability to regain culturability after temperature up-shift is also remarkable. As shown in paper IV, the survival of the bacteria was significantly higher when the latter were added to sterile seawater (shown by qPCR) than to microcosms containing sediment (Fig. 4), with Table 4 illustrating the regain in culturability of the clinical strain very clearly. Strains were screened for a set of virulence genes and as mentioned previously, the only difference found was the harboring of the T3SS genes which were carried solely by the marine strain. This might suggest that this particular secretion system is primarily involved in ecological fitness (Dziejman*, et al.*, 2005). However in an unpublished pilot study performed at our laboratory, several *V. cholerae* strains, both clinical and marine, were screened, with the

results showing the genes encoding T3SS to be randomly distributed between the different strains.

These results suggest that the ability of *Vibrio* strains to quickly adapt to temperature changes and to regain culturability after a temperature up-shift may be more important than the harboring of virulence genes in determining their pathogenicity to humans. Concerning the hibernation of *V. cholerae*, the ability to inhabit marine sediment in order to survive periods of very low water temperatures seems to be a possible survival strategy for pathogenic *V. cholerae* clones. Sediment may also provide much-needed protection against UV-light during warmer periods, which is a more probable reason why the bacteria were found in the bottom sediment of Maputo Bay.

It was shown earlier that hemolysins of an El Tor pandemic strain and a diarrhea-causing nonO1/O139 strain were identical, while both clinical and environmental nonO1/O139 strains may exhibit pathogenic activity (Datta-Roy*, et al.*, 1986, Yamamoto*, et al.*, 1986). It is thus plausible that other characteristics determine the pathogenicity and persistence of the strains.

Statistics
Comparison of the levels of *Vibrio* spp. found in clams during the different seasons and microcosms was carried out using One Way Analyses of Variance, with the level of significance set at p= 0.05. Data were $\log_{10}$ transformed if not initially normally distributed. In paper IV, regression analyses were used to investigate the correlation between CFU and qPCR numbers.

GENERAL DISCUSSION OF THE AIMS OF THE STUDIES

Investigate the seasonal distribution of potential human pathogenic vibrios in clams and water samples from Maputo Bay, Mozambique.

This aim was fulfilled in paper I, with a high number of culturable vibrios present in clams throughout the year. The four sampling occasions were spread out during the year and represent seasons characterized by different water temperatures and salinities. These factors have been stated to affect the number of vibrios found in the water, although we should also take the data presented in paper III into consideration when evaluating these results. Paper III includes an *in vivo* study of mussels exposed to *V. cholerae* of various origins. Mussels were seemingly able to distinguish the different strains and did not accumulate highly pathogenic bacteria, which suggests that the bacteria, which were accumulated in the clam tissue, may not belong to strains most harmful to humans. However, the results of the study presented in paper II, showing that hemocytes were not able to kill *Vibrio*, suggest that Maputo clam tissue may provide a suitable reservoir for the bacteria if ingested.

Characterize the Mozambican strains in terms of their antibiotic resistance, virulence and biochemical diversity and to compare these strains with those of strains from tropical (Indian) and boreal (Swedish) waters.

This aim was fulfilled in paper I. The *V. parahaemolyticus* strains isolated from Mozambique (sub tropical) and Sweden (boreal) were characterized in terms of their antibiotic resistance, virulence and biochemical diversity, with these characteristics then compared with those of strains isolated from India (tropical). Results showed all three groups to be very similar, both biochemically and genetically, regarding screened virulence genes, but their respective antibiotic resistance patterns differed significantly, with higher sensitivity observed among the Mozambican strains. However, many strains exhibited resistance or intermediate sensitivity to several broad-spectra antibiotics, which is alarming. These antibiotics are commonly used across the world as they are less expensive, which may generate even higher resistance in the near future. Moreover, the functional hemolytic activity of the strains differed. Out of the Mozambican strains, 70% had this capability, while only 40% of the Swedish and Indian strains were positive.

Investigate the presence of potential human pathogenic vibrios in the sound between Sweden and Denmark (Öresund) and the cell toxicity of isolated strains to eukaryotic cells.

This was investigated in paper II, which was performed during the summer of 2006. Sampling commenced one month prior to the first reported fatal case of "bathing wound fever" on the 16th of July. We isolated and identified *V. cholerae, V. parahaemolyticus* and *V. vulnificus* from mussels and screened the tissue for a set of vibrio-related bacterial virulence genes. Positive results for virulence genes were achieved on 89% of sampling occasions when seawater temperatures exceeded 17°C. The cell toxicity of the environmental strains to CHO-cells was also investigated and compared with that of clinical strains plausibly derived from the same area. The results suggest that there are characteristics other than virulence genes which determine the pathogenicity of the strains. This theory is strengthened by the data presented in paper I, which show that a large number of the *V. parahaemolyticus* strains exhibited hemolytic activity, even if they were not carrying the genes associated with hemolysin (*tdh* and *trh*).

Study the uptake and persistence of marine and clinical (isolated from a wound infection) V. cholerae *strains when exposed to the common blue mussel* M. edulis

Our aim to study *in vivo* the persistence of marine and clinical *V. cholerae* strains when exposed to *M. edulis* was accomplished in paper III. Analysis revealed that the mussels did not filter when exposed to the highly pathogenic El Tor strain throughout the experiment, even after the exposure time was extended from 6h to 18h. Nevertheless, the mussels were seen to accumulate both the clinical and marine strains in their tissue, with the former to a lesser degree. We could also conclude that the mussels eliminated the clinical strain significantly less efficiently than they did the marine strain. As mentioned above, the inability of mussel hemocytes to kill *Vibrio* strengthens the idea that mussel tissue can act as an ecological niche for potential human pathogenic strains (paper II), where the latter may be protected from predation and UV-radiation.

Study, in laboratory experiments, the persistence of clinical V. cholerae *strains when exposed to low water temperature and natural sediment*

Finally, we aimed to study the persistence of *V. cholerae* in low temperature conditions. This was performed in paper IV. We found that the clinical strain economized well under the given conditions; when environmental factors were unfavorable, the bacteria rapidly decreased in culturability and when these factors improved, the bacteria rapidly regained metabolic activity. In comparison to the tested marine strain, the clinical strain exhibited a more successful survival strategy, which may represent a virulence characteristic lacking in the marine strain. No other difference was observed between the two strains which indicated why the clinical strain causes severe wound infections – even after comparing the two strains in terms of a set of potential virulence genes.

THIS LEADS US TO ANSWERING THE OVERALL QUESTIONS RAISED FOR THE EXPERIMENTS:

Are human virulent strains favored by a higher water temperature?

The screening study presented in paper II showed that the occurrence of human virulent and non-virulent strains was equal; independent of water temperature, virulence genes and species-specific genes were equally distributed throughout the sampling period. However, when strains of different origins were compared in microcosms, the results suggested clinical strains to be favored by lower temperatures, as these bacteria were able in some way to economize and regain culturability better than the tested marine strain.

Is a low water temperature always unfavorable for vibrio strains?

No, not directly. The bacteria may go into a non-culturable state when temperatures decrease, but as shown in paper IV, not all strains have the ability to resuscitate when environmental conditions improve. However, a lower temperature may relocate the bacteria from the water column to the bottom sediment, where the bacteria seem to be found when winter conditions prevail. Some strains enter this non-culturable state, and thereby economize well and regain culturability when water temperatures increase. As suggested in paper IV, the clinical strains may have an advantage over the marine

strains in this regard. Low water temperatures also go hand in hand with lower levels of UV-radiation, which in high levels are very harmful to bacteria.

In paper I, a higher water temperature improved the cell culturability of strains obtained from clam tissue, but as the molecular screening of water revealed significantly higher numbers of bacteria during periods of lower temperatures, there may be additional factors responsible. For instance, higher temperatures may stress mussels and thereby impair their immune response.

Can strains isolated from aquatic environments be harmful to eukaryotic cells?
We have shown that strains isolated from aquatic environments may be just as or even more virulent than clinical strains to CHO-cells. This indicates that we have not yet identified the actual virulence genes which may distinguish human pathogenic from non-pathogenic strains – and that there may be other characteristics which determine pathogenicity than the tested virulence genes.

Is the virulence and antibiotic resistance patterns different in strains of the same species from different areas of the world?
Yes and no. We showed that the investigated *V. parahaemolyticus* strains from different aquatic environments were very similar biochemically. In terms of their virulence only one strain harbored the virulence genes *tdh*. However, functional hemolytic activity was more pronounced among Mozambican strains (70% of the isolated strains) than the Swedish and Indian strains (40%). Antibiotic resistance was much more common among Swedish and Indian strains.

Do the blue mussel accumulate and eliminate bacteria independently of the latter's level of pathogenicity?
No. *M. edulis* can distinguish highly virulent *V. cholerae* strains at the gills and do not accumulate the most virulent. Less virulent strains may be accumulated, but to a lower level. We could see that the mussels eliminated the clinical strains significantly less efficiently than they did the marine strain. This latter strain was accumulated in mussel tissue and was almost totally eliminated after 24 hours, which is the recommended depuration time (EU-standard regulation). Overall this indicates that mussel tissue may be a suitable ecological niche for human pathogenic *V. cholerae.*

Are human clinical strains persistent in aquatic environments?
Yes. As shown in paper IV, the clinical strain was more persistent in the aquatic environment, under low temperature conditions and when accumulated in mussel tissue. This indicates that the features of vibrios which cause human illness may originate in their environmental adaptation.

MAJOR FINDINGS

The occurrence of vibrio in clams from Maputo Bay peaked during the late rainy season, when the water temperature was high. Although the virulence of isolated *V. parahaemolyticus* strains was low (*tdh, trh*), hemolytic activity indicates that additional genes should be involved when screening for the occurrence of potential human pathogens. The observed antibiotic resistance pattern among strains is alarming and should be highlighted when discussing antibiotic treatments.

Potential human pathogenic *Vibrio* strains were present along the Swedish south coast on 100% of sampling occasions when water temperatures exceeded 17°C. We also found that a highly human pathogenic *V. cholerae* strain may be more persistent at low temperatures (4°C), both in the water column and in untreated sediment, and may resuscitate quickly when temperatures are increased to 20°C. This quick change in metabolic activity may be an important virulence factor. The up-shift from 4°C to 20°C may be comparable to that from 20°C to 37°C, i.e. from the temperature of seawater during a typical Swedish summer to that of the human body.

Mussels may have the ability to regulate the filtration and accumulation of *Vibrio* of the same species but different pathogenicity, when exposed to strains of different origin. As the studied strains were also of different serotypes, their cell surface proprieties may be of significance during filtration by molluscs. Even though uptake of the more virulent bacteria was relatively low, mussels were not able to eliminate this strain effectively and thus mussel tissue may constitute an appropriate niche for highly human pathogenic *Vibrio*.

When *V. cholerae* strains of different origins were exposed to low water temperatures, the clinical strain was more persistent than that isolated from a marine environment. As these strains were genetically very similar, some other feature may be the actual virulence marker, such as the ability to adapt quickly to new environmental factors and/or economize well when needed.

FUTURE PERSPECTIVES

In concluding my research as a PhD-student, I have many ideas regarding future studies. For example, I think it would be of great importance to investigate the factors that make *Vibrio* spp. pathogenic to humans from an ecological perspective. The occurrence of potential human pathogenic *Vibrio* in Swedish waters indicates that there is a reservoir of human pathogens from which these bacterial strains may emerge, when conditions are optimized. In Mozambique we isolated a *tdh⁺ V. parahaemolyticus* strain, and it would be interesting to investigate whether there are any environmental factors that induce the human pathogenicity/expression of this virulence gene. Could factors associated with climate change, such as increasing water temperature, favor human pathogenic *Vibrio* strains? Does a change in pH and salinity increase the persistence of vibrios in the water, potentially by making the mussel immune system less effective in eliminating the bacteria? The list of questions to answer may be long...

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