Potentially pathogenic *Vibrio* species in the Norwegian marine environment, characterisation of virulence and antibiotic resistance determinants

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Abstract

Vibrio species are indigenous to the marine environment, and the genus includes 12 human pathogenic species which has shown a strong seasonality and are most abundant when the sea temperature exceeds 18 °C. The last two decades, an increased number of infections have been reported in areas where this previously have been rare, and this has been linked to increased temperatures due to global warming. Furthermore, the World Health Organization (WHO) has ranked antimicrobial resistance as one of the biggest threats to global health, food security and development. This is the first larger study on the occurrence of potentially pathogenic *Vibrio* spp. in the Norwegian marine environment since 2006, and to my knowledge the first on Norwegian pelagic fish.

A number of 20 water samples, 60 fish and 16 bivalve molluscs were examined for the presence of *Vibrio* spp., and isolated bacteria were characterised morphologically, biochemically and by molecular methods. Antimicrobial susceptibility testing was conducted by a disk diffusion method. Isolates belonging to *V. anguillarum* were additionally tested for the ability to hydrolyse imipenem. All isolated *V. metschnikovii* and *V. alginolyticus* were examined for hemolytic activity on blood agar. A selection of 23 isolates were subjected to whole genome sequencing (WGS).

A total of 64 *V. alginolyticus,* 38 *V. metschnikovii,* 24 *V. anguillarum,* seven *V. aestuarianus* and two presumptive *V. cincinnatiensis* were isolated. A high prevalence of phenotypic resistance to ampicillin (74 %), reduced susceptibility or resistance to oxolinic acid (32 %), aztreonam (19 %) and tobramycin (18 %), was observed. Preliminary analysis of assembled genomes revealed the presence of different classes of putative β-lactamase genes in examined isolates, topoisomerase IV subunit B (*parE)* conferring resistance to quinolones and quinolone resistance B proteins (QnrB) were detected in *V. alginolyticus*. Resistance-Nodulation cell Division (RND) efflux pumps were detected in all examined species. A *tlh*- and *hlyA* related gene was detected in all *V. metschnikovii* examined by WGS and a *tlh* related gene was found in all *V. alginolyticus.*

The results from this study indicates a risk of exposure to potentially pathogenic vibrios through bathing or other marine recreational activities. Additionally, the occurrence of *Vibrio* spp. in both fish and bivalve molluscs indicates a risk of infection through consumption and handling of raw seafood, and the transfer of resistant bacteria to the human food chain.

Since the temperature is expected to increase further as a result of global warming, it is necessary to monitor the occurrence of these bacteria in the Norwegian marine environment.

Abbreviations

API = Analytical Profile Index

APW = Alkaline Peptone Water

bp = Base Pair

CARB = Carbenicillin-hydrolysing

CARD = The Comprehensive Antibiotic Resistance Database

CCUG = Culture Collection University of Gothenburg

cfu = Colony Forming Units

CLSI = Clinical and Laboratory Standards Institute

CTX = Cholerae Toxin

DDD = Defined Daily Doses

ECOFF = Epidemiological Cut-off

EDTA = Ethylendiaminetetraacetic Acid

EEA = European Environment Agency

EUCAST = European Committee on Antimicrobial Susceptibility Testing

GyrA = DNA gyrase subunit A

GyrB = DNA gyrase subunit B

HGT = Horizontal Gene Transfer

HlyA = Hemolysin A

IMR = Institute of Marine Research

KOH = Potassium Hydroxide

LPS = Lipopolysaccharide

MALDI-TOF MS = Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry

MHA = Mueller Hinton Agar

MIC = Minimum Inhibitory Concentration

NFSA = Norwegian Food Safety Authority

NIPH = Norwegian Institute of Public Health

NMKL = Nordic Committee on Food Analysis

NSC = Norwegian Sequencing Centre

NVI = The Norwegian Veterinary Institute

ParC = Topoisomerase IV Subunit A

ParE = Topoisomerase IV Subunit B

PBP = Penicillin-binding protein

PCA = Plate Count Agar

PCR = Polymerase Chain Reaction

PCU = Population Correction Unit

PMF = Peptide Mass Fingerprint

QC = Quality Control

QnrB = Quinolone resistance B protein

RAST = Rapid Annotation using Subsystem Technology

rcf = Relative Centrifugal force

RND = Resistance-Nodulation cell Division

RTX = Repeats-In-Toxin

SST = Sea Surface Temperature

TCBS = Thiosulfate Citrate Bile Sucrose

TDH = Thermostable Direct Hemolysin

TLH = Thermolabile Direct Hemolysin

TRH = TDH-Related Hemolysin

TSA = Tryptic Soy Agar

UoB = University of Bergen

VBNC = Viable but non-culturable

VCS = *Vibrio* ChromoSelect Agar

VFDB = Virulence Factors Database

WGS = Whole Genome Sequencing

WHO = World Health Organization

Contents

1.Introduction

1.1 Aim of the study

The aim of this study was to increase the knowledge on the occurrence of human pathogenic *Vibrio* species in the Norwegian marine environment, and the occurrence of virulence- and antibiotic resistance genes in isolated strains. This is the first larger study on the occurrence of potentially pathogenic *Vibrio* spp. in the Norwegian marine environment since 2006, and to my knowledge the first on Norwegian pelagic fish. The work will add to the general knowledge on *Vibrio* bacteria, both those of relevance for seafood safety and non-human pathogenic species, and the role of the marine environment in the development and the spread of antibacterial resistance. The practical work in this project included sampling, isolation, obtaining pure cultures, characterisation, identification, antimicrobial susceptibility testing, determination of hemolytic activity, extraction of DNA and molecular methods to determine the genetic basis of pathogenicity and antimicrobial resistance (Figure 1).

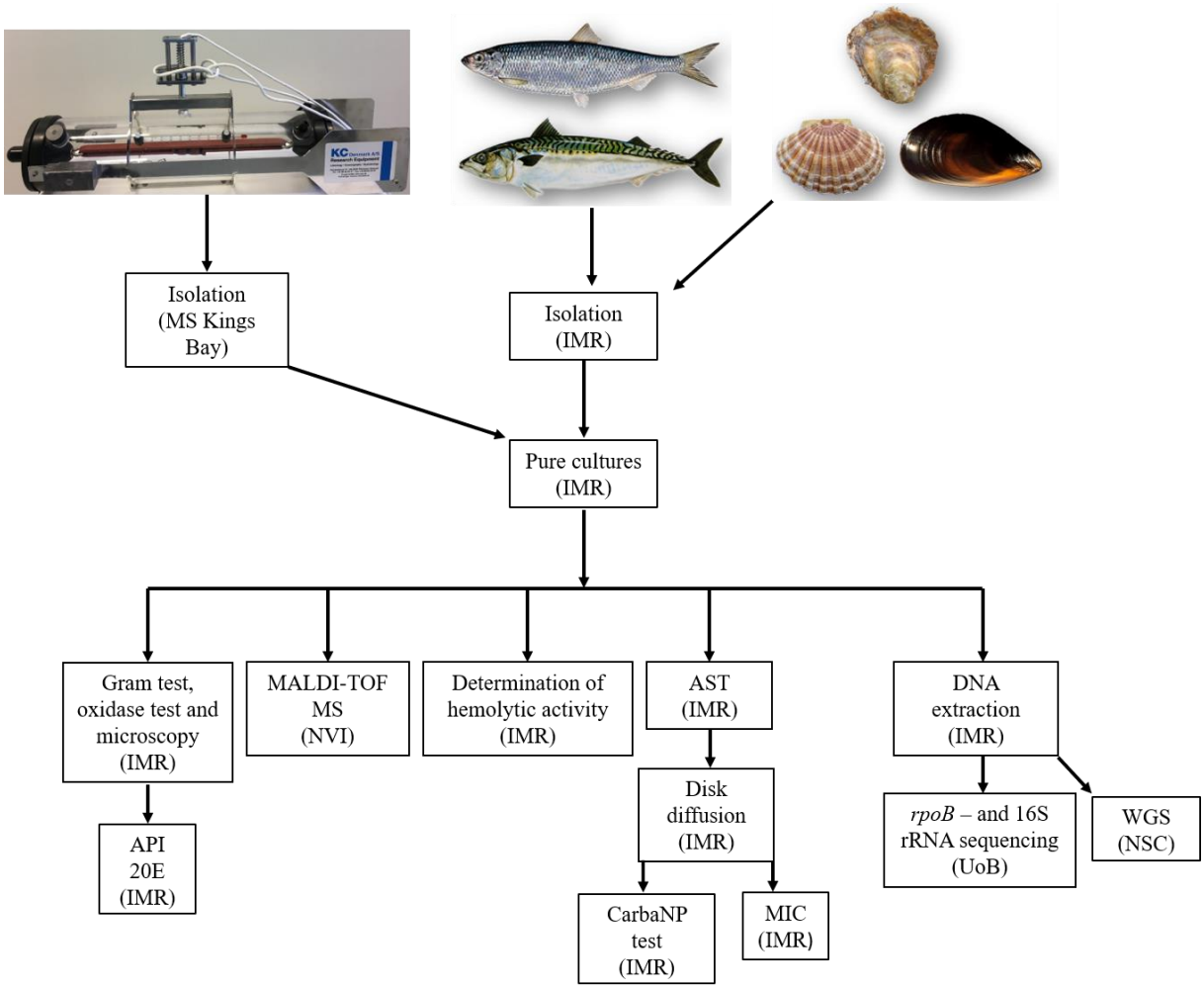

Figure 1: Sampling and analysis carried out throughout the project. Isolation of Vibrio *spp., pure cultures, Gram test, oxidase test, microscopy, analytical profile index (API) 20E, determination of hemolytic activity, antimicrobial susceptibility testing (AST), determination of minimum inhibitory concentration (MIC), carbaNP test, extraction of DNA and bioinformatical analysis was conducted at the Institute of Marine Research (IMR). Identification by MALDI-TOF MS was done at the Norwegian Veterinary Institute (NVI),* rpoB- *and 16S rRNA sequencing was done at the University of Bergen (UoB) and Whole genome sequencing (WGS) was conducted at the Norwegian Sequencing Centre (NSC).*

1.2 *Vibrio* species

1.2.1 Genus *Vibrio*

The genus *Vibrio* consists of Gram-negative bacteria belonging to the phylum Proteobacteria, class Gammaproteobacteria and family Vibrionaceae (Farmer, 2006;Williams et al., 2010). Bacteria belonging to the genus *Vibrio* are non-spore forming and typically appear as curved rods (Lunestad and Rosnes, 2008). All *Vibrio* species are oxidase positive, apart from *Vibrio metschnikovii* and *Vibrio gazogenes* (Lee et al., 1978;Farmer et al., 1988;Lunestad and Rosnes, 2018). Vibrios have the marine and estuarine environment as their natural habitat (Lunestad and Rosnes, 2008). They are also common on the surface of marine plants and animals, and they occur naturally in the intestine of marine animals (Lunestad and Rosnes, 2018). Vibrios are facultative anaerobic, and NaCl stimulates the growth of all species and is an obligate requirement for some of the species (Adams, 2008). Their genomes are divided between two chromosomes, and recombination and horizontal gene transfer events are frequent and contribute to the evolution of *Vibrio* spp. (Baker-Austin et al., 2018).

Vibrios are among the most common bacteria found in surface waters in the world and some species can cause infections in humans and animals (Vezzulli et al., 2015). At present, the genus *Vibrio* consists of 142 species (Bonnin-Jusserand et al., 2017). Among these, more than twenty *Vibrio* spp. have been described as pathogenic to animals (Lunestad and Rosnes, 2018), and twelve have been recognized as human pathogens (Kokashvili et al., 2015). The human pathogens are *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, V. fluvialis, V. alginolyticus, V. damsela, V. furnissii, V. hollisae, V. mimicus, V. cincinnatiensis, V. metschnikovii* and *V. carchariae* (Lunestad and Rosnes, 2018).

The vibrios preferentially grow in warm (>18 °C), low salinity (<25 $^{o}/_{oo}$) seawater and brackish waters (Vezzulli et al., 2013). The human pathogenic *Vibrio* spp. show strong seasonality, and they are most abundant during the summer months when the waters are warmer (Vezzulli et al., 2015). Warming of low salinity marine environments is likely to support larger numbers of *Vibrio* populations and therefore increase the risk of infections (Baker-Austin et al., 2016). However, in the environment, vibrios can enter a state where they only retain basic metabolic processes when the conditions become less favourable, and can thus persist with low activity for an extended time period in the environment. This state is called viable but non-culturable (VBNC), and in this state they fail to grow on standard laboratory media and an enrichment step is required for isolation of the vibrios in this state

(Jorgensen and Pfaller, 2015;Fernández-Delgado et al., 2015). During warmer conditions, these cells will resuscitate. As a result, the cells can evade unfavourable conditions while they can return to their active state when the conditions again become favourable (Ayrapetyan et al., 2014).

Human *Vibrio* infections typically occur as a result of ingestion of contaminated seafood, by exposure of wounds to seawater or through handling of seafood (Iwamoto et al., 2010). *V. cholerae, V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* are most frequently causing disease in humans (Stavric and Buchanan, 1997;Baker-Austin et al., 2018).

1.2.2 Human pathogenic *Vibrio* spp. and virulence factors

Vibrio spp. are responsible for the majority of human infections attributed to the natural microbiota of aquatic environments and seafood (Baker-Austin et al., 2018).

The pathogenicity of *V. parahaemolyticus* and *V. cholerae* is highly correlated to the presence of specific virulence genes.

Throughout history *V. cholerae* has caused several pandemics. The epidemic capability of *V. cholerae* is linked to the presence of genes encoding the cholerae toxin (CTX), and the main serotypes possessing these genes are designated *V. cholerae* O1 and *V. cholerae* O139. Today, outbreaks of cholerae are often related to natural disasters and/or war-like situations where the hygienic and sanitary conditions are poor (Lunestad and Rosnes, 2008). An example of a recent outbreak of cholerae includes Yemen, where an ongoing war has damaged the water supply infrastructure, leading to water shortage and increased prices on clean water (Camacho et al., 2018). As of 1 March 2018, the total of suspected cholera cases in Yemen stands at 1 063 786 cases with 2 258 associated death since April 2017 (WHO, 2018).
Strains within the species *V. cholerae* can be subdivided into more than 200 serotypes based on the O antigen in the cell envelope (Lunestad and Rosnes, 2008;Baker-Austin et al., 2018). Strains not possessing the *ctx* genes are designated non-O1/non-O139 (Lunestad and Rosnes, 2018).

V. cholerae non-O1/non-O139 can cause both intestinal and extra intestinal infections, including wound and ear infections. The illness caused by these strains are often self-limiting and may include diarrhoea but can sometimes result in septicaemia. The virulence

mechanisms by *V. cholerae* non-O1/nonO-139 are mostly unknown, but in intestinal disease a heat stable enterotoxin has been described, in addition to a thermostable hemolysin (TDH) (Ellingsen, 2008).

Food poisoning caused by *V. parahaemolyticus* is exclusively associated with fish and other seafood. Occasional outbreaks have been reported in USA and Europe, while in Japan it is the most common cause of food poisoning (Adams, 2008). The pathogenicity of *V. parahaemolyticus* strains is strongly linked to their ability to produce a thermostable direct hemolysin (TDH), or a TDH-related hemolysin (TRH), which are encoded by *tdh* - and *trh* genes. TDH is a pore forming toxin with several biological activities, including hemolytic activity, cytotoxicity, cardiotoxicity, and enterotoxicity (Raghunath, 2014). TDH targets epithelial and intestinal cells and alters the ion flux in intestinal cells, causing a secretory response (Ghenem et al., 2017;Nishibuchi and Kaper, 1995). This is the cause of diarrhoea in patients with *V. parahaemolyticus* infections. TRH is a heat labile toxin and immunologically similar to TDH. Both TDH and TRH also activates Cl^- channels, resulting in altered ion flux (Raghunath, 2014). However, during a study in 2005, 6 % of clinical isolates lacked both *tdh* - and *trh* genes, suggesting the presence of other virulence determinants in *V. parahaemolyticus* (Lynch et al., 2005). In rare cases, *V. parahaemolyticus* can cause wound infections, ear infections or septicaemia that can be life threatening to vulnerable individuals (Letchumanan et al., 2014).

Together with *V. cholerae* and *V. parahaemolyticus, V. vulnificus* is a normal inhabitant of the marine environment. *V. vulnificus* is divided into three biotypes, 1, 2 and 3. All three biotypes are opportunistic human-pathogens, but biotype 2 is also a fish pathogen (Roig et al., 2018). There are two major manifestations of *V. vulnificus* infections, wound infection and septicaemia. This opportunistic pathogen primarily cause infection in people with a compromised immune system and/or underlying disease. Especially people suffering from liver or blood related disorders, often resulting from alcoholism are at risk. The ability to cause disease in humans is related to the production of a polysaccharide capsule that enables the bacterium to resist phagocytosis, lipopolysaccharide (LPS) which is to cause tissue damage by stimulation of nitric oxide synthase, which then release nitric oxide (Ellingsen, 2008). The genetic basis for human virulence is only partial known, although a number of studies suggest that all strains of *V. vulnificus,* regardless of their origin, may be able to cause infections in humans (Roig et al., 2018). A number of other virulence factors, such as hemolysin, proteases and flagella have been examined, but none of these have been

conclusively linked with pathogenesis (Ellingsen, 2008). The ability of *V. vulnificus* to infect fish is dependent on a virulence plasmid (PVvBt2) that is only present in biotype 2 strains (Roig et al., 2018).

However, several other *Vibrio* spp., such as *V. alginolyticus, V. fluvialis, V. mimicus, V. metschnikovii, V. furnissii, V. hollisae* and *V. damsela,* are considered to be lower risk organisms, but can cause infections in humans (Austin, 2010;Adams, 2008).
The species *V. alginolyticus* can cause ear - and wound infections, as well as gastroenteritis (Austin, 2010;Mustapha et al., 2013;Baker-Austin et al., 2016;Adams, 2008). This species can produce three different hemolysins, TRH, TDH and Thermolabile Direct Hemolysin (TLH) (Hernández-Robles et al., 2016;Ellingsen, 2008). *V. metschnikovii* have been implicated in different types of infections, such as wound infections, pneumonia, bacteremia and gastroenteritis (Linde et al., 2004;Dalsgaard et al., 1996;Wallet et al., 2005;Hardardottir et al., 1994). Hemolysin and verotoxin are suggested to be the virulence factors of this *Vibrio* sp. (Matté et al., 2007). Other vibrios, such as *V. fluvialis, V. furnissi* and *V. hollisae* can cause diarrhoea, while *V. mimicus* and *V. damsela* in addition to diarrhoea, can cause wound infections (Adams, 2008). The infections caused by the human pathogenic *Vibrio* spp. are summarized in Table 1.

Table 1: Human pathogenic Vibrio *spp. and the infections they can cause. GI tract: Gastro intestinal tract. ++: most common site of infection. +: other sites of infection. (+): rare sites of infection. -: no reported infections (Adams, 2008;A. Daniels and Shafaie, 2000;West, 1989;Austin, 2010;Deshayes et al., 2015;Oliver, 2005;Chowdhury et al., 2016;Hao et al., 2015;Glenn Morris, 2018;Roig et al., 2018;Chiang and Chuang, 2003;Kim et al., 2003;Katz, 1988;Mizuno et al., 2009;Linde et al., 2004;Hardardottir et al., 1994;Dalsgaard et al., 1996;Macarena Pariente et al., 2008;Jensen and Jellinge, 2014;Jean-Jacques et al., 1981;Mustapha et al., 2013;Hansen et al., 1993;Baker-Austin et al., 2018;Lunestad and Rosnes, 2018)*

Species	**GI tract**	**Wound**	**Ear**	**Sepsis**	**Lung**	**Meningitis**
V. cholerae O1/O139	++	+	-	-	-	-
*V. cholerae non-*O1/O139	++	+	+	+	-	(+)
V. parahaemolyticus	++	+	+	(+)	(+)	(+)
V. vulnificus	+	++	-	++	(+)	(+)
V. alginolyticus	+	++	+	(+)	-	-
V. metschnikovii	(+)	(+)	-	(+)	(+)	-
V. fluvialis	++	(+)	(+)	-	-	-
V. damsela	-	++	-	-	-	-
V. furnissii	+	-	-	-	-	-
V. mimicus	++	+ +	+	-	-	-
V. hollisae	++	+	-	(+)	-	-
V. cincinnatiensis	-	-	-	(+)	-	(+)
V. carchariae	-	+	-	-	-	-

1.2.3 The occurrence of *Vibrio* spp. in Europe and Norway

During the last two decades, reported infections have increased in areas not usually associated with these bacteria, including temperate and cold regions, such as US Pacific Northwest, South America, and Northern Europe (Baker-Austin et al., 2016). Previous studies have shown that a regime shift warming has caused increased occurrence of *Vibrio* spp. in the

environment, and that Sea Surface temperatures (SST) > 18 ˚C is a critical threshold for triggering infections (Martinez-Urtaza et al., 2018).

According to the European Environment Agency (EEA), one of the primary effects of climate change in the European seas are increased SST, and this may facilitate the spread of water-borne diseases, such as Vibriosis (EEA, 2017). It has been estimated that the global SST in average is 1 °C higher today than it was 140 years ago (Vezzulli et al., 2015).

There are no common mandatory notification systems for *Vibrio* infections, and accurate estimates of the number of *Vibrio* infections occurring in Europe is thus not available (Leroux et al., 2015).

The increase in outbreaks of *Vibrio*-associated human illness caused by *V. parahaemolyticus, V. vulnificus* and *V. cholerae* non-O1/non-O139 in several European countries, including reported infections in Sweden and Denmark, has been linked to the increase in SST. Warming of low salinity marine environments, which can occur in Norway during the summer, is shown to support larger numbers of *Vibrio* spp. and consequently increase the risk of infections.

Domestically acquired *Vibrio* infections are rare in northern Europe, however during a heat wave in northern Europe the summer of 2014, an unusually high number of *Vibrio* infections was reported in Finland and Sweden. A total of 89 infections was reported, of which 70 were caused by *V. cholerae* non-O1/non-O139, four by *V. parahaemolyticus*, three by *V. alginolyticus*, two by *V. vulnificus*, one by *V. mimicus*, and eight cases were caused by unspecified *Vibrio* sp. (Baker-Austin et al., 2016).

Potentially pathogenic *Vibrio* spp. have been detected in biofilm communities on microplastics in the oceans, and these particles can be spread over long distances by waves and currents. The composition of these biofilm communities varies with season and geographical location. It has been suggested that these particles can serve as vectors for the dispersal of human pathogenic *Vibrio* spp. between distant geographical locations (Kirstein et al., 2016). It has also been suggested that pathogenic *V. parahaemolyticus* have been spread over large geographical areas via ballast water and transport of shellfish (Baker-Austin et al., 2018).

Little is known regarding the occurrence of *Vibrio* species in Norwegian waters, but *V. parahaemolyticus, V. alginolyticus, V. cholerae* and *V. vulnificus* have all previously been isolated. During a study by Gjerde and Bøe conducted in 1981, *V. parahaemolyticus* was isolated from mussels in July and August, with sea temperature from 15-16 °C. In this period 16 mussels where examined and *V. parahaemolyticus* was isolated from five of these (30 %). In the same period *V. parahaemolyticus* were also isolated from four (25 %) of 16 samples from bottom sediments. *V. alginolyticus* were regularly isolated from mussels, seawater, bottom sediments and fish, with the highest number of bacteria isolated in July, August and September (Gjerde and Bøe, 1981). During a study conducted in the period 2001 – 2006, a total of 1280 seafood and water samples were examined with respect to the presence and level of *V. parahaemolyticus, V. cholerae* and *V. vulnificus.* During this study, the three species were all detected in blue mussels, almost exclusively during summer months, but the levels were <100 cfu/g. One crab and one oyster sample tested positive for the presence of the three species. *V. parahaemolyticus, V. cholerae* and *V. vulnificus* were also detected in water samples with maximum levels of 30 cfu/ml. In 13 of the *V. parahaemolyticus* positive samples the *trh* gene was detected, while all of the *V. cholerae* isolates were non-O1/non-O139 (Ellingsen, 2008). However, during the summer of 2018, several bathing associated *Vibrio* infections were reported in Southern Norway. A total of six serious *Vibrio* infections and 27 mild cases of wound- and ear infections caused by *Vibrio* spp. were reported to the Norwegian Institute of Public Health (NIPH) (NIPH, 2018a). Four of the serious infections were caused by *V. vulnificus* (NIPH, 2018b). All the reported infections occurred after bathing along the South coast of Norway and in areas along Oslofjorden (NIPH, 2018a). The summer of 2018 was unusually warm in Norway, with an average temperature 1.8 ˚C above normal. The highest average temperature was recorded in the eastern parts of Norway with an average temperature 3-4 ˚C above the normal average temperature (MET, 2018;ECDC, 2019). This corresponds with literature showing an increase in reported domestically acquired *Vibrio* infections in northern Europe in "heatwave" years (Leroux et al., 2015). During the summer of 2018, the Norwegian Food Safety Authority issued a warning against eating raw oysters caught in Oslofjorden, along the coast of Telemark and the South coast of Norway due to the increased risk of foodborne *Vibrio* infections. The warning applied until the water temperature dropped below 20 °C, and remained below for a few days (NIPH, 2018b).

1.3 Bacteria of fish and bivalve molluscs

The aquatic environment represents the largest part of the planet and the oceans contain several species of microorganisms adapted to these habitats. Sea water contain approximately 10^6 bacterial cells/ml water (Bergh et al., 1989;Azam et al., 1983). Bacteria isolated from the open oceans often have a physiological requirement for salt, grow best at relatively low temperatures and are adapted to the low concentrations of available organic and nitrogenous compounds present in the oceans (Adams, 2008).

Fish caught from the open sea will have a microbiota which reflects their environment, and consequently, fish caught from cold water will predominantly contain psychrophilic and psychrotrophic bacterial species (Adams, 2008). Live fish in temperate waters have bacteria present in the mucoid layer of their skin (10^2-10^7 cfu/cm^2), their gills (10^3-10^7 cfu/g) and the highest concentration is found in their intestine (10^7-10^9 cfu/g) (Lunestad and Rosnes, 2018).

The majority of shellfish feed by filtering out particles from the seawater, and therefore bacteria present in their environment will be concentrated. The seas around the coasts are also influenced by inputs of terrestrial and freshwater bacteria, and by human activities. The sea has been a convenient dump site for sewage and waste products, and this has had a detrimental effect on coastal waters. Many shellfish used for food grow in these coastal waters, and if these waters have been contaminated by sewage, the filter feeders may contain high concentrations of enteric bacteria (Adams, 2008). In warmer waters, unpolluted water may even contain high numbers of *V. parahaemolyticus* and these may be concentrated by the shellfish and form a stable part of the enteric flora of some shellfish (Adams, 2008).

1.4 Antimicrobial agents

1.4.1 The history of antibiotics

The development of antibiotics has been one of the major medical achievements in the 20th century, and since they were first introduced on a larger scale in the 1940`s, millions of lives have been saved (Marti et al., 2014). The first true antibiotics introduced were penicillin and streptomycin, antimicrobial agents produced by microorganisms. Penicillin was first isolated from the fungus *Penicillium chrysogenum*, and streptomycin from the soil bacterium *Streptomyces griseus* (Madigan et al., 2015;Marti et al., 2014;Yazdankhah et al., 2013). The period between the 1950`s and 1970`s was the golden era of discovery of antibiotics, with no

new classes discovered since then, and one-half of the antibiotics commonly used today were discovered in this period (Aminov, 2010;Davies, 2006). More than 350 agents have reached the marked as antimicrobials, these are natural and semisynthetic antibiotics, and strictly synthetic chemicals (Demain, 2009).

1.4.2 Mechanisms of action

Antibiotics can be ranged from a narrow to a broad spectrum of activity, where the broad-spectrum antibiotics are effective against a larger group of bacteria, while the narrow-spectrum antibiotics are effective against a limited range of bacteria. These agents can again be either bacteriostatic or bactericidal. Bacteriostatic agents are typically inhibitors of important biochemical process, e.g. protein synthesis, by binding weakly to their target. If the agent is removed, the cells can keep growing. Bactericidal agents bind tightly to their target and kills the cell (Madigan et al., 2015). Antibiotics are categorized in groups based on their mechanism of action (Kapoor et al., 2017):

Antibiotics targeting the cell wall:

The bacterial cell is surrounded by a cell wall which amongst other consists of peptidoglycan. Peptidoglycan is a polysaccharide composed of *N-acetylglucosamine* and *N-acetylmuramic acid*, and a few amino acids. This forms a stiff layer and is primarily responsible for the strength of the cell wall. Long chains of peptidoglycan are synthesized next to each other and forms a sheet that surrounds the cell. The chains are connected by cross-links between short peptides (Madigan et al., 2015;Kapoor et al., 2017;Vollmer et al., 2008). The Gram-positive cell wall mainly consists of peptidoglycan, while the Gram-negative cell wall only consists of a small amount of peptidoglycan as most of the cell wall is composed of an outer membrane. In Gram-negative cells, a region called the periplasm is located between the outer- and the cytoplasmic membrane, and the periplasm contains several classes of proteins (Madigan et al., 2015). Some classes of antibiotics work by inhibiting the cell wall synthesis. Penicillin-binding proteins (PBPs) are enzymes involved in the polymerization of glycan strands and the cross-linking between the glycan strands (Sun et al., 2014). Antibiotics which inhibit the cell wall synthesis can work by binding to the PBPs, and this inhibits the synthesis of new peptidoglycan. This disruption of the peptidoglycan layer leads to cell lysis (Kapoor et al.,

2017). Penicillins and cephalosporins are classes of β-lactam antibiotics that work by inhibiting the synthesis of a functional bacterial cell wall (Madigan et al., 2015)

Inhibitors of the protein synthesis:

In the protein synthesis, the information in the bacterial DNA is first used to synthesize mRNA. This process is called transcription. Next, in the translation, the ribosome synthesizes the proteins in mRNA (Kapoor et al., 2017). The protein synthesis is a continuous process, but can be broken down to three steps, initiation, elongation and termination.
The prokaryotic ribosome, 70S, consists of 30S- and 50S subunits which makes up intact 70S ribosomes (Madigan et al., 2015). Both the 30S- and 50S subunits are essential for the biosynthesis of proteins, and different agents can inhibit this process by targeting either one of the subunits. Among the classes of antibiotics which work by inhibiting this process are the tetracyclines (30S), aminoglycosides (30S) and the macrolides (50S) (Kapoor et al., 2017).

Inhibitors of DNA replication:

The quinolones can disturb the bacterial DNA replication by interfering with bacterial DNA gyrase (topoisomerase II), an enzyme responsible for the supercoiling of DNA (Hawkey, 2003). This step is required for packaging of DNA in the bacterial cell (Madigan et al., 2015). DNA gyrase consists of two subunits, A (GyrA) and B (GyrB). The A subunit carries out the nicking of DNA, while the B subunit introduces negative supercoils, and the A subunit reseals the strands. The quinolones binds the A subunit, and by this mechanism they disrupt the cutting and resealing of its strand (Kapoor et al., 2017). DNA gyrase is the primary target for quinolones in Gram-negative bacteria, while a structurally similar protein, topoisomerase IV, is the primary target for quinolones in Gram-positive bacteria (Kohanski et al., 2010). Topoisomerase IV consists of the subunits A (ParC) and B (ParE) (Madigan et al., 2015).

Inhibitors of essential metabolic steps:

Growth factors are specific chemical substances required by an organism that is unable to synthesize them. These organisms are therefore dependent on an outside source for these substances. Growth factor analogues are synthetic compounds that are structurally similar to a growth factor, but the differences between the analogue and the growth factors prevent the analogue from functioning in the cell. As a result, the cell metabolism is disrupted (Madigan et al., 2015). The sulphonamides and trimethoprim are antibacterial agents which inhibit steps in the folic acid metabolism. Sulphonamide is an analogue of *p*-aminobenzoic acid

(PABA), a part of folic acid and a nucleic acid precursor. By this mechanism, the sulphonamides block the synthesis of folic acid and thereby inhibit the synthesis of nucleic acid (Madigan et al., 2015;Kapoor et al., 2017). Trimethoprim is a structural analogue which binds to bacterial dihydrofolate reductase, and thereby blocking the reduction of dihydrofolate to tetrahydrofolate, the active form of folic acid (D Byron May et al., 2018;Gleckman et al., 1981). Sulphonamide and trimethoprim are often used in a combination as they block two sequential steps in the folic acid synthesis pathway (Madigan et al., 2015).

1.4.3 The usage of antibiotics in Norway

The use of antimicrobial agents in Norway is low compared to many other countries. Norway is in a unique position with low levels of resistant bacteria in animals, in food and in feed. Only 10 % of all antibiotics used in Norway is used for terrestrial animals and only 0.5 % for aquaculture. However, the situation may change if the use of antimicrobial agents in Norway increases or resistant strains develop or are imported from abroad (NORM/NORM-VET, 2016).

The total sale of antibiotics in Norway in 2017 was 13.8 defined daily doses (DDD)/1000 inhabitants/ day, in both primary care and in institutions. The major classes of antibacterial agents for clinical use in Norway is penicillins with extended spectrum, β-lactamase sensitive penicillins and tetracyclines (NORM/NORM-VET, 2017).

In 2017, the overall sales of antibacterial agents for use in veterinary medicine was 5 587 kg active substance (NORM/NORM-VET, 2017). For terrestrial food producing animals, penicillins is the most selling class, and almost exclusively benzylpenicillin. In aquaculture, florfenicol (269 kg active substance), oxolinic acid (343 kg active substance) and flumequine (<0.05 kg active substance) are the only antibacterial agents used (NORM/NORM-VET, 2017).

From 1987 to 2017 the sales of antibacterial agents used in aquaculture has decreased by 99.9 %, from 876 mg/PCU to 0.5 mg/PCU in 2017. This reduction is mainly attributed to the introduction of effective vaccines against bacterial infections in Atlantic salmon and rainbow trout, along with the prevention of bacterial infections and the spread of these (NORM/NORM-VET, 2017).

In 2015, the Norwegian government launched a national strategy to reduce the use of antibiotics in humans by 30 % and by 10 % in food producing animals within 2020. The 10 % reduction of antibiotics used in food producing animals was reached in 2017 (Ministry of Agriculture and Food, 2018).

1.5 Antimicrobial drug resistance

1.5.1 Development of antimicrobial drug resistance

Antimicrobial drug resistance is defined as the acquired ability of a microorganism to resist the effects of an antimicrobial agent to which it is normally susceptible to (Madigan et al., 2015).

Shortly after the introduction of penicillin in the 1940`s resistance against this antibiotic became a clinical problem, and in the 1950`s many of the advances made in the treatment of bacterial infections became threatened (Ventola, 2015). However, antibiotic resistance is not a modern phenomenon, bacteria originated over 3.8 billion years ago, and as antibiotics are natural products produced by bacteria and fungi, antibiotics are at least hundreds of millions of years old (Wright and Poinar, 2012;von Wintersdorff et al., 2016). As a result, bacteria must have been exposed to antibiotics and their derivates directly or indirectly for the same amount of time. Antibiotic producers must co-evolve mechanisms to protect them self from the toxic effect of their self-produced antibiotics (Wright and Poinar, 2012). Most antibiotics used are derived from the Actinomycetes, with the most significant genus for antibiotic production being *Streptomyces*. This genus is responsible for the production of antibiotics such as streptomycin, tetracycline, chloramphenicol, erythromycin and vancomycin. These organisms have to be able to avoid the toxic effect of their own metabolites, and therefore, they are resistant to these antibiotics (Perry et al., 2016).

Today, antimicrobial resistance is an emerging problem worldwide, leading to increased morbidity and mortality (NORM/NORM-VET, 2016). There is a strong correlation between the usage of antibiotics and the occurrence of resistant bacteria (NORM/NORM-VET, 2016), and the extensive use of antibiotics, both clinically (Llor and Bjerrum, 2014;Shallcross and Davies, 2014;Webster, 2017;Marti et al., 2014) and in food producing animals (Martin et al., 2015;Santos and Ramos, 2018;Tang et al., 2017;Marti et al., 2014), is a driving force of the evolution of resistant bacteria. This leads to drug-sensitive competitors being removed, while

the resistant strains are left behind to re-produce as a result of natural selection (Ventola, 2015).
The World Health Organization (WHO) has ranked antimicrobial resistance as one of the biggest threats to global health, food security and development. Resistance against antimicrobial agents is rising to dangerously high levels all over the world, and a growing list of infections, such as pneumonia, tuberculosis, septicaemia, gonorrhoea and food poisoning are becoming harder to treat as antimicrobial agents become less effective (WHO, 2017a).

1.5.2 Dissemination of antimicrobial resistance

Both resistant bacteria, antibiotic residues and genetic resistance determinants are spread to the adjacent environments. The majority of consumed antibiotics are excreted unchanged and can then be introduced to the environment, either directly or through e.g. waste streams (von Wintersdorff et al., 2016). New methods for high-throughput sequencing tools have revealed that an intrinsic resistome exists, including sequences normally belonging to bacterial metabolic networks that can participate in resistance towards antimicrobial agents. These "pre-resistance" genes can evolve to new resistance mechanisms if they reach an environment with a high concentration of antibiotics (Marti et al., 2014).
Resistance to antibiotics can be disseminated through the spread of resistant bacteria themselves, among bacteria through vertical transmission (the genome is transferred to daughter cells during binary fission) or by horizontal gene transfer (HGT). HGT is not limited to closely related bacteria, it can also take place between bacteria from different species and/or ecological niches (NORM/NORM-VET, 2016). At least three mechanisms for HGT are known, transformation, conjugation and transduction (Madigan et al., 2015).
Acquisition of foreign DNA from HGT is one of the most important drivers for bacterial evolution, and hence it is frequently responsible for the development of antimicrobial resistance.

The role of the aquatic environment in the development and dissemination of antimicrobial resistance is largely unknown. A large portion of antibiotics consumed by humans, and used for livestock, is released in an active biological form through urine and feces (Osunla and Okoh, 2017). Human and animal potentially pathogenic bacteria are constantly released with waste water into the marine environment, and many of these organisms harbour genes encoding antibiotic resistance. These are able to spread among the members of microbial

communities in water and soil (Baquero et al., 2008). The use of antimicrobial agents in agriculture leads to discharge of residual antibiotics and resistance genes via run-off from land to the marine environment (Berglund, 2015). Waste streams and treatment plants are considered to be hotspots for the dissemination of antimicrobial resistance, since resistance genes, mobile genetic elements and antibiotic selection pressure are introduced. The antibiotic compounds are poorly removed in the treatment plants, and as a result, they can spread further (von Wintersdorff et al., 2016). Even though the antibiotic concentrations in non-clinical settings are generally low, the selection of resistant bacteria can occur at extremely low antibiotic concentrations, like those concentrations found in natural environments as soil and the aquatic environment (Marti et al., 2014). Therefore, the use of antimicrobial drugs and resistance in one ecological compartment can have consequences for the occurrence of resistance in another compartment (NORM/NORM-VET, 2016). Multidrug resistant strains have been detected in coastal areas dedicated to fish farming (Labella et al., 2013).

1.5.3 Biochemical and molecular resistance mechanisms

Bacteria possess three main strategies for resistance. Individual bacterial isolates often possess more than one resistance mechanism, and individual antimicrobials can be affected by different mechanisms in different bacteria. Bacteria may also have intrinsic resistance to certain agents from inherent structural or metabolic properties of the bacterial species (Munn, 2011).

Modification of the target binding site:

Bacteria can develop antimicrobial resistance by modification of the agents binding site, and in this way avoid the action of the antibiotic. These alterations often result from spontaneous mutations of a bacterial gene on the chromosome and leads to reduced affinity for the antibiotic molecule (Kapoor et al., 2017;Munita and Arias, 2016). An example of this is alteration in the PBPs, which results in reduced affinity for β-lactam antibiotics (Kapoor et al., 2017).

Reduced permeability and Efflux pumps:

Many antibiotics have intracellular targets, or for Gram-negative bacteria, in the periplasm. Because of this, the antibiotic molecule must pass the outer and/or cytoplasmic membrane to exert its effect (Munita and Arias, 2016). These molecules can be transferred by diffusion

through porins, diffusion through the bilayer and by self-uptake. The porin channels are located in the outer membrane in Gram-negative bacteria, and the small hydrophilic molecules, such as β-lactams can only cross the outer membrane through porins. The decrease in number of porins leads to decreased influx of β-lactams into the periplasm, and thereby resistance against such antibiotics (Kapoor et al., 2017).
Alterations in the membrane transport proteins can also lead to active efflux of the antibiotics, the molecule is removed from the cell in the same speed as they enter before they reach their target (Kapoor et al., 2017). The efflux pumps can be either substrate specific (specific for a particular antibiotic), or they can have a broad substrate specificity (Munita and Arias, 2016).

Enzymatic degradation:

One of the most successful strategies to avoid the effect of antibiotics, is by producing enzymes that can inactivate the antimicrobial agent. These enzymes can break down the antibiotic molecule, and in this way the agent become ineffective (Munita and Arias, 2016). One of the main enzymes that inactivate antibiotics are β-lactamases, of which some, such as New Delhi metallo-β-lactamase (NDM-1) are able hydrolyse nearly all β-lactam antibiotics with ester- and amide bonds (e.g. penicillins, cephalosporins and carbapenems). There are about 300 known β-lactamases. Other enzymes with the ability to inactivate antibiotics are aminoglycoside-modifying enzymes and chloramphenicol acyltransferases (Kapoor et al., 2017).

1.5.4 Antibiotic resistance and *Vibrio* spp.

As vibrios are naturally occurring in the marine environment, these bacteria can acquire resistance genes from other bacteria in the environment. Many of the genetic determinants that confer resistance to antibiotics are located on plasmids and transferable to other bacteria in the environment through vertical gene transfer or HGT (Osunla and Okoh, 2017). For this reason, the environmental vibrios may serve as a reservoir for the spread of antibiotic resistance due to HGT (Zhang et al., 2012). The abundance of *Vibrio* spp. in the marine environment makes them interesting for monitoring the challenges that the ecosystem faces, such as the impact of using antibiotics and chemicals in aquaculture, agriculture and clinical facilities due to the spread of residues to the environment (Banerjee and Farber, 2018).

In some regions, excessive use of antibiotics in the aquaculture and agriculture industry, has led to the development of multidrug resistance and high levels of resistance to several antibiotics among pathogens associated with seafood, such as *Vibrio* spp. In the recent years the presence of antibiotic resistance genes detected in *Vibrio* species have increased, including the β-lactam and penicillin resistance genes *penA* and *blaTEM-1,* genes encoding resistance against chloramphenicol, such as *floR, catI* and *catII*, and several *tet* genes encoding resistance against tetracycline (Letchumanan et al., 2015).
In 2004, *V. anguillarum* strains showing reduced susceptibility to quinolones was isolated from Atlantic cod reared in Norway. The examination of these strains revealed mutations in the *gyrA* and *parC* genes and these mutations were responsible for the quinolone resistance (NORM/NORM-VET, 2007).

Environmental *Vibrio* spp. isolated from retail shrimp and reared fish have also shown resistance or reduced susceptibility to cefepime, tetracycline, aztreonam, streptomycin, gentamicin, tobramycin, cefazolin, cefuroxime and trimethoprim, and *V. vulnificus, V. alginolyticus, V. harveyi* and *V. parahaemolyticus* isolated from seafood have shown high prevalence of resistance to ampicillin (Hernández-Robles et al., 2016;Pan et al., 2013;Li et al., 1999).
An intrinsic resistance mechanism against penicillins have been detected in *Vibrio* spp. In *V. parahaemolyticus,* a histidine kinase/response regulator pair (VbrK/VbrR) that controls the expression of a β-lactamase have been detected. VbrK detects β-lactam antibiotics via direct binding and the signal is transmitted to VbrR, to control the expression of a carbenicillin-hydrolysing (CARB) β-lactamase gene ($bla_{carb-17}$). VbrK is present in almost all *Vibrio* spp. and the residues responsible for specific recognition of lactams are conserved in different *Vibrio* species. The gene coding β-lactamase have also been found in many *Vibrio* spp., including *V. harveyi, V. alginolyticus* and non-O1/non-O139 *V. cholerae*, and the direct recognition of β-lactam antibiotics by Vbrk can be a well conserved mechanism to induce the β-lactamase gene in *Vibrio* spp. (Chiou et al., 2015;Li et al., 2016).

The correlation between the use of antimicrobial agents and development of resistance leads to differences in the frequency of resistant strains depending on geographical use of antibiotics.

Little recent data regarding the antimicrobial susceptibility of *Vibrio* species in Northern Europe has been found. However, in 2015 Bier et al. performed a study were the antimicrobial susceptibility of *V. vulnificus* and *V. cholerae* isolated in Germany was

examined. The susceptibility of environmental strains obtained from German coastal and estuarine waters, clinical strains and isolates from retail seafood were investigated. The results showed that most of the antimicrobial agents recommended for treatment of *V. vulnificus* and *V. cholerae* non-O1/non-O139 were effective. However, among *V. vulnificus* isolates, resistance against aminoglycosides was found, and for *V. cholerae* resistance against aminopenicillins and aminoglycosides was observed. In this study, the results indicated the presence of a β-lactamase with carbapenem hydrolysing activity in four environmental *V. cholerae* non-O1/non-O139 isolates. The author of this publication highlights the need for systematic monitoring of antimicrobial susceptibility in potentially pathogenic *Vibrio* spp. in Europe, as carbapenems are considered last line agents for treatment of multidrug resistant Gram-negative bacteria (Bier et al., 2015).

During an investigation of the antimicrobial susceptibility of 99 strains of *V. cholerae* non-O1/non-O139 isolated from wastewater and shellfish in 2000-2001 in France, resistance towards one antimicrobial agent (streptomycin, sulphonamides or ampicillin) was detected in 55 % of the tested strains, while multidrug resistance was detected in 6 % of the strains. All the multidrug resistant strains were isolated from cockles and showed resistance to all three agents (Baron et al., 2017).

1.6 Identification of *Vibrio* spp.

1.6.1 Analytical Profile Index 20E (API 20E)

The API 20E test kit is used for identification of Enterobacteriaceae and other non-fastidious Gram-negative bacteria (bioMèrieux, 2002). The system consists of dehydrated chemicals in a set of 20 reaction wells which are inoculated with a bacterial suspension. During incubation, bacterial metabolism produces colour changes. These colour changes are either spontaneous or revealed by addition of reagents to the wells. Based on the results from the biochemical tests, the tested isolates are given a seven-digit number which is used to identify the isolates using the apiweb^{TM} database. The API 20E database contains six *Vibrio* spp.: *V. alginolyticus, V. cholerae, V. fluvialis, V. mimicus, V. parahaemolyticus* and *V. vulnificus* (bioMèrieux, 2015).

1.6.2 Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry (MALDI-TOF MS) is used for the analysis of biomolecules, such as proteins and peptides (Bishop et al., 2013). The samples for analysis by MALDI-TOF MS is prepared by mixing with a matrix, an energy absorbent, organic compound, on a stainless-steel target plate. When the matrix is dried, it becomes crystalized, and the sample within the matrix will co-crystallize (Singhal et al., 2015). The sample is then ionized and vaporized by a laser. This generates singly protonated ions from analytes in the sample. The protonated ions generated are accelerated along the column toward a detector by an electric field. The time of flight (TOF) for each ion depends on their mass-to-charge-ratio (m/z). The charged analytes are then detected and measured (Madigan et al., 2015). Based on the TOF information, a characteristic spectrum, a peptide mass fingerprint (PMF), is generated for the analytes in the sample. Identification of microbes by MALDI-TOF-MS is done by either comparing the PMF of an unknown organism with the PMFs in a database, or by matching the masses of biomarkers of unknown organisms with the proteome database (Singhal et al., 2015).

1.6.3 *rpoB* sequencing

Sequencing of the 16S ribosomal RNA (rRNA) gene is widely used in microbiology to identify prokaryotic organisms. This small subunit rRNA gene is the recognized gold standard for estimating the diversity in microbial communities (Vos et al., 2012). However, the genus *Vibrio* contains a large number of closely related species with 16S rRNAs differing in nucleotide sequence from less than 1 % up to 6 % (Moreno et al., 2002), rendering 16s rRNA sequencing less reliable for identification to the species level. The number of known *Vibrio* spp. continues to rise, thus it becomes more likely that sequence variation in the 16s rRNA gene will no longer be sufficient alone as a target for differentiation of closely related *Vibrio* spp. (Hoffmann et al., 2010).

The *rpoB* gene has been suggested as a potential biomarker to overcome the high conservation of the 16S rRNA gene. *rpoB* is a gene encoding the beta subunit of RNA polymerase. This gene is homogeneous within cells because it is a single copy gene, and therefore it has advantages over the 16S rRNA gene (Ki et al., 2009). Sequencing of the *rpoB*

gene has proved to be a valuable tool for identification of *Vibrio* spp. (Schirmeister et al., 2014;Tarr et al., 2007;Schwartz et al., 2017).

1.7 Antimicrobial susceptibility testing

1.7.1 Disk diffusion

Disk diffusion is a method used for antimicrobial susceptibility testing in many clinical laboratories (Balouiri et al., 2016).

When an antibiotic disk with a known concentration of an antibiotic agent is placed on a Mueller Hinton agar (MHA) plate inoculated with a bacterial suspension, the agent will start to diffuse into the agar and inhibit the growth of the microorganism. The speed of the diffusion into the agar is not as quick as the speed of extraction of the agent from the disk. As a result, the concentration of the antibiotic will be highest closest to the disk and decrease with increasing distance, and an inhibition zone will form around the agent. How fast the agent diffuses in the agar depends on the agents solubility in the medium and the molecular weight of the agent. This results in unique breakpoints for each antimicrobial agent, and this makes it possible to determine the tested isolates susceptibility towards the agents by measuring the inhibition zones (Hudzicki, 2009).

International organizations, such as Clinical (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), have developed standardized protocols for the disk diffusion method for different groups of bacteria, and breakpoints which makes it possible to determine the test organisms susceptibility towards the tested agents. Based on these breakpoints, the tested bacteria can be categorized as sensitive (S), intermediate susceptible (I) or resistant (R) to the tested agents.
The tested bacteria can also be classified as wild-type (WT) or non-wild-type (NWT) to antibacterial agents. A WT strain can be defined as a bacterium without an acquired resistance mechanism or mutation causing resistance to the tested antibacterial agent, while an NWT strain has an resistance mechanism to the tested agent (Turnidge and Paterson, 2007). The categorization as WT/NWT is based on epidemiological cut-off values (ECOFF). ECOFFs are useful when clinical breakpoints not yet have been defined and could detected development of resistance early. These values are not necessarily clinically significant (Toutain et al., 2017).

As many of the vibrios are halophilic bacteria, they may have a higher salt requirement than the 0.86 % found in MHA, and it is recommended by the CLSI to test obligate halophilic bacteria on MHA supplemented with 1 % NaCl (Guérin-Faublée et al., 1995;CLSI, 2006).

1.8 Genomic analysis

1.8.1 Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is a method that enables analysis of the complete genomic DNA sequence of a cell. This method provides the most comprehensive genotypic characterisation of microbes, including the identification of antimicrobial resistance determinants and virulence determinants (Thomas et al., 2017).

The first step in many methods used for WGS is DNA fragmentation, where the DNA is enzymatically cut to small random fragments. The fragments are then amplified and sequenced. After sequencing, computer software is used to piece the reads together to longer continuous stretches of sequences (contigs). The contigs are then joined together to form longer stretches of sequences (scaffolds) (Ekblom and Wolf, 2014). The assembled genome can then be uploaded to databases, such as the Rapid Annotation using Subsystem Technology server (RAST) (RAST, 2008), for annotation. In this process, the function of the genomes features are identified (Dunn et al., 2019).

2. Materials and methods

2.1 Sampling

Samples for this project included herring, mackerel, water and bivalve molluscs.

Initial Sampling was conducted on a North Sea herring cruise which I attended from 29.05 to 02.06 2018, on board the pelagic purse seine/trawler “MS Kings Bay”.

Additional samples from herring and mackerel were obtained from research cruises which took place in September and November 2018.

Bivalve molluscs were collected through the annual surveillance programme on *Escherichia coli* in bivalves, where samples are collected and sent to the institute by the Norwegian Food Safety Authority (NFSA).

Locations for collection of water samples, sampling sites for bivalve molluscs, herring and mackerel are included in Figure 2.

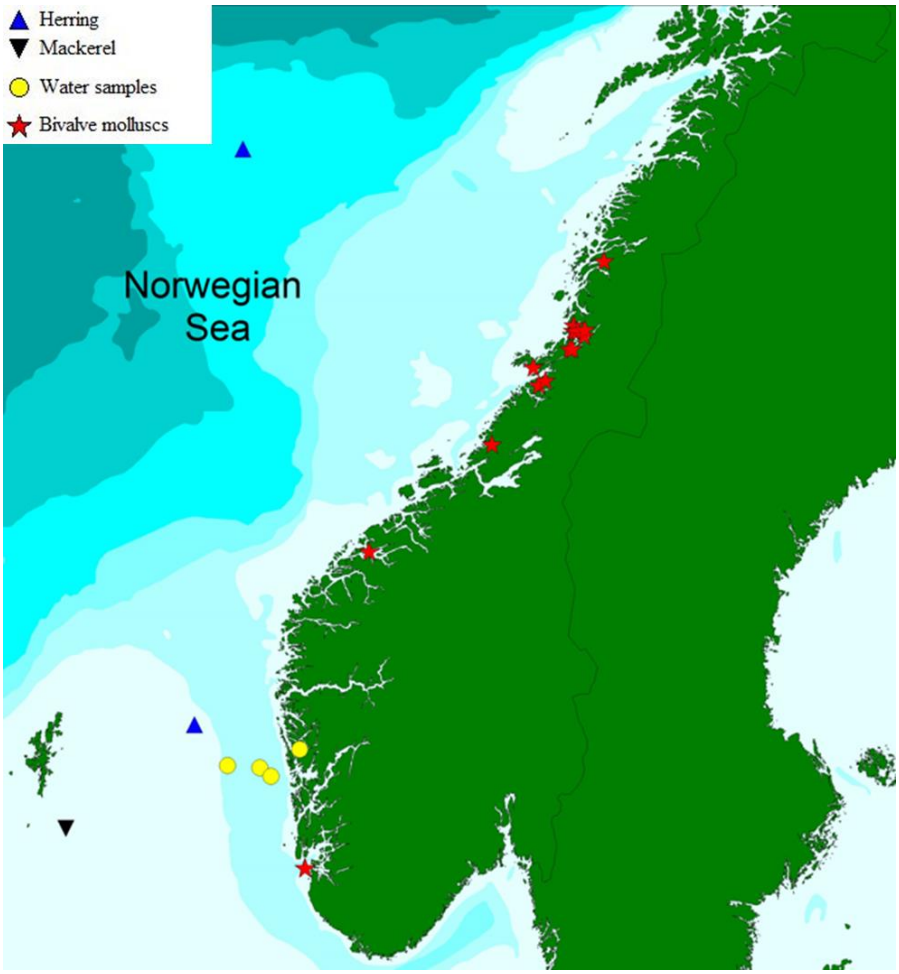

Figure 2: The locations for collection of water samples, herring, mackerel and bivalve molluscs.

2.1.1 water samples

Water samples were collected from four different locations (Figure 2) at five different depths from each location: surface, 2 m, 5 m, 7 m and 10 m respectively using a Van Dorn water sampler (KC Denmark, Denmark) collecting 3 l for each sample. The water temperature was recorded at each sampling site.

From each sample, aliquots of 100 ml were collected in sterile plastic cups for pH and salinity measurements.

For the detection of *Vibrio* spp., aliquots of 100-250 ml water were filtered through a filter with 0.45 µm pores (Merck Millipore, Germany) using the EZ-fit Manifold 3-place system (Merck Millipore, Germany) connected to a vacuum pump. The filters were transferred to Thiosulfate Citrate Bile Sucrose (TCBS) agar (Oxoid, UK) and incubated at 37 °C for 24-48 hours. Five parallels were set up from each sample. For two of the parallels an enrichment

step was performed on approximately 500 ml water. For the enrichment step, the samples were added 50 mL concentrated (360 mg/ml) Alkaline Peptone Water (APW) (Oxoid, UK) and incubated at 42 °C for 18±2 hours. After incubation, 100 µl was streaked onto TCBS agar and incubated at 37 °C for 24-48 hours. The agar plates were stored at refrigerated temperature on board the ship.
The TCBS plates were brought back to the laboratory at the IMR and stored at 4 °C. At the lab, all plates were counted to estimate the number of colony forming units (cfu) per 100 ml. Single colonies were picked and sub cultured on to new TCBS plates at least three times to obtain pure cultures.

2.1.2 Fish

A total of 60 fish were sampled: 40 herring and 20 mackerel.

At the last day of the research cruise in June 2018, 40 herring were collected from the storage tank on board the ship. Using gloves, the fish were picked by hand and placed in sterile plastic bags (WWR, USA). Two fish were placed in each plastic bag, comprising one sample. The herring was stored at chilled temperature and brought back to the lab at the IMR and stored at 4 °C until further processing.

From the herring, samples were collected from the muscle with skin, the intestine and the gills. Isolation of *Vibrio* spp. was based on the Nordic Committee on Food Analysis (NMKL) method no. 156 (NMKL, 1997). Using sterile scalpels, 20 g was cut from each tissue type and enriched in 180 ml APW with 2 % NaCl. Samples for detection of *V. parahaemolyticus* were enriched in APW with 2 % NaCl supplemented with polymyxin B (250 IU/ml). The enrichment cultures were incubated at 42±1 °C for18±2 hours. After incubation, a 10 µl loop of the enrichment culture was streaked on to TCBS plates and incubated at 37±1 °C for 24±3 hours. From the TCBS plates, single colonies corresponding to *Vibrio* characteristics were picked and sub cultured on new TCBS plates at least three times to obtain pure cultures.

From the mackerel cruise in September 2018, an additional 40 fish, comprising 20 samples, were obtained. Samples were collected from the muscle with skin and enriched in APW with and without polymyxin B. The enrichment cultures were incubated at 42±1 °C for18±2 hours. After the enrichment step, 10 µl from enrichment cultures were spread on TCBS agar and incubated at 37±1 °C for 24±3 hours.
Samples were also collected from gut content and homogenized in phosphate buffered saline

(PBS) (Sigma-Aldrich, USA), and a ten-fold dilution series was made. The following dilutions were prepared: 10^{-1}, 10^{-2} and 10^{-3}. From each sample, 0.1 ml was transferred to TCBS agar and spread using an L-shaped spreader. Both undiluted and diluted samples were cultured on TCBS agar. The plates were incubated in 37±1 °C and read after 24- and 48 hours (NMKL, 1997).

Samples were also collected from herring collected in November 2018. A total of 40 fish, comprising 20 samples, were brought to the lab and samples collected from the muscle with skin.

2.1.3 Bivalve molluscs

Analyses were based on NMKL method no. 156 (NMKL, 1997). Samples comprised batches of 10-15 individual bivalve molluscs from 16 different locations, where 14 were blue mussels (*Mytilus edulis*), one flat oyster (*Ostrea edulis*), and one scallop (*Pecten maximus*).
The bivalves were cleaned in cold water and opened using a sterile knife. From each sample approximately 100 g soft tissue and intra-valvular fluid was weighed in and homogenized using a stomacher for 2-3 min. Aliquots of 20 g from each sample was transferred to new sterile bags and enrichment followed the same protocol as samples from fish.
After incubation, 10 µl of the enrichment cultures were transferred to TCBS plates and to *Vibrio* ChromoSelect agar (VCS) (Sigma-Aldrich, USA) plates, and spread using a sterile loop. The plates were incubated at 37±1 °C for 24±3 hours.

Before the enrichment step, approximately 1 ml of the homogenate was transferred to sterile tubes and the following dilutions were made using peptone water (bioMerièux, France): 10^{-1}, 10^{-2} and 10^{-3}. From each dilution and the undiluted sample, 100 µl were transferred to TCBS plates and to VCS plates and spread using an L-shaped spreader. The plates were incubated 37±1 °C for 48 hours and read after 24 and 48 hours.

From both the TCBS plates and the VCS plates, single colonies corresponding to *Vibrio* characteristics were picked and sub-cultured on to new plates at least three times to obtain pure cultures.

2.2 Characterisation of the isolates

Before characterisation of the isolates, they were re-streaked on to plate count agar (PCA) (Oxoid, UK) supplemented with 2 % NaCl.
Characterisation was done by Gram testing, testing for the production of cytochrome c oxidase, light microscopy, Analytical Profile Index (API) 20 E (bioMèrieux, France), and by Matrix Assisted Laser Desorption Time Of Flight Mass spectrometry (MALDI-TOF MS).

2.2.1 Gram test

Gram-staining was performed on all isolates using the potassium hydroxide (KOH) method (Buck, 1982). This method depends on the lysis of Gram-negative cells in KOH releasing DNA from the cell to turn the suspension viscous. Colonies were transferred from the agar plates to a slide with a drop of 3 % KOH. The viscosity was determined by raising the loop from the slide. If a string was observed, the isolate was Gram-negative. If no string was observed, the isolate was Gram-positive.

2.2.2 Oxidase test

Oxidase testing was performed by dispensing a drop of remel Bactidrop ^{TM} oxidase (Thermo Fisher Scientific, USA) reagent on to a filter paper. Using a sterile loop, a colony was smeared on to the filter paper. Development of a violet colour within 10-30 sec. was interpreted as a positive reaction (Thermo Fisher Scientific, 2006).

2.2.3 Cell morphology

A drop of sterile water was transferred on to a slide before a colony was smeared on to the slide and the cell morphology of the isolates were studied by phase contrast microscopy with an Olympus BX40 microscope (Olympus optical, Japan) under the 1000x magnification using the oil immersion objective. The isolates were grouped according to their morphology as rods or cocci.

2.2.4 Analytical Profile Index 20E (API 20E)

All isolates were preliminary identified using the API 20E test kit (bioMèrieux, France). A bacterial suspension corresponding to 0.5 McFarland standard was prepared by transferring well isolated, fresh colonies to 5 ml sterile saline (2 % NaCl). Using a sterile pipette, the bacterial suspension was transferred to the reaction wells on the API 20E strip. Depending on the substrate, the wells were filled with different volumes. For the following wells, both the well and couple were filled: CIT (citrate), VP (sodium pyruvate) and GEL (gelatine). For the remaining wells, only the lower part of the well was filled with the bacterial suspension. The wells containing arginine (ADH), lysine (LDC), ornithine (ODC), sodium thiosulfate (H_2S) and urea (URE) were overlayed with mineral oil to create an anaerobic environment (bioMèrieux, 2002). The strip was placed in the tray filled with distilled water and a lid was placed over the tray to create a humid atmosphere. The API 20E strips were incubated at 36±2 ˚C for 18-24 hours.

After incubation, the strip was read according to the reading table provided by the manufacturer. The VP-well was added one drop of VP1 (bioMèrieux, France) and one drop of VP2 (bioMèrieux, France), the TDA well was added one drop of TDA reagent (bioMèrieux, France) and the IND well was added one drop of James reagent (bioMèrieux, France). A colour change in the VP well within 10 min. was interpreted as a positive reaction, while the TDA- and IND well was read immediately after addition of the reagents (bioMèrieux, 2002). Based on the results from the biochemical tests, each isolate was given a seven-digit number which was used to identify the isolates using the apiweb^{TM} database.

2.2.5 Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry (MALDI-TOF MS)

All Gram-negative rod-shaped isolates and three Gram-positive isolates were cultured on PCA with 2 % NaCl, incubated at 37 ˚C over night and sent to the Norwegian Veterinary Institute (NVI) for identification by MALDI-TOF MS. The obtained Peptide Mass Fingerprints (PMFs) were compared to spectra in the commercial MALDI-TOF MS database (MALDI Biotyper, Bruker, Germany) and to spectra in an in-house generated database (NVI) containing spectra from vibrios associated with fish.

2.3 Antimicrobial susceptibility testing

2.3.1 Disk diffusion

Antimicrobial susceptibility testing was conducted according to The Clinical and Laboratory Standards Institute (CLSI) method M42-A, Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals (CLSI, 2006).

The day before testing, the isolates were re streaked on to PCA supplemented with 2 % NaCl and incubated overnight in 28±2 °C or 22±2 °C, depending on the species to be tested. After incubation, fresh colonies were picked and suspended in 5 ml sterile saline (0.85 % NaCl) to the density of McFarland standard 0.5. A sterile cotton swab was dipped in the suspension and pressed against the inside of the tube to avoid over-inoculation. Using an automatic plate rotator, the inoculum was spread evenly with the cotton swab on plates with cation adjusted Mueller-Hinton agar (MHA) (Oxoid, UK).
Each isolate was tested for their susceptibility against 18 antibiotics belonging to 10 different classes (Table 2) using antibiotic disks (Oxoid, UK). Six disks were placed on each plate, giving a total of three plates for each isolate. After application of the antibiotic disks, the MHA plates were stacked with five plates in each and incubated at 28±2 °C for 24-28 hours.

To avoid erroneous reading of the inhibition zones, the 15-15-15 minute rule was applied: the inoculum suspension was used within 15 min. of preparation, the antibiotic disks were applied within 15 min. of inoculation of the MHA plates and the plates were incubated within 15 min. of disk application (EUCAST, 2017). *E. coli* CCUG17620 was used to assure the quality of the MHA plates and the antibiotic disks.

V. aestuarianus has a lower optimum growth range (Tison and Seidler, 1983) then the rest of the isolated species, and for antimicrobial susceptibility testing of this species an incubation temperature of 22 °C was used. The inhibition zones were read after 24-28 hours and 44-48 hours.

After incubation, the inhibition zone for each antibiotic was measured using a digital calliper on the MHA plates flipped bottom-up and aligned against a dark background (Bauer et al., 1966). To determine the isolates susceptibility against the antibacterial agents tested, the measured zone diameters were compared to breakpoints for Enterobacteriaceae from CLSI method M100, Performance Standards for Antimicrobial Susceptibility Testing 27th edition (CLSI, 2017). For the agents oxolinic acid, florfenicol and erythromycin, the measured zone

diameters were compared to breakpoints and epidemiological cut-off (ECOFF) values for *Aeromonas salmonicida* from CLSI method VET03/VET04-S2, Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Second Informational Supplement (CLSI, 2014). Based on the breakpoints and ECOFF values, the isolates were classified as sensitive (S), intermediate (I) or resistant (R), or wildtype/non-wildtype for each antibiotic tested. An inhibition zone equal to the disk diameter (6 mm) was regarded as no zone (Bauer et al., 1966).

Table 2: The antibacterial agents used for antimicrobial susceptibility testing. All the agents tested are considered to have a broad spectrum of activity.

Class	**Agent**	**Concentration (µg)**
Penicillins	Mecillinam (MEL)	10
	Ampicillin (AMP)	10
Cephalosporins	Cefotaxime (CTX)	5
	Ceftazidime (CAZ)	10
Tetracyclines	Doxycycline (DO)	30
	Tetracycline (TE)	30
Quinolones	Ciprofloxacin (CIP)	5
	Oxolinic acid (OA)	2
Carbapenems	Imipenem (IPM)	10
	Meropenem (MEM)	10
Macrolides	Erythromycin (E)	15
	Azithromycin (AZM)	15
Sulphonamide and Trimethoprim	Sulfamethoxazole/Trimethoprim (STX)	25
	Trimethoprim (W)	5
Aminoglycosides	Gentamicin (CN)	10
	Tobramycin (TOB)	10
Amphenicols	Florfenicol (FFC)	30
Monobactams	Aztreonam (ATM)	30

Five isolates from each species were selected for antimicrobial susceptibility testing on MHA supplemented with 1 % NaCl as recommended by CLSI for obligate halophilic strains (group 2 organisms) (CLSI, 2006) to study differences in zone diameter with and without addition of NaCl to the medium. The diameter of inhibition zones was read after 24-28 hours of incubation at 28 °C. Isolates belonging to the species *V. aestuarianus* were incubated at 22 °C, and inhibition zones were read after 24 and 48 hours.

2.3.2 Minimum Inhibitory Concentration (MIC) for imipenem

As imipenem is one of the agents considered last line for treating multidrug resistant Gram-negative bacteria (Bier et al., 2015), isolates showing reduced susceptibility to this agent were selected for determination of Minimum Inhibitory Concentration (MIC) using M.I.C Evaluator Strips (Oxoid, UK).

Fresh colonies were picked and suspended in sterile saline (0.85 % NaCl) to the density of McFarland standard 0.5. A sterile cotton swab was dipped in the suspension and pressed against the inside of the tube to avoid over-inoculation. Using an automatic plate rotator, the inoculum was spread evenly on MHA plates with the cotton swab. The M.I.C evaluator strip was placed on the plate using sterile tweezers and the plates were incubated aerobically at 28±2 °C for 24-28 hours. The MIC was read where the growth touched the strip. If the growth was inhibited between the sections on the strip, the MIC was read as the value in the white section above (Oxoid, 2008). The isolates tested were categorized as resistant, intermediate or susceptible to imipenem using breakpoints for Enterobacteriaceae from CLSI method M100 (CLSI, 2017).

E. coli CCUG17620 was included to assure the quality of the MHA and the M.I.C evaluator strips.

2.3.3 CarbaNP (Carbapenemase Nordmann-Poirel) test

Isolates showing reduced susceptibility to imipenem were examined for production of carbapenemase by the carbaNP test. The carba NP test is based on a reduction in pH due to the hydrolysis of carbapenems. The pH change will result in a colour change from red to yellow in a phenol red solution containing the carbapenem (Dortet et al., 2012).
A phenol red solution was prepared by mixing 2 ml phenol red solution (Sigma-Aldrich, USA) (0.5 %) with 16.6 ml dH_2O. The pH was adjusted to 7.8 with 1 M NaOH and 180 µl 10 mM $ZnSO_4 \cdot 7H_2O$ were added to the solution. From each isolate, one 10 µl loop of colonies were transferred to 1.5 ml Eppendorf tubes containing 240 µl 5 M NaCl and vortexed for 5 sec. The phenol red solution was added imipenem (Sigma-Aldrich, USA) to a final concentration of 6 mg/ml and vortexed. For each isolate, two 1.5 ml Eppendorf tubes were prepared, one tube containing phenol red solution with imipenem and one tube containing

only the phenol red solution as a negative control. Both tubes were inoculated with 100 µl of the bacterial solution and incubated at 37 °C. The results were read after 10, 20 and 30 min., and a colour change to yellow was interpreted as a positive reaction, while no colour change was interpreted as a negative result.

OXA 48 and NDM1 were included as positive controls, and AmpC porin and TEM552 as negative controls.

2.4 Determination of hemolytic activity

Hemolytic activity of *V. alginolyticus* and *V. metschnikovii* on blood agar was determined on Tryptic Soy Agar (TSA) with 5 % sheep blood (VWR) or TSA with 5 % human blood. Blood agar plates containing human blood was prepared using TSA (Merck, Germany) as base. Blood was drawn from myself to EDTA tubes (BD Diagnostics, U.S.) by the help of an approved biomedical laboratory scientist. TSA was prepared by dissolving 40 g in 1000 ml distilled water. The pH was adjusted to 7.3±0.2, and the agar was autoclaved at 121 °C for 15 min. The agar was chilled to 45-50 °C and 5 % blood was added under stirring. Approximately 12-15 ml agar was poured into sterile petri dishes. The plates were stored at 4 °C until use.

Isolates were streaked on to blood agar plates and incubated at 37 °C for 24 hours. Isolates showing a green zone around the colonies were classified as α-hemolytic, while isolates showing a clear zone around the colonies were classified as β-hemolytic (Engelkirk et al., 2011).

2.5 Molecular methods

2.5.1 Extraction of DNA

Bacterial cells were harvested by transferring 3-4 10 µl loops of colonies to 1.5 ml Eppendorf tubes containing 500 ml PBS. The tubes were centrifuged at 16860 rcf for 1 min. before the supernatant was removed. The pellet was dissolved in 300 ml PBS and centrifuged at 16860 rcf for 1 min. The supernatant was removed, and the pellet was resuspended in 500 ml sterile water. Working solutions were prepared by transferring 200 µl of the bacterial suspension to

new 1.5 ml Eppendorf tubes, and centrifuging the tubes at 16990 rcf for 1 min. The supernatant was removed, and the pellets were stored at -20 ° until use.

Extraction of DNA from bacterial isolates was done using the DNeasy Blood & Tissue kit (Qiagen, Germany). An additional lysis step was performed by resuspending the samples in 180 µl lysis buffer and incubating them at 37 °C over night. The samples were then completely lysed by adding 25 µl proteinase K and 200 µl AL buffer to the samples. The samples were then incubated at 56 °C for 30 min. After incubation, 200 µl 96 % ethanol was added to each sample, before the solution was transferred to the DNeasy spin column. The column was centrifuged at 5900 rcf for 1 min. before the flow-through was discarded. The column was transferred to a new collection tube and added 500 µl AW1 buffer and centrifuged at 5900 rcf for 1 min. After centrifugation, the spin column was placed in a new collection tube and added 500 µl buffer AW2. The spin column was then centrifuged at 16100 rcf for 3 min. The flow-through was discarded and the column placed in a 1.5 ml Eppendorf tube. To elute DNA from the column, 50 µl AE buffer was pipetted directly on to the DNeasy membrane before the tubes were centrifuged at 5900 rcf for 1 min. The elution was repeated by transferring the spin column to a new 1.5 ml Eppendorf tube and adding 30 µl buffer AE on to the membrane (Quiagen, 2006).The tubes were then centrifuged at 5900 rcf for 1 min. The purity (260/280 and 260/230 ratios) and concentration of the elute was measured using Nanodrop ND-1000 (NanoDrop Technologies, USA). The DNA concentrations were also measured using Qubit 2.0 (Invitrogen, USA). The DNA elute was stored at -20 °C.

2.5.2 Taxonomy sequencing

Isolates characterised as Gram-negative, oxidase positive, able to grow well on PCA with 2 % NaCl and not identified by MALDI-TOF MS were subjected to *rpoB* sequencing.

A PCR mix was prepared by mixing 5 µl 10x HiFi buffer, 2 µl $MgSO_4$ (50 mM), 1 µl dNTP mix, 0.5 µl primer 1110-F (5`-GTAGAATCTACCGCATGATG-3`), 0.5 µl primer CM32b-R (5`-CGGAACGGCCTGACGTTGCAT-3`), 0.2 µl Platinum® Taq HiFidelity polymerase and approximately 100 ng of the template in a 200 µl PCR tube. The final volume was adjusted to 50 µl with nuclease free H_2O. The PCR tubes were placed in a GeneAmp PCR system 9700 (Applied Biosystems, USA), and the following programme was used; 94 °C for 5 min., then 35 cycles of 94 °C for 30 sec. and 68 °C for 30 sec, followed by 72 °C for 30 sec. A final step

on 72 °C for 7 min. and a hold on 4 °C ended the program. The PCR products were checked by gel electrophoresis (1 % agarose gel stained with Gel Red Nucleic Acid Stain (Biotium, USA)) and UV illumination.

The resulting 984 bp PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany). For sequencing of the products, the PCR primers and additional sequencing primers were used: 1661-F (5`-TTYATGGAYCARAACAACCC-3`) and 1783-R (5`-GGACCTTYAGGNGTTTCGAT-3`). The following reaction was prepared in 200 µl PCR tubes, four tubes per sample: 1 µl Big Dye®, 1 µl sequencing buffer, 5-20 ng template, and 0.5 µl primer, one primer per PCR tube. The total volume was adjusted to 10 µl with MilliQ H_2O. A PCR was run with the following programme: 96 °C for 5 min, then 25 cycles of denaturation at 96 °C for 10 sec., primer hybridization at 50 °C for 5 sec. and elongation at 60 °C for 4 min. The reaction was ended with at hold at 4 °C.

The PCR products were sent to The sequencing facility at the University of Bergen (UoB) for sequencing. The obtained *rpoB* sequences were analysed using the BLAST tool and compared to sequences in the Nucleotide collection (nr/nt) database.

To assure that isolates intended for Whole Genome Sequencing (WGS) were in pure cultures, a total of 23 isolates were subjected to 16s rRNA sequencing.

The PCR reaction was prepared as described for *rpoB* sequencing with the exception of the primers used and the PCR program. For 16s rRNA, the primers BA338-F (5′ACT CCT ACG GGA GGC AGC AG 3′) and UN518-R (5′ATT ACC GCG GCT GCT GG 3′) were used. The following program was used: 94 °C for 2 min., then 30 cycles of 94 °C for 30 sec. and 54 °C for 30 sec, followed by 68 °C for 1 min. A final step on 68 °C for 7 min. and a hold on 4 °C ended the program. The resulting approximately 200 bp PCR products were purified using the QIAquick PCR purification kit. For sequencing of the products, only primer BA338-F was used. The PCR mix and program followed the same protocol as for *rpoB* sequencing.

The PCR products were sent to The sequencing facility at the UoB for sequencing.
The finished 16s rRNA sequences were analysed using the BLAST tool and compared to sequences in the 16S rRNA sequence database.

2.5.3 Whole Genome Sequencing (WGS)

A selection of 23 isolates, eight *V. anguillarum*, six *V. metschnikovii*, seven *V. alginolyticus* and two *V. cincinnatiensis*, were sent to the Norwegian Sequencing Centre (NSC) for WGS. Before the samples were sent to the NSC, they were run on a 0.8 % agarose gel to examine if the DNA was degraded.

The obtained raw sequence data was processed at the IMR by using BBDuk to remove the adaptor sequences from the reads and to trim low-quality ends. The genomes were assembled from the reads using SPAdes and uploaded to the Rapid Annotation using Subsystem Technology server (RAST) (RAST, 2008) for annotation. The assembled genomes were also uploaded to the Virulence Factors Database (VFDB) (VFDB, 2018) for detection of virulence factors and to the Comprehensive Antibiotic Resistance Database (CARD) (CARD, 2019) for identification of resistance genes.

3. Results

3.1 Locations, samples and amount of bacteria

A total of 20 water samples were collected and depth (m), temperature (°C) and salinity (‰) were registered, prior to analysis of cfu/100 ml on TCBS agar.
The highest temperatures were measured at the location closest to shore (Location 1), in Sample 1 (surface), 16 °C, and Sample 2 (2 m), 15 °C. From all other sampling locations temperatures ≤ 15 °C were measured (Figure 3).

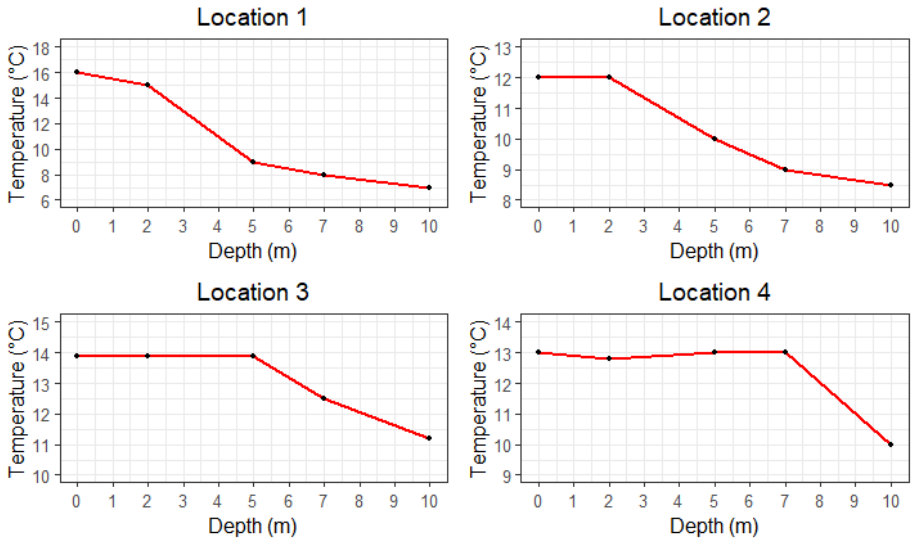

Figure 3: Measured temperature (°C) in each sample from each location. Note: different values on y-axis on each graph.

The lowest salinity was measured in the samples collected closest to shore (Location 1) in the surface and at 2 m depth, while it increased with increasing depth at this location. The highest salinity was measured at Location 3 from the sample collected at 10 m depth (Figure 4).

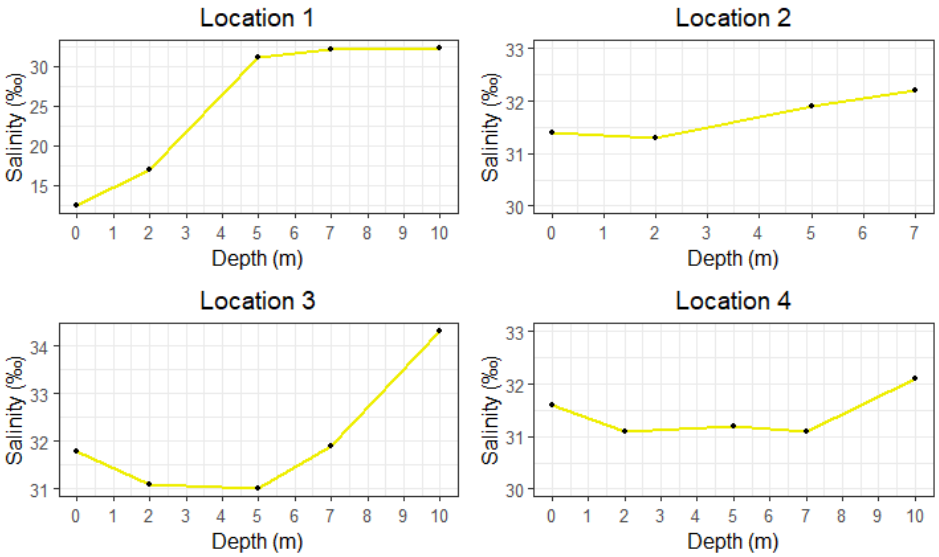

Figure 4: Measured salinity (‰) in each sample from each location. Note: different values on both axis on each graph.

The highest number of cfu/100 ml was found in Sample 1 (surface) and 2 (2 m) from the sampling location closest to shore (Location 1). From the samples collected further from shore and at lower depths, a lower cfu/100 ml was counted (Figure 5). The highest number of cfu/100 ml was found were the water temperature was ≥ 15 °C and salinity ≤ 17 ‰.

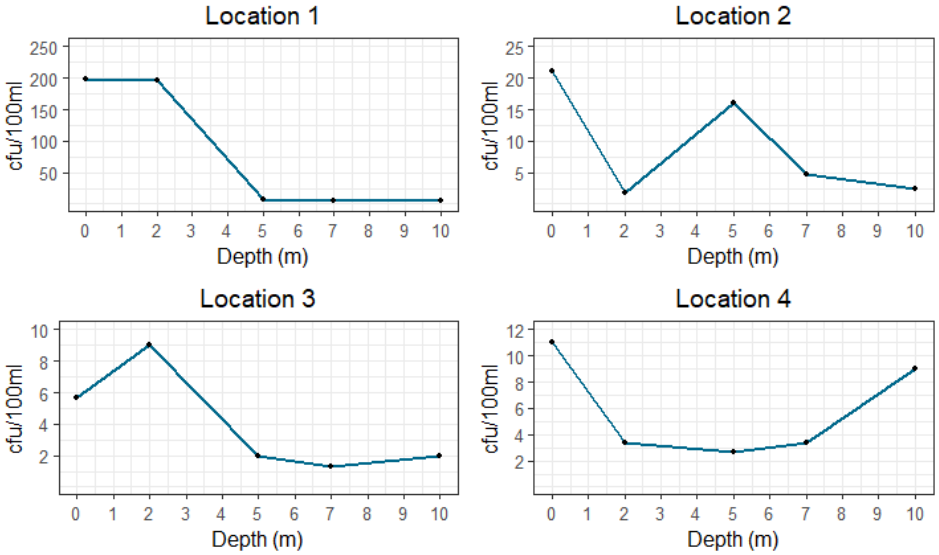

Figure 5: Number of colony forming units (cfu)/100 ml water on TCBS plates incubated at 37 °C for 24-48 hours counted in each sample from each location. Note: different values on y-axis on each graph.

In addition to the water samples, 60 fish samples and 16 bivalve molluscs were collected and analysed.

3.2 Characterisation of the isolates

From the samples, a total of 177 colonies were picked from TCBS- and VCS plates and isolated in pure cultures for further analysis. Of these, a total of 83 of these were isolated from water samples, 70 from fish samples and 24 from bivalve molluscs. From the water samples, 88 % of the colonies were isolated from Location 1, 7 % from Location 2 and 5 % from Location 3. From the fish samples, 41 % of the colonies were picked from samples from muscle with skin (n=60), 31 % from intestine (n=40) and 27 % from gills (n=20). From the bivalves, 67 % of the isolates came from Sample 4.

A first characterisation of the total 177 isolates was done by Gram testing, oxidase testing, microscopy and identification by API 20E. A total of 174 were Gram-negative, rod-shaped bacteria, while three were identified as Gram-positive cocci. Based on the results from the oxidase test, 102 of the Gram-negative isolates were found to be oxidase positive and 72 were oxidase negative. Among the 177 isolates, 65 were identified as *Vibrio* spp. using the API 20E system, while the remaining isolates were identified as other species or yielded an unacceptable profile. Several of the obtained profiles gave low identification scores and T values (Table 3).

Table 3: Isolated colonies identified by API 20E. Excellent identification: %ID≥99.9, T≥0.75, Very good identification: %ID≥99.0, T≥0.50, Good identification: %ID≥90.0, T≥0.25, Acceptable identification: %ID≥80.0, T≥0.
A. hydrophila/caviae/sobria: Aeromonas hydrophila/caviae/sobria, P. pneumotropica /M. haemolytica: Pasteurella pneumotropica/Mannheimia haemolytica, S. putrefaciens: Shewanella putrefaciens, P.damsela: Photobacterium damsela, P. vulgaris: Proteus vulgaris.

No. of isolates	**Bacterium**	**%ID – range**	**T value - range**
54	*V. alginolyticus*	85.9 - 99.3 %	0.66 - 0.81
8	*V. cholerae*	89.8 - 98.5 %	0.41 - 0.71
2	*V. fluvialis*	56.5 - 93.5 %	0.51 - 0.52
1	*Vibrio* sp.	49.7 %	0.53
49	*A. hydrophila/caviae/sobria* 1/2	37.3 - 98.8 %	0.28 - 0.84
28	*P. pneumotropica/M. haemolytica*	34.4 - 99.2 %	0.42 - 0.69
5	*S. putrefaciens*	>99 %	0.42 - 0.9
1	*P. damsela*	88 %	0.11
2	*P. vulgaris*	99.2 - 99.9 %	0.93
27	Unacceptable profile	-	-

All 177 isolates were sent for identification by MALDI-TOF MS. The results from MALDI-TOF MS identified 133 of the isolates as *Vibrio* spp., eight were species within the genus *Proteus,* four isolates were identified as *Shewanella putrefaciens* and three isolates were identified as *Staphylococcus* spp. Four *Vibrio* species were identified using MALDI-TOF MS, including 64 *V. alginolyticus*, 38 *V. metschnikovii*, 24 *V. anguillarum* and seven *V. aestuarianus*. Two of the 64 *V. alginolyticus* had a score value < 2.0, placing them in category B (Low-confidence identification), while the remaining 62 isolates had a score >2.0, placing them in category A (High-confidence identification). All the isolated *V. metschnikovii* strains were given a score value between 1.70 – 1.90, placing them in category B, while all the *V. anguillarum* and *V. aestuarianus* strains had score values > 2.0. A total of 29 isolates failed to be identified by the MALDI-TOF MS.

Comparing the results from the API 20E system and MALDI-TOF MS, 83 % of the *V. alginolyticus* strains were identified to the same species using both methods, while 11 % were identified as other *Vibrio* spp. using the API 20E system. None of the 38 *V. metschnikovii* were identified correctly by API 20E, but 11 % were identified as members of the genus *Vibrio.* None of the isolated *V. anguillarum* and *V. aestuarianus* were identified to the correct species by API 20E, while 14 % of the *V. aestuarianus* strains were identified as other *Vibrio* spp.

3.3 *rpoB* sequencing

Two of the isolates not identified by MALDI-TOF MS were characterised as Gram-negative, oxidase positive and halophilic isolates, and were subjected to *rpoB* sequencing for identification. The *rpoB* sequences were analysed using the BLAST tool and compared to sequences in the Nucleotide collection (nr/nt) database. Both isolates were identified as *V. cincinnatiensis* from their *rpoB* sequences, with an identity of 89.09 % and 89.32 % respectively.

3.4 Prevalence of *Vibrio* spp.

Based on the results from identification by MALDI-TOF MS and *rpoB* sequencing, *Vibrio* spp. was detected in 45 % of the water samples (n=20), 43 % of fish samples (n=60) and in 31 % of the samples from bivalve molluscs (n=16). Only 11 % of the vibrios isolated from the water samples were recovered from enrichment cultures, the remaining were isolated from cultivation of filters without an enrichment step. From fish, *Vibrio* spp. was isolated from 40 % of samples from gills, 20 % of samples from the intestine and 32 % in samples from skin and flesh. A total of 87.5 % of *Vibrio* spp. from bivalve molluscs were isolated by direct plating of undiluted samples, while the remaining isolates from bivalve molluscs were recovered from enrichment cultures.

From the water samples, *V. alginolyticus* was the most frequently isolates species followed by *V. aestuarianus*, *V. metschnikovii*, *V. anguillarum* and *V. cincinnatiensis* (Table 4). *V. metschnikovii* was the most frequently detected species in fish, with 85 % of all *V. metschnikovii* isolated from fish. The only other *Vibrio* sp. isolated from fish was *V. alginolyticus* (Table 4). Both *V. anguillarum* and *V. alginolyticus* were isolated from bivalve molluscs. Totally 87.5 % of the isolated *V. anguillarum* were recovered from bivalve molluscs, while 5 % of the isolated *V. alginolyticus* were detected from the same samples (Table 4).

Table 4: Vibrio spp. *isolated from water samples, fish and bivalve molluscs.*

		Fish			
Species	**Water (n=20)**	Muscle with skin(n=60)	Intestine (n=40)	Gills (n=20)	**Bivalve molluscs (n=16)**
V. alginolyticus	57	4	0	0	3
V. metschnikovii	6	15	9	8	0
V. anguillarum	3	0	0	0	21
V. aestuarianus	7	0	0	0	0
*V. cincinnatiensis**	2	0	0	0	0

*: *uncertain identification.*

3.5 Antimicrobial susceptibility testing

3.5.1 Disk diffusion

The antimicrobial susceptibility of all isolated *Vibrio* spp. was tested against 18 antimicrobial agents belonging to 10 classes according to CLSI method M42-A. An additional six

V. metschnikovii isolated from Atlantic mackerel at the IMR during the spring of 2017 were included in the antimicrobial susceptibility testing.

Resistance to ampicillin was observed in all examined *V. alginolyticus* (n=64), *V. anguillarum* (n=24), in 27.3 % of the isolated *V. metschnikovii* (n=44) and in 28.6 % of examined *V. aestuarianus* (n=7). A total of 12 isolates were susceptible to all antibiotics tested, all identified as *V. metschnikovii*. All isolates, regardless of species, were susceptible to tetracycline, doxycycline, meropenem, mecillinam, florfenicol, ciprofloxacin, azithromycin and sulfamethoxazole/trimethoprim.

All *V. anguillarum* isolates tested showed phenotypic resistance to ampicillin and were resistant or intermediate susceptible to imipenem. Two isolates were resistant to aztreonam, while 14 isolates showed intermediate susceptibility for this agent. Seven of the tested isolates were categorized as resistant to erythromycin. Resistance to ampicillin, imipenem and aztreonam was observed in two isolates (Figure 6).

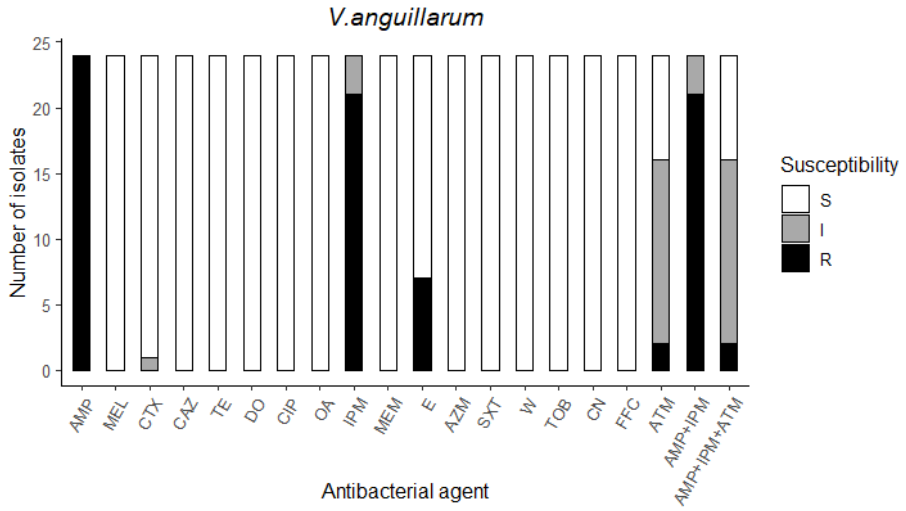

Figure 6: Antimicrobial resistance patterns among V. anguillarum (n=24). *AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam, AMP+IPM+ATM: Sum of susceptible, intermediate susceptible or resistant isolates to the individual agents AMP, IPM and ATM. S: Susceptible, I: intermediate, R: Resistant.*

Among the *V. metschnikovii* isolates, 12 were resistant to ampicillin, 11 were resistant to tobramycin and 12 intermediate susceptible to this antibiotic, 12 showed intermediate

susceptibility to gentamicin, four isolates were intermediate susceptible to ceftazidime and three isolates showed intermediate susceptibility to cefotaxime. Two *V. metschnikovii* isolates were intermediate susceptible to aztreonam and one of the tested strains were intermediate susceptible to oxolinic acid (Figure 7). Two of the isolates tested were resistant against ampicillin and tobramycin, and intermediate susceptible against cefotaxime, ceftazidime, gentamicin and aztreonam.

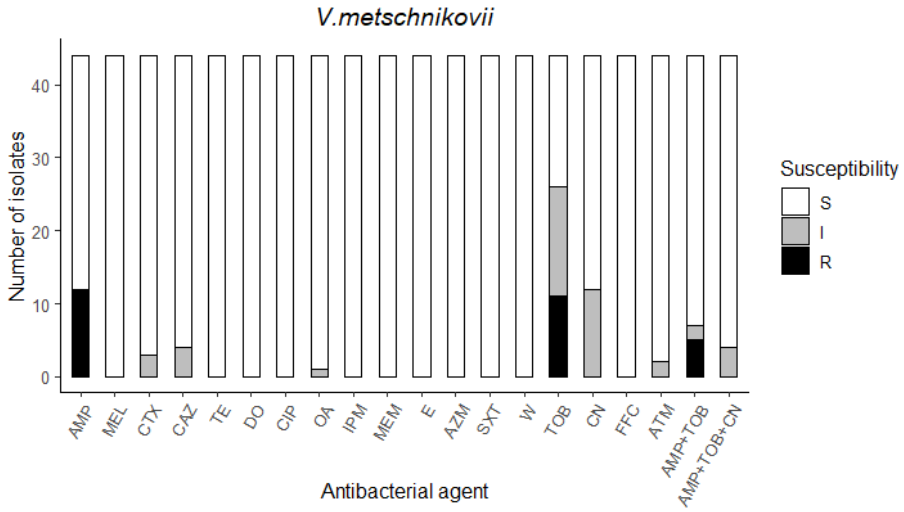

Figure 7: Antimicrobial resistance patterns among V. metschnikovii (n=44). *AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam, AMP+TOB: Sum of susceptible, intermediate susceptible or resistant isolates to individual the individual agents AMP and TOB, AMP+TOB+CN: Sum of susceptible, intermediate susceptible or resistant isolates to the individual agents AMP, TOB and CN. S: Susceptible, I: intermediate, R: Resistant.*

All *V. alginolyticus* isolates showed resistance against ampicillin. Three of the tested isolates were resistant to oxolinic acid, while 41 showed intermediate susceptibility to this agent. Three isolates did not grow on MHA and were therefore tested on MHA supplemented with 1 % NaCl. Two of the tested isolates were intermediate susceptible to trimethoprim and two showed intermediate susceptibility against aztreonam. The two isolates showing reduced susceptibility towards aztreonam were tested on MHA with 1 % NaCl (Figure 8)

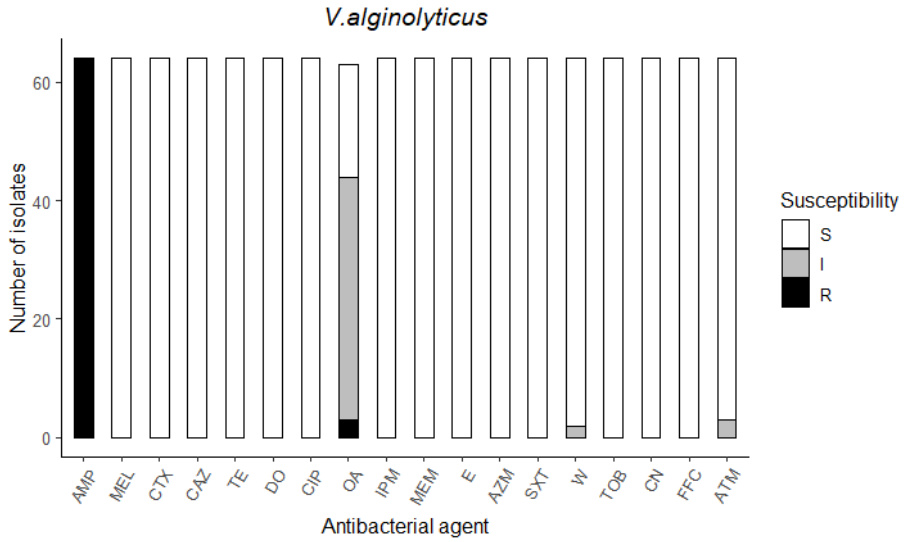

Figure 8: Antimicrobial resistance patterns among V. alginolyticus (n=64). *AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam. S: Susceptible, I: intermediate, R: Resistant.*

Resistance against aztreonam was observed in all *V. aestuarianus* isolates. Two of the isolates were resistant to ampicillin, while two were intermediate susceptible to the same agent (Figure 9).

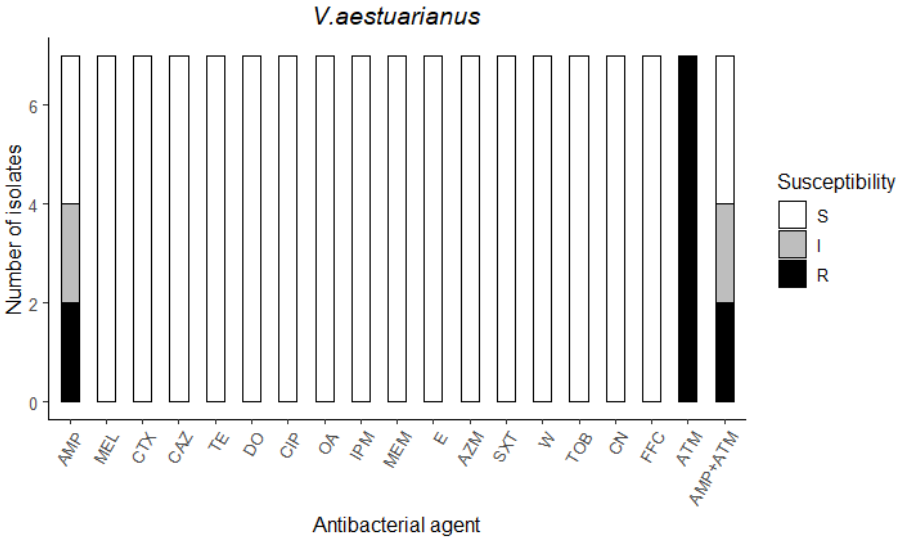

Figure 9: Antimicrobial resistance patterns among V. aestuarianus (n=7). *AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam, AMP+ATM: Sum of susceptible, intermediate susceptible or resistant isolates to the individual agents AMP and ATM S: Susceptible, I: intermediate, R: Resistant.*

Since most *Vibrio* spp. have higher salt requirements than the approximately 0.86 % NaCl found in MHA, a selection of five isolates from each of the identified *Vibrio* spp. were subjected to antimicrobial susceptibility testing by the same disk diffusion method only on MHA supplemented with 1 % NaCl. Of the five *V. anguillarum* isolates tested on MHA supplemented with 1 % NaCl, one of the isolates changed from susceptible to resistant for cefotaxime, and from susceptible to intermediate for ceftazidime. Two of the isolates changed from susceptible to intermediate susceptible for cefotaxime, while one isolate previously susceptible to ceftazidime were classified as intermediate. A reduction in zone diameter for aztreonam was observed for all the tested isolates, classifying three isolates previously susceptible as resistant when tested on media containing 1 % NaCl. A reduced mean inhibition zone was measured for all agents, with the exception of trimethoprim (Figure 10).

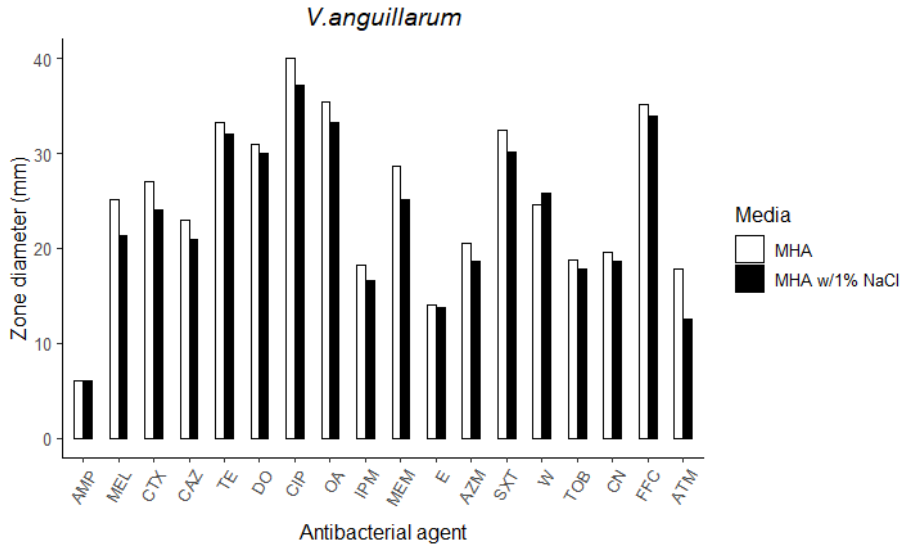

Figure 10: Mean zone diameter (mm) around antibiotic disks for V. anguillarum (n=5) *when tested on Mueller Hinton agar (MHA) and MHA supplemented with 1 % NaCl (MHA W/ 1 % NaCl). AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam*

When tested on MHA with 1 % NaCl, one *V. metschnikovii* isolate previously susceptible to ceftazidime became resistant, while three previously intermediate susceptible changed to resistant to this agent. One isolate showed intermediate susceptibility to ceftazidime on MHA, but when tested on MHA w/ 1 % NaCl, it was classified as susceptible. A change in susceptibility was also observed for the aminoglycosides. Three isolates changed from

resistant to intermediate susceptible to tobramycin, one from intermediate susceptible to susceptible and one isolate went from intermediate to resistant. Three *V. metschnikovii* isolates were classified as intermediate susceptible to gentamicin on MHA, and sensitive on MHA w/ 1 % NaCl. Four of the tested isolates were resistant to aztreonam when tested on MHA w/ 1 % NaCl, of these, three were sensitive and one was intermediate susceptible on MHA. One *V. metschnikovii* changed from intermediate susceptible to susceptible to aztreonam. However, a reduced mean inhibition zone was observed for all agents, except for the aminoglycosides and sulfamethoxazole/trimethoprim, where similar or increased zones were seen (Figure 11).

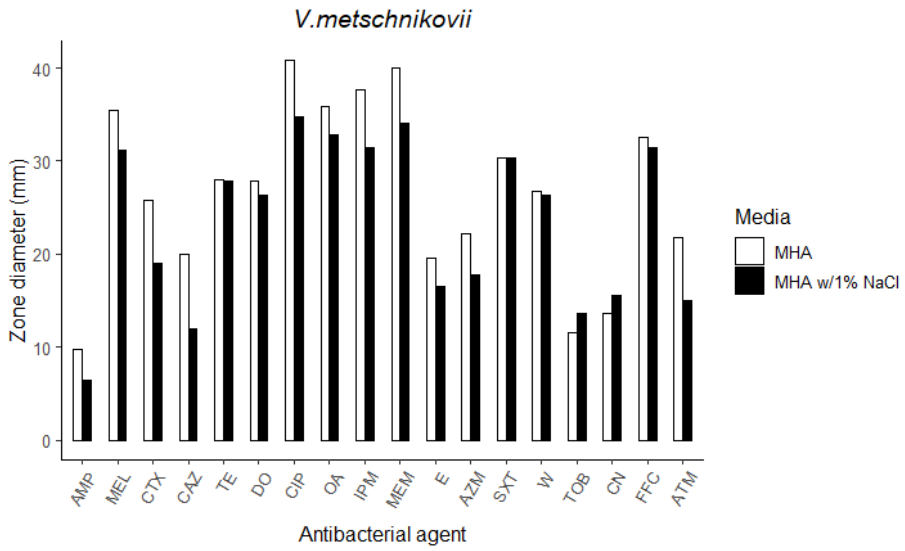

Figure 11: Mean zone diameter (mm) around antibiotic disks for V. metschnikovii (n=5) *when tested on Mueller Hinton agar (MHA) and MHA supplemented with 1 % NaCl (MHA W/ 1 % NaCl). AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam*

Antimicrobial resistance among the tested *V. alginolyticus* was observed for only a few antimicrobial agents. When tested on media containing 1 % NaCl, one isolate was intermediate susceptible to cefotaxime. Two isolates became intermediate susceptible to trimethoprim and tobramycin. Two isolates changed from intermediate susceptible to resistant to oxolinic acid and two isolates from sensitive to intermediate susceptible to the same agent. One of the tested strains categorized as wild-type to erythromycin on MHA became non-wild-type when tested on media supplemented with NaCl, while one of the isolates previously sensitive to aztreonam, was categorized as intermediate susceptible to this agent on MHA

with 1 % NaCl. All the tested isolates showed smaller inhibition zones to all agents when tested on media supplemented with NaCl (Figure 12).

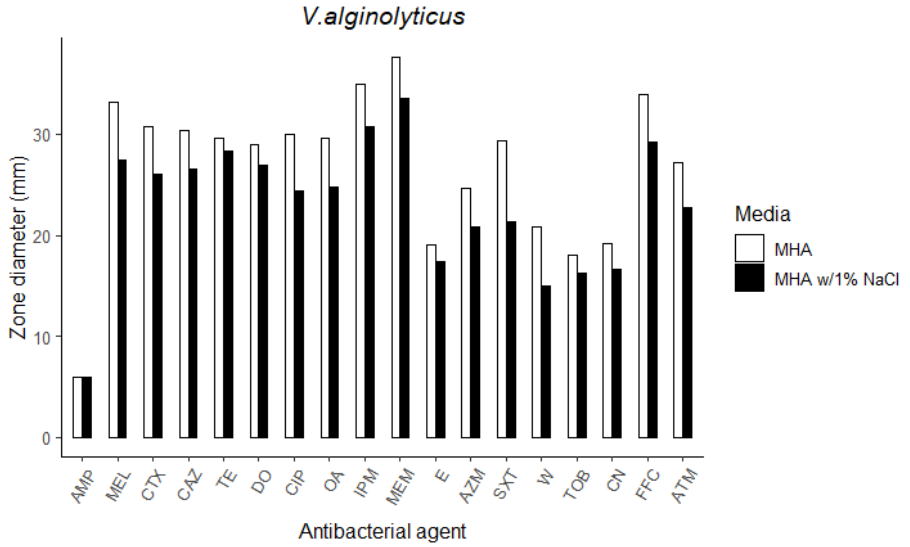

Figure 12: Mean zone diameter (mm) around antibiotic disks for V. alginolyticus (n=5) *when tested on Mueller Hinton agar (MHA) and MHA supplemented with 1 % NaCl (MHA W/ 1 % NaCl). AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam*

Among the tested *V. aestuarianus*, a change in susceptibility pattern for ceftazidime was observed for two isolates. One of the isolates changed from susceptible to resistant, and one isolate from susceptible to intermediate susceptible for this agent. An increased inhibition zone was measured for ampicillin, while a reduced or similar inhibition zone was measured for the rest of the tested agents (Figure 13).

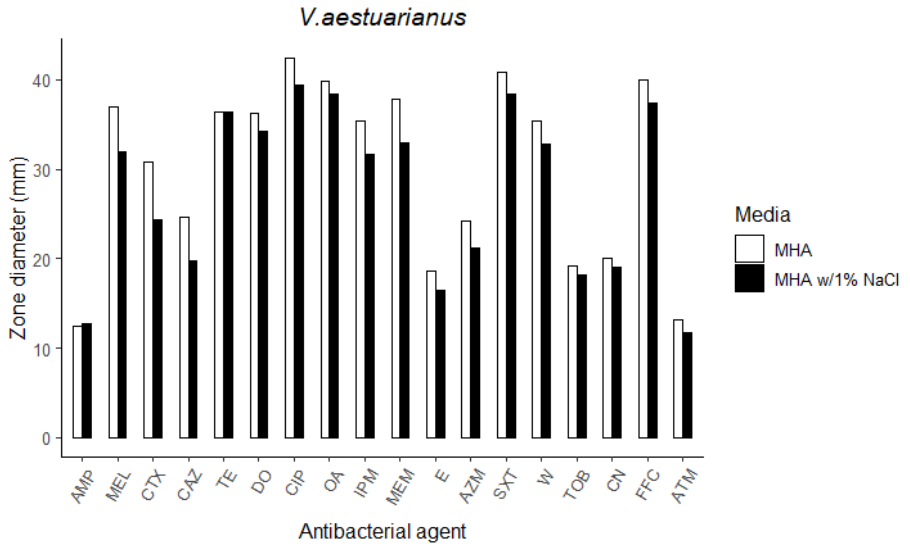

Figure 13: Mean zone diameter (mm) around antibiotic disks for V. aestuarianus (n=5) *when tested on Mueller Hinton agar (MHA) and MHA supplemented with 1 % NaCl (MHA W/ 1 % NaCl). AMP: Ampicillin, MEL: Mecillinam, CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, DO: Doxycycline, CIP: Ciprofloxacin, OA: Oxolinic acid, IPM: Imipenem, MEM: Meropenem, E: Erythromycin, AZM: Azithromycin, SXT: Sulfamethoxazole/Trimethoprim, W: Trimethoprim, TOB: Tobramycin, CN: Gentamicin, FFC: Florfenicol, ATM: Aztreonam*

3.5.2 MIC for imipenem

All 24 *V. anguillarum* isolates showed resistance to, or reduced susceptibility to imipenem. A MIC test was performed on these isolates and showed that MIC values ranged from 2 µg/ml to 8 µg/ml, classifying them as either resistant or intermediate susceptible to imipenem. A total of 21 isolates were classified as resistant, while the remaining three isolates were classified as intermediate susceptible.

3.5.3 CarbaNP test

All *V. anguillarum* isolates showing reduced susceptibility or resistance against imipenem was examined for production of carbapenemase by the carbaNP test. No positive reaction was observed for any of the tested isolates.

3.6 Determination of hemolytic activity

All isolated *V. alginolyticus* (n=64) and *V. metschnikovii* (n=44) were screened for hemolytic activity on TSA with 5 % sheep blood and TSA with 5 % human blood. None of the *V. alginolyticus* showed hemolytic activity on neither TSA with sheep – or human blood. For *V. metschnikovii,* α hemolysis was observed for all isolates on TSA with 5 % human blood. When grown on TSA with 5 % sheep blood, 39 of the *V. metschnikovii* isolates were α hemolytic, while five isolates were β hemolytic (Table 5).

Table 5: Results from determination of hemolytic activity in V. metschnikovii *and* V. alginolyticus. *TSA: Tryptic Soy Agar, α: alpha hemolysis, β: beta hemolysis.*

	TSA w/ 5% sheep blood		**TSA w/ 5% human blood**	
Bacterium	**α**	**β**	**α**	**β**
V. alginolyticus (n=64)	0	0	0	0
V. metschnikovii (n=44)	39	5	44	0

3.7 Whole genome sequencing

A total of eight *V. anguillarum*, seven *V. alginolyticus*, six *V. metschnikovii* and one *V. cincinnatiensis* were whole genome sequenced. The assembled genomes were annotated in RAST and uploaded to the VFDB- and CARD database. Genes encoding a thermolabile hemolysin (TLH) and hemolysin A (HlyA) was detected in all examined *V. metschnikovii*, and one of these isolates had an additional hemolysin and genes encoding repeat-in-toxin (RTX) was detected. All *V. alginolyticus* were positive for a *tlh* associated gene. Among *V. anguillarum*, TLH, HlyA and RTX was detected in all but one strain (Table 6).

Table 6: Known virulence factors detected using VFDB and RAST. HlyA: Hemolysin A, TLH: Thermolabile hemolysin, RTX: Repeat-in-Toxin

Species	**Hemolysins**			**Toxins**
V. alginolyticus (n=7)	-	TLH (100%)	-	-
V. metschnikovii (n=6)	HlyA (100%)	TLH (100%)	Hemolysin III (17%)	RTX (17%)
V. anguillarum (n=8)	HlyA (87%)	TLH (87%)	-	RTX (87%)
V. cincinnatiensis * (n=1)	-	-	-	-

*: *uncertain identification.*

Several resistance determinants were detected in the isolates subjected to WGS (Table 7). A class A CARB β-lactamase gene was detected in all *V. alginolyticus* and all but one *V. metschnikovii*. In *V. alginolyticus* and one *V. anguillarum* a putative class C β-lactamase gene was found. Most of the examined *V. alginolyticus* also had an additional putative subclass B3 β-lactamase gene and in one of the *V. anguillarum* strains a putative *V. cholerae varG* gene encoding a class B β-lactamase was detected. In all *V. alginolyticus* both a quinolone resistance B protein (QnrB) and *parE* conferring resistance to quinolones were detected. Resistance-Nodulation cell Division (RND) efflux pumps were detected in all examined isolates.

Table 7: Resistance determinants detected in vibrio *isolates subjected to whole genome sequencing using the RAST server and CARD database. CARB: Carbenicillin-hydrolysing, RND: Resistance-Nodulation Cell Division. QnrB: Quinolone resistance B protein.*

Mechanism	***V. alginolyticus* (n=7)**	***V. metschnikovii* (n=6)**	***V. anguillarum* (n=8)**	***V. cincinnatiensis**** **(n=1)**
Inactivation	Class A CARB B-lactamase 100%)	Class A CARB B-lactamase (83%)	VarG subclass B1-like β-lactamase (12%)	-
	Subclass B3 β-lactamase (71%)		Class C β-lactamase (12%)	-
	Class C β-lactamase (57%)			-
Target protection	QnrB (100%)	-	-	-
Target alteration	ParE (100%)	-	-	-
Efflux	RND efflux pump (100%)	RND efflux pump (100%)	RND efflux pump (100%)	RND efflux pump (100%)

**: uncertain identity.*

4. Discussion

4.1 Identification of *Vibrio* spp.

Identification and differentiation of *Vibrio* spp. can be difficult. During this project, a first characterisation of the isolates was conducted by Gram testing, oxidase testing and microscopy. Further identification was done using API 20 E, MALDI-TOF MS and *rpoB* sequencing.

The API 20E system identified 68 of the 177 isolates as members of the genus *Vibrio*, whereas examination by MALDI-TOF MS increased the number of identified *Vibrio* isolates to 133. Comparing the results of the two methods, 84 % of *V. alginolyticus* were identified to the correct species. None of the other species were identified correctly using API 20E. This is likely because the API 20E database only contain six *Vibrio* species: *V. alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* (bioMèrieux, 2015), and not the other four *Vibrio* spp. that were identified by MALDI-TOF MS. The API system is primarily designed for clinical application (Viña-Feas et al., 2006) and therefore not ideal for identification of environmental vibrios. A total of 16 % of the isolated *V. alginolyticus* were not identified correctly using API 20E. When this test was done, an inoculum with 2 % NaCl was used. The concentration of NaCl can affect the biochemical profile and lead to erroneous identification with this system (Tarr et al., 2007). Other factors which can lead to misreading of the results is the formation of air bubbles in the anaerobic wells, and thereby cause false positive results, or misinterpretation of weak positive or negative reactions. Some of the isolated *V. metschnikovii* were attempted identified using the KB007 HiVibrio Identification Kit (HiMedia, India), but the few tested isolates were not identified to the correct species and no further work was done with this system.

All the isolated *Vibrio* spp. were oxidase positive with the exception of *V. metschnikovii*, which together with *V. gazogenes* is the only known *Vibrio* spp. lacking cytochrome c oxidase (Farmer et al., 1988). This feature may cause this species to be overlooked when analysing raw fish and shellfish, and samples from the environment, as most *Vibrio* spp. are oxidase positive (Matté et al., 2007;NMKL, 1997).

MALDI-TOF MS proved to be a valuable method for the identification of *Vibrio* spp. A total of 133 isolates were identified as *Vibrio* spp. All the identified vibrios, except the isolated

V. metschnikovii and two *V. alginolyticus*, were placed in category A (High-confidence identification). However, this method was not able to identify 29 of the isolates. Of these, two were oxidase positive and able to grow well on PCA with 2 % NaCl, hence categorized as slight halophilic bacteria. These two isolates were selected for identification by sequencing of the *rpoB* gene. A method which has been shown valuable in the identification of and differentiation of *Vibrio* spp. The obtained sequences were analysed using the BLAST tool, and identified as *V. cincinnatiensis*, but with a low score. The BLAST database only contains two *rpoB* sequences from this species, and this may explain the relatively low score. However, these isolates were subjected to WGS, but one failed the library preparation. From the assembled genome, the 16s rRNA and other *rpo-* sequences were extracted and analysed using BLAST. All these genes gave different identification, and as a consequence the identification of this isolate remains unclear. These isolates are referred to as *V. cincinnatiensis* throughout this thesis. This illustrates the difficulties in identifying *Vibrio* spp., and shows that the available identification tools for vibrios are suboptimal and may yield conflicting results.

4.2 Prevalence of *Vibrio* spp. in the Norwegian marine environment

During the study period, a total of 20 water samples, 60 fish samples and 16 samples from bivalve molluscs were examined for the presence of potentially pathogenic *Vibrio* spp. A total of 135 vibrios were isolated from these samples using a method based on NMKL no. 156. Among the species considered to be human pathogenic, *V. alginolyticus* was isolated from all sources, while *V. metschnikovii* was isolated from fish- and water samples. Two *V. cincinnatiensis* were isolated from water samples. Of the non-human pathogenic vibrios, *V. anguillarum* was isolated from both bivalves and water samples, whereas *V. aestuarianus* was only isolated from water samples. However, both *V. anguillarum* and *V. aestuarianus* can cause disease in marine animals and are known to cause problems in the aquaculture industry (Travers et al., 2017;Roux et al., 2015;Chatterjee S, 2012)

From the water samples, the highest number of *Vibrio* isolates were recovered from the first sampling location, closest to shore and were the measured temperature was the highest. A total of 69 % of the isolated *V. alginolyticus* were isolated from the samples collected closest to shore at surface and 2 m depth, were the temperature was measured to ≥15 °C and the salinity to ≤25 ‰, which are the preferred conditions for the vibrios (Vezzulli et al.,

2013;Vezzulli et al., 2015). Totally, 50 % of all *Vibrio* spp. isolated from water were recovered from the first sampling location. *V. alginolyticus* is usually the dominating species in *Vibrio* communities (Fu et al., 2016), as seems to be in accordance with the results from this study.

Only seven *Vibrio* isolates were from enrichment cultures from the water samples, one *V. metschnikovii* and six *V. alginolyticus*, possibly indicating a sub-optimal enrichment method for the water samples. As APW is not specific for *Vibrio* spp., other bacteria present in the samples may overgrow the vibrios (Huq et al., 2012). Previous studies have indicated better success when isolating *V. cholerae* from seawater by filtering of the samples before enrichment, and enrichment of bacteria on the filter (Ellingsen, 2008). After cultivation of the filters on TCBS plates, the plates were stored at 4 °C for approximately two weeks before further analysis. If the TCBS plates are not examined immediately after incubation, sucrose positive colonies may revert to green. This may have caused problems as almost all colonies had the same colour when single colonies were picked.

Among the *Vibrio* spp. isolated from fish, *V. metschnikovii* was the most prevalent species. A total of 32 *V. metschnikovii* were isolated from fish, and only three *V. alginolyticus.* All *V. metschnikovii* from fish were isolated from enrichment cultures, with the exception of one isolate. *V. metschnikovii* was detected in both mackerel and herring, while *V. alginolyticus* was detected only in herring collected in November 2018. As the temperature in Norwegian waters in November normally are lower than the preferred temperature for this species, the isolation of *V. alginolyticus* at this time of year may indicate a successful resuscitation of viable but non-culturable cells.

Among the examined bivalve molluscs *V. anguillarum* was the most prevalent species isolated, as 87.5 % of the isolates from these samples were identified as *V. anguillarum*. A total of 67 % were isolated from Sample 4, which were blue mussels reared in Nordland. From the bivalves, all *V. anguillarum* were isolated from direct plating of undiluted samples, primarily on VCS agar (81 %) after 48 hours of incubation at 37 °C. The remaining three isolates were from TCBS agar. Enrichment cultures were set up for each sample, and only one sample was positive, and three *V. alginolyticus* were isolated from this sample. A higher diversity of vibrios were expected from these samples because of their filter feeding, but as the samples were collected in November, the seawater temperature might have been lower than the preferred temperature for vibrios.

None of the three major human pathogenic vibrios (*V. cholerae, V. vulnificus* and *V. parahaemolyticus*) were isolated during this project. However, according to Ellingsen (2008) all three have been isolated from shellfish, and from seawater in the South of Norway when the seawater temperature was >17 °C. In the same study, *trh* positive *V. alginolyticus* was isolated from seawater.

4.3 Antimicrobial resistance

Resistance or reduced susceptibility to one of the tested agents was observed in 79 % of the examined vibrios and 60 % showed resistance or reduced susceptibility to two or more agents.

The most frequently observed resistance among the tested *Vibrio* spp. was to ampicillin, where 74 % were phenotypically resistant to this agent. This included all *V. alginolyticus* and *V. anguillarum*, 27.3 % of the *V. metschnikovii* and 28.6 % of the *V. aestuarianus.* These results are consistent with previous studies demonstrating high prevalence of resistance to this agent in *Vibrio* spp. (Hernández-Robles et al., 2016;Pan et al., 2013;Li et al., 1999;Banerjee and Farber, 2018;Chiou et al., 2015). The widespread resistance to ampicillin among the tested isolates may be explained by the VbrK/VbrR system described in *V. parahaemolyticus*, which controls the expression of a CARB β-lactamase gene which also has been found in many *Vibrio* spp. (Chiou et al., 2015;Li et al., 2016). In this study, a class A CARB β-lactamase was detected in all *V. alginolyticus* subjected to WGS and in five *V. metschnikovii*. However, one *V. metschnikovii* showing phenotypic resistance to ampicillin did not harbour this gene and among *V. anguillarum*, a β-lactamase was only detected in one of the examined isolates. This indicates the presence of a resistance determinant to ampicillin not detected in the databases used in this study.

All *V. anguillarum* tested during my project displayed reduced susceptibility to imipenem. Although imipenem resistant *V. anguillarum* have been reported (Garcia-Aljaro et al., 2014), no literature on the resistance mechanism have been found. When uploading the assembled genomes from the eight strains subjected to WGS to the CARD database, one of the isolated *V. anguillarum* showed the presence of a putative *V. cholerae varG* gene encoding a subclass B1-like β-lactamase. The hit gave a 93 % identity to the matching region of *varG* and 96 % identity to the reference sequence. This enzyme belongs to the metallo-β-lactamase class B

which can hydrolyse most β-lactam antibiotics, including newer cephalosporins and carbapenems (Lin et al., 2017). Carbapenemase producing *V. cholerae* have also been isolated from the marine environment (Bier et al., 2015) and retail shrimp (Mangat et al., 2016). One of the isolates showed the presence of a class C β-lactamase gene, encoding enzymes known to hydrolyse cephalosporins and penicillins (Jacoby, 2009). This may explain resistance to ampicillin in this isolate, but only one isolate was intermediate susceptible to cefotaxime. Reduced susceptibility to ampicillin, imipenem and aztreonam was detected in 16 isolates tested in this project, one of these also showed reduced susceptibility to cefotaxime. However, all of the strains tested during this study were susceptible to meropenem. Additionally, all isolates yielded a negative reaction in the carbaNP test, indicating a resistance mechanism other than production of carbapenemase. Furthermore, high prevalence of reduced susceptibility to aztreonam (67 %) and erythromycin (25 %) was detected. An RND efflux pump was detected in the genomes of the sequenced isolates. However, the specificity of these pumps to antibacterial agents have not been examined and it is uncertain if these are involved in reduced susceptibility to these agents.

Among *V. metschnikovii*, reduced susceptibility was most frequently observed for aminoglycosides. The results from this study are in accordance with previous studies of the antimicrobial susceptibility of clinical isolates of *V. metschnikovii*, where resistance to ampicillin (Linde et al., 2004), aminoglycosides and third generation cephalosporins have been described (Macarena Pariente et al., 2008;Wallet et al., 2005). However, clinical isolates have also been shown to respond well to treatment with ampicillin (Hardardottir et al., 1994) and to be susceptible to cephalosporins and aminoglycosides (Linde et al., 2004). In environmental isolates, resistance to ampicillin, tetracycline, cefoxitin, streptomycin, nitrofurantoin and sulfamethoxazole have been described (Garcia-Aljaro et al., 2014;Rebouças et al., 2011), as well as intermediate susceptibility to aztreonam (Rebouças et al., 2011) has been reported. In this project, reduced susceptibility was observed to ampicillin, ceftazidime, cefotaxime, gentamicin, tobramycin and aztreonam. However, newer literature regarding the antimicrobial susceptibility profile of environmental *V. metschnikovii* is quite scarce and published studies on the antimicrobial susceptibility of environmental vibrios often only include few tested *V. metschnikovii*. Reduced susceptibility to the highest number of antibacterial agents were observed within this species. However, from the sequenced isolates from this species no determinants explaining the phenotypic resistance to other agents than ampicillin was detected.

It is important to note that the control strain was never within the accepted quality control (QC) range for gentamicin (appendix D). It is therefore uncertain if the results for the aminoglycosides are reliable.

Reduced susceptibility to oxolinic acid was observed in several of the isolated *V. alginolyticus*. Resistance to this agent have been described within this species (Scarano et al., 2014), however the frequency of reduced susceptibility was quite high in my study. All the *V. alginolyticus* subjected to WGS showed the presence of a QnrB protein and mutations in *parE*, factors known to mediate resistance to quinolones (Hopkins et al., 2005;Jacoby et al., 2014). Two of the isolates were these determinants were detected displayed phenotypic susceptibility to oxolinic acid. No resistance towards ciprofloxacin was observed in this study. It is known that only one mutation is not enough to yield resistance to some newer quinolones (VKM et al., 2018), such as ciprofloxacin. Based on this study, it seems that mutations in *parE* in combination with QnrB is not sufficient to cause resistance to ciprofloxacin in *V. alginolyticus*. Also, it is worth noting that three of the isolated *V. alginolyticus* did not grow on MHA and susceptibility testing had to be done on MHA supplemented with 1 % NaCl. This indicates a requirement for higher NaCl concentrations in these three strains than the rest of the strains.

All *V. aestuarianus* were resistant to aztreonam, while two isolates were resistant to ampicillin and to intermediate susceptible to the same agent. Available literature on the susceptibility of environmental vibrios includes only few *V. aestuarianus*, but resistance to ampicillin, oxolinic acid, florfenicol and trimethoprim was seen in one isolate, whereas two isolates were resistant to erythromycin (Scarano et al., 2014). All isolates tested during this study was susceptible to these agents with the exception of ampicillin. None of the isolated *V. aestuarianus* were whole genome sequenced.

One or more RND efflux pumps was detected in all the isolates subjected to WGS. It is uncertain if these efflux pumps are involved in the reduced susceptibility observed during this study. To determine their role, it is necessary to examine their specificity to individual antibacterial agents. Furthermore, three different classes of putative β-lactamase genes were detected. Class C β-lactamases are known to hydrolyse cephalosporins (Jacoby, 2009). This was not seen in the disk diffusion test in the isolates were this β-lactamase gene was found. A putative subclass B3 β-lactamase gene was detected in five *V. alginolyticus*, and these are active against penicillins, cephalosporins and carbapenems (Palzkill, 2013). This does not correspond to the results from phenotypic susceptibility testing. Differences between

phenotypic and genetic susceptibility has been reported and may be caused by variable expression in tested isolates, leading to difficulties in phenotypic detection (Sundsfjord et al., 2004). Further studies should be done to examine the presence of these, their activity against antibacterial agents, and significance in antibacterial resistance in the aquatic environment.

Of the tested agents in this study, florfenicol and oxolinic acid are the only ones used in aquaculture in Norway the last ten years. Penicillins are the most sold antibacterial class for food producing animals, and for human use, narrow spectrum penicillins and tetracycline are the most sold agents (NORM/NORM-VET, 2017). Of these antibiotic classes, resistance was observed against oxolinic acid in *V. alginolyticus* and *V. metschnikovii*, and broad-spectrum penicillins in all the tested species, thus only to ampicillin. Resistance to oxolinic acid have previously been reported in aquatic bacteria (Ervik et al., 1994;Guardabassi et al., 2000). However, it is important to note that the breakpoints used to determine the susceptibility to this agent applies for *A. salmonicida* and a lower incubation temperature and longer incubation time. Further studies are necessary to determine if these breakpoints are applicable to *Vibrio* spp. and the conditions used during this study.

The results in this study shows differences in resistance patterns within the species, especially among the isolated *V. metschnikovii*. There are several sources for the spread of antibiotics to the aquatic environment, such as through wastewater and run-off from land (Berglund, 2015;Baquero et al., 2008), and resistant bacteria other than vibrios can be disseminated by the same routes. This can result in the spread of resistance among bacteria, possibly explaining the differences in susceptibility within the examined species.

The most interesting resistance observed during this project was against imipenem in isolated *V. anguillarum*. Imipenem is a carbapenem antibiotic, a class considered to be critically important in human medicine by the WHO (WHO, 2017b). All resistant isolates were recovered from bivalves, which may function as a hotspot for exchange of genetic material (Grevskott et al., 2017). These findings may indicate a possibility of exchange of determinants conferring resistance to imipenem between bacteria, including human pathogenic bacteria, present in the marine environment, and the transfers between different niches through bivalves. These bacteria can eventually cause foodborne infections in humans. Even though a carbapenem hydrolysing β-lactamase was detected in one of the isolates, this does not comply with - or explain the results seen in this study. It is unknown if resistance to

this agent is caused by an intrinsic mechanism or one acquired and transferable through HGT. Further studies are necessary to determine the underlying mechanism and significance of this discovery. Furthermore, the detection of a putative class B β-lactamase gene in several isolated *V. alginolyticus* is highly interesting as this species is considered human pathogenic.

The breakpoints in the standard method used to assess the antimicrobial susceptibility of the isolates tested in this study applies for Enterobacteriaceae and an incubation temperature of 35 °C for 16-18 hours. For the agents oxolinic acid, florfenicol and erythromycin, the breakpoints and ECOFF values applies for *A. salmonicida* when tested at 22 °C and incubated for 44-48 hours. This means that the method used for susceptibility testing in this project is not specific for the breakpoints, and the results from phenotypic susceptibility testing should be interpreted with some precautions. Further analysis of the genomes is necessary to determine the activity and significance of detected mechanisms, and to locate undetected antibiotic resistance determinants.

When reading the inhibition zones, double zones were observed for erythromycin and azithromycin in several of the tested strains of *V. metschnikovii* and *V. alginolyticus*. This caused difficulties in the precision when measuring the zones. This phenomenon was particularly observed for several agents when testing the antimicrobial susceptibility of *V. alginolyticus*. This species swarms on solid media, and caused difficulties in determining the exact zone diameter, and the outer zone was regarded as the inhibition zone. Weak growth was observed on MHA plates for several of the tested species, and this caused difficulties in measuring of inhibition zones. This is a factor possibly affecting the results, and may be due to a to low NaCl concentration or to low density in the inoculum spread on the plates.

When tested on MHA supplemented with 1 % NaCl reduced inhibition zones were observed for most of the agents for all species. The most notable change was observed for the cephalosporins and aztreonam. For cefotaxime, a change from susceptible to intermediate or resistant, or intermediate susceptible to resistant, was observed within all the tested species. The same pattern was observed for ceftazidime among *V. metschnikovii* and *V. anguillarum*. For aztreonam, members of all the species showed reduced inhibition zones and isolates previously susceptible or intermediate susceptible were categorized as resistant or intermediate susceptible to this agent. However, one of the *V. metschnikovii* changed from intermediate to sensitive for ceftazidime, cefotaxime and aztreonam, when examined on MHA with 1 % NaCl. This may indicate that this isolate was in a mixed culture.

The effect of a higher NaCl concentration in MHA on both the growth of bacteria and the activity of the antimicrobial agents has not been studied in detail (CLSI, 2006). Addition of NaCl will also change the osmolarity in the medium and could affect the diffusion rate of the antibiotics into the agar (CLSI, 2006). This is known to have a marked effect on the aminoglycosides (Medeiros et al., 1971;Lamb et al., 1972). As no determinants explaining resistance to these agents in isolates subjected to WGS, it is likely that the increased frequency of resistance to aztreonam and cephalosporins was at least partially caused by the effects of NaCl on the media and/or the antibacterial agents. However, further studies on susceptibility testing of halophilic bacteria is necessary. Currently, no QC data exists for antimicrobial susceptibility testing using MHA supplemented with NaCl, and it is therefore not possible to assure the quality of the agar and disks when testing on media supplemented with NaCl. No specific protocols exist for interpretation of inhibition zones when using media with additional salt.

A recently published study concluded that antimicrobial susceptibility testing of *Vibrio* spp., including *V. alginolyticus*, should be done using unmodified MHA (Smith and Egan, 2018). MHA contains hydrolysate from casein which has enough NaCl to allow the growth of most halophilic *Vibrio* species (Dworkin et al., 2006;Smith and Egan, 2018). However, during this project not all isolated *V. alginolyticus* did grow on MHA without the addition of 1 % NaCl. This may lead to problems when conducting large studies on antimicrobial susceptibility of *Vibrio* spp., as it is important that all testing is performed under standardized conditions. Thus, it is necessary to establish a standardized protocol for antimicrobial susceptibility testing of vibrios which enables testing of all members of a species using the same protocol. For future studies, it should be considered performing broth dilution susceptibility testing in Mueller Hinton broth (MHB), as this will allow the determination of an MIC value. Using MHB would also remove the problem with double zones, and the swarming of *V. alginolyticus* on solid media.

4.4 Hemolytic activity

Hemolysins are considered virulence factors in several *Vibrio* spp., including *V. metschnikovii*. The hemolysin produced by this species has been showed to lyse cells from several animals, including humans, sheep and horse (Miyake et al., 1988). Hemolytic activity was observed in all isolated *V. metschnikovii* on both TSA with 5 % sheep- and human blood.

The results indicate that sheep erythrocytes are more susceptible to this hemolysin as β-hemolysis was observed on agar containing sheep blood only. This is conflicting to previous studies showing that this species seems to have low activity on sheep blood, and that human erythrocytes are more susceptible to hemolysins produced by *V. metschnikovii* (Matté et al., 2007). During the present study, *tlh*- and *hlyA* related genes were detected in all strains subjected to WGS, and in one isolate two *tlh*- and *hlyA* related genes, an additional hemolysin and RTX encoding genes were detected. TLH is a hemolysin previously described as virulence factor in *Vibrio* spp., such as in environmental *V. alginolyticus* (Hernández-Robles et al., 2016). HlyA, also known as *V. cholerae* cytolysin (VCC), is a pore forming hemolysin and cytolysin with activity against a range of eukaryotic cells (Ruenchit et al., 2017) and is found in both *V. cholerae* O1 and nonO1/nonO139 (Gutierrez et al., 2007;Elluri et al., 2014). Cytotoxic activity has previously been described in *V. metschnikovii* isolated from a leg wound (Linde et al., 2004). Even though *V. metschnikovii* have caused infections in humans, this species is poorly described with regards to virulence factors. However, the presence of these genes may indicate a pathogenic potential. RTX is a pore forming toxin found in several pathogenic Gram-negative bacteria (Lee et al., 2008) possibly indicating a higher pathogenic potential in this strain. No difference in hemolytic activity was observed from isolated *V. metschnikovii* from water and fish.

No hemolytic activity was observed on agar containing neither sheep- or human blood among the tested *V. alginolyticus*. In all *V. alginolyticus* a *tlh* related gene was identified. The role of TLH in human infections is unknown, but it is upregulated under conditions simulating those in the human intestine (Gutierrez West et al., 2013;Schwartz et al., 2019). A previous study have demonstrated that sheep erythrocytes are not very sensitive to TLH (Jia et al., 2010), possibly explaining the lack of hemolysis observed on blood agar. Based on the results from this study, the TLH detected seems to have low or no activity to human erythrocytes and that HlyA was responsible for the hemolysis on blood agar caused by *V. metschnikovii*.
In *V. anguillarum*, *hlyA*-, *tlh*- and *rtx* related genes were detected, but this species is not considered to be human pathogenic.

5 Conclusion

Totally, 135 *Vibrio* spp. was isolated from seawater, fish and bivalves, 64 *V. alginolyticus*, 38 *V. metschnikovii*, 24 *V. anguillarum* and seven *V. aestuarianus*. Also, two *V. cincinnatiensis* was isolated, but the identity of these needs confirmation.

API 20E was not ideal for identification of environmental vibrios, while MALDI-TOF MS proved to be a valuable tool for identification of members of this genus. The *rpoB* sequencing gave identification with low confidence.

High prevalence of phenotypic resistance or reduced susceptibility was observed to ampicillin, aztreonam, oxolinic acid, tobramycin and imipenem. Addition of 1 % NaCl to MHA resulted in smaller inhibition zones for all agents. WGS revealed the presence of different classes of putative β-lactamase genes in the isolated vibrios, QnrB proteins, *parE* conferring resistance to quinolones and RND efflux pumps. These does however not explain the results from phenotypic susceptibility testing and further studies are necessary to determine the activity and significance of these resistance mechanisms. Resistance to clinically important antibacterial agents, such as tetracycline and cephalosporins, in vibrios have been reported (Hernández-Robles et al., 2016;Letchumanan et al., 2015;Lee et al., 2018). However, this study demonstrates a low prevalence of resistance to these agents in vibrios isolated from the Norwegian marine environment.

All isolated *V. metschnikovii* were hemolytic on agar containing sheep- or human blood and contained *tlh*- and *hlyA* related genes. *V. alginolyticus* was not hemolytic on blood agar, but a *tlh* related gene was identified.

The results from this study indicates a risk of exposure to potentially pathogenic vibrios through bathing, consumption- and handling of raw seafood, as well as the transfer of resistant bacteria to the human food chain. Furthermore, a link between SST, salinity and occurrence of *Vibrio* spp. was seen. The prevalence of human pathogenic vibrios are shown to be highly dependent on SST, and as the sea temperature is expected to keep increasing due to global warming (EEA, 2017), the conditions in the ocean will become more favourable for *Vibrio* spp. Thus, there is a need to establish a surveillance programme of public bathing areas and of bivalve molluscs intended for human consumption when the water temperatures exceed 18-20 °C, as there is no correlation between commonly measured fecal indicator bacteria and *Vibrio* spp. (Lunestad and Rosnes, 2018).

The role of the aquatic environment in the spread of antibiotic resistance is largely unknown. Thus, it is necessary to establish standardized protocols for antimicrobial susceptibility testing of aquatic bacteria, such as *Vibrio* spp., to monitor the spread of antibacterial resistance in this environment.

6. Further studies

For future studies regarding the occurrence and characteristics of *Vibrio* spp. in Norwegian waters, the study should be conducted over a longer period of time, and cover larger parts of the Norwegian coast, especially along the South coast where these bacteria have caused infections when temperatures exceeds 20 °C. A standardized method for the analysis and enrichment of water samples from public bathing areas and locations used for farming of bivalve molluscs, should be developed to monitor the occurrence of these bacteria in such areas.

Further studies on the pathogenic potential of *V. metschnikovii* should be conducted, as this was a frequently isolated species which previously have caused infections in humans.

This study has shown that antimicrobial susceptibility testing of environmental vibrios can be challenging as the test media used in standard protocols contains an insufficient concentration of NaCl to support the growth of all isolated strains. Further work should therefore focus on establishing standardized methods for antimicrobial testing of *Vibrio* spp., and not only those considered to be major human pathogens. It is necessary to standardize test conditions, as well as establishing breakpoints, MIC- and ECOFF values to both monitor resistance within in the genus *Vibrio*, as well as the role of the marine environment in the dissemination of antibiotic resistance. Future work on the antimicrobial susceptibility of vibrios should be conducted using the broth dilution method as this method allows the determination of each tested isolates MIC value to each of the tested agents. Experiments to examine the ability of these bacteria to both transfer and acquire genes conferring resistance towards antibacterial agents through horizontal gene transfer should be performed.

Due to time limitations, only a preliminary analysis of the sequenced genomes was conducted. Further in-depth studies should be done on these genomes to try to explain the phenotypic resistance observed in this study. Furthermore, more work should be done to examine the significance of the putative class B β-lactamase gene detected in *V. alginolyticus* as this may indicate an acquired- and clinically significant resistance mechanism in these isolates which are considered to be human pathogenic. As all isolated *V. anguillarum* were phenotypic resistant or intermediate susceptible to imipenem, further studies should be conducted to identify the mechanism of resistance to this agent.

7. References

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8. Appendix

8.1 Appendix A. Measured temperature, pH and Salinity in water samples

-: Missing measurement

Sample no.	Location	Depth	Temperature (°C)	pH	Salinity (‰)
1	1	Surface	16	7.721	12.4
2	1	2 m	15	7.734	16.9
3	1	5 m	9	7.821	31.1
4	1	7 m	8	7.849	32.1
5	1	10 m	7	7.860	32.2
6	2	Surface	12	7.982	31.4
7	2	2 m	12	7.862	31.3
8	2	5 m	10	7.890	31.9
9	2	7 m	9	7.878	32.2
10	2	10 m	8.5	-	-
11	3	Surface	14	7.880	31.8
12	3	2 m	14	7.883	31.1
13	3	5 m	14	7.887	31
14	3	7 m	12.5	7.893	31.9
15	3	10 m	11	7.956	34.3
16	4	Surface	13	7.886	31.6
17	4	2 m	13	7.878	31.1
18	4	5 m	13	7.964	31.2
19	4	7 m	13	7.893	31.1
20	4	10 m	10	7.872	32.1

8.2 Appendix B. Identification by API 20E

API 20E			
Isolate	**Bacterium**	**Identification (%)**	**T-value**
1-1 (4)	*V. alginolyticus*	97.8	0.74
1-1 (4-a)	*V. alginolyticus*	97.8	0.74
1-1 (7)	*A. hydrophila/caviae/sobria* 1	37.3	0.33
1-1 (8)	*V. alginolyticus*	97.8	0.74
1-2 (2)	*A. hydrophila/caviae/sobria* 1	63.4	0.69
1-2 (3)	*A. hydrophila/caviae/sobria* 2	68.7	0.67
1-2 (3-a)	*V. cholerae* (presumptive ID)	98.5	0.71
1-2 (4)	*V. cholerae* (presumptive ID)	98.5	0.71
1-2 (5)	Identification not valid		
1-2 (6)	*A. hydrophila/caviae/sobria* 1	63.4	0.64
1-2 (7)	*V. alginolyticus*	85.9	0.81
1-2 (7-a)	*V. alginolyticus*	97.8	0.74
1-2 (8)	*P. pneumotropica/M. haemolytica*	94.8	0.39
1-2 (9)	*A. hydrophila/caviae/sobria* 2	63.4	0.69
1-3 (1)	*V. alginolyticus*	85.9	0.81
1-3 (1-a)	*V. alginolyticus*	85.9	0.81
1-3 (2)	*V. alginolyticus*	85.9	0.81
1-3 (2-a)	*V. alginolyticus*	95.4	0.74
1-3 (3)	Unacceptable profile		
1-3 (4)	*V. fluvialis*	56.5	0.52
1-3 (4-a)	*V. alginolyticus*	85.9	0.81
1-3 (4-b)	*V. alginolyticus*	85.9	0.81
1-3 (5)	*V. alginolyticus*	95.4	0.74
1-3 (6)	*V. alginolyticus*	97.8	0.74
1-3 (6-a)	*V. alginolyticus*	97.8	0.74
1-3 (7)	*V. alginolyticus*	97.8	0.74
1-3 (8)	Low discrimination. *Vibrio* sp.	49.7	0.53
1-3 (10)	*V. alginolyticus*	97.8	0.74
1-4 (1)	*V. cholerae* (presumptive ID)	98.5	0.71
1-4 (2)	*V. cholerae (*presumptive ID)	94.6	0.43
2-1 (2)	*A. hydrophila/caviae/sobria* 2	68.7	0.67
2-1 (2-a)	*V. cholerae* (presumptive ID)	98.5	0.71
2-1 (5)	*V. alginolyticus*	97.8	0.74
2-1 (6)	*V. alginolyticus*	99.7	0.81
2-1 (6-a)	*V. alginolyticus*	99.3	0.66
2-1 (7)	*V. alginolyticus*	97.8	0.74
2-1 (7-a)	*V. alginolyticus*	99.3	0.66
2-1 (9)	*V. alginolyticus*	97.8	0.74
2-2 (2)	*V. alginolyticus*	85.9	0.81
2-2 (2-a)	*V. alginolyticus*	85.9	0.81
2-2 (3)	*V. alginolyticus*	85.9	0.81
2-2 (3-a)	*V. alginolyticus*	85.9	0.81

2-2 (7)	*V. alginolyticus*	85.9	0.81
2-2 (8)	Identification not valid		
2-2 (9)	*V. alginolyticus*	97.8	0.74
2-3 (1)	*V. alginolyticus*	85.9	0.81
2-3 (3)	*P. pneumotropica/M. haemolytica*	84.1	0.69
2-3 (3-a)	*P. damselae*	99.2	0.44
2-3 (5)	*V. alginolyticus*	97.8	0.74
2-3 (5-a)	*V. alginolyticus*	97.8	0.74
2-3 (6)	*V. alginolyticus*	99.3	0.66
2-3 (6-a)	*V. alginolyticus*	97.8	0.74
2-3 (9)	*V. alginolyticus*	99.3	0.66
2-3 (9-a)	*V. alginolyticus*	85.9	0.81
3-1 (1)	*V. alginolyticus*	97.8	0.74
3-1 (1-a)	*V. alginolyticus*	99.3	0.66
3-2 (1)	*A. hydrophila/caviae/sobria* 2	69.86	0.28
4-1 (2)	*V. alginolyticus*	97.8	0.74
4-2 (2)	*V. alginolyticus*	97.8	0.74
4-2 (5)	*P. pneumotropica/M. haemolytica*	57.7	0.69
4-3 (1)	*V. alginolyticus*	97.8	0.74
4-3 (1-a)	*P. pneumotropica/M. haemolytica*	34.4	0.64
4-3 (2)	*V. alginolyticus*	97.8	0.74
4-5 (1)	*V. alginolyticus*	95.4	0.74
4-5 (1-a)	*V. alginolyticus*	97.8	0.74
4-5 (2)	*V. alginolyticus*	85.9	0.81
5-1 (1)	Unacceptable profile		
5-1 (2)	Unacceptable profile		
5-1 (4)	*V. Cholerae*	89.8	0.41
5-2 (1)	*A. hydrophila/caviae/sobria* 2	89.3	0.49
5-2 (2)	Unacceptable profile		
5-2 (3)	*A. hydrophila/caviae/sobria* 2	89.3	0.49
5-3 (1)	Unacceptable profile		
6-2 (1)	*A. hydrophila/caviae/sobria* 2	89.3	0.49
6-3 (1)	Unacceptable profile		
7-5 (1)	Unacceptable profile		
7-5 (1-a)	*V. alginolyticus*	85.9	0.81
8-1 (1)	*V. alginolyticus*	85.9	0.81
8-1 (1-a)	Unacceptable profile		
11-4 (1)	*V. alginolyticus*	85.9	0.81
11-4 (1-a)	Unacceptable profile		
12-2 (3)	Unacceptable profile		
12-2 (3-a)	Unacceptable profile		
A2-1	*V. fluvialis*	93.5	0.51
A2-2	Identification not valid		
A5-1	*P. pneumotropica/M. haemolytica*	99.2	0.47
A5-2	*P. pneumotropica/M. haemolytica*	96.3	0.42
A6	*A. hydrophila/caviae/sobria* 2	89.3	0.49

A7	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A8-1	Identification not valid		
A8-2	*P. pneumotropica/M. haemolytica*	85.1	0.44
A9	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A10	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A11	Unacceptable profile		
A12	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A13	*P. pneumotropica/M. haemolytica*	96.2	0.42
A14	*P. pneumotropica/M. haemolytica*	96.3	0.42
A15	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A17	*A. hydrophila/caviae/sobria* 2	89.3	0.49
A19	*P. pneumotropica/M. haemolytica*	96.3	0.42
TA 2	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 4-1	Unacceptable profile		
TA 4-2	Unacceptable profile		
TA 5	Unacceptable profile		
TA 6	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 7	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 8	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 11	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 12	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 13	Unacceptable profile		
TA 14	*K. pneumoniae ssp rhinoscleromatis*	85.1	0.42
TA 16	*A. hydrophila/caviae/sobria* 2	89.3	0.49
TA 17	*P. pneumotropica/M. haemolytica*	96.2	0.42
TA 20	*P. pneumotropica/M. haemolytica*	96.2	0.42
TS 2	Unacceptable profile		
TS 4	*A. hydrophila/caviae/sobria* 2	94.3	0.26
TS 6	*A. hydrophila/caviae/sobria* 2	89.3	0.49
TS 8	Unacceptable profile		
TS 18-1	*Proteus vulgaris* group	99.9	0.93
TS 18-2	*Proteus vulgaris* group	99.2	0.93
TS 20	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 2	*A. hydrophila/caviae/sobria* 2	89.3	0.49
GA 3	Unacceptable profile		
GA 4	*P. pneumotropica/M. haemolytica*	96.3	0.42
GA 5	*A. hydrophila/caviae/sobria* 2	89.3	0.49
GA 6	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 7	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 8	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 9	*V. Cholerae*	89.8	0.41
GA 10	*V. Cholerae*	89.8	0.41
GA 11	*P. pneumotropica/M. haemolytica*	85.4	0.28
GA 13	*A. hydrophila/caviae/sobria* 2	89.3	0.49
GA 14	*A. hydrophila/caviae/sobria* 2	87.2	0.23
GA 15	*P. pneumotropica/M. haemolytica*	96.2	0.42

GA 16	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 17	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 18-2	Unacceptable profile		
GA 19	*P. pneumotropica/M. haemolytica*	96.2	0.42
GA 20	*A. hydrophila/caviae/sobria* 2	89.3	0.49
GS 15	Unacceptable profile		
T.2 A12	Unacceptable profile		
T.2 A16	*A. hydrophila/caviae/sobria* 2	89.6	0.49
T.2 A17	*Aeromonas hydrophila/caviae/sobria* 1	51.8	0.28
T.2 T7	*A. hydrophila/caviae/sobria* 2	98.8	0.49
T.3 A1	*S. putrefaciens* group	99.7	0.9
T.3 A2	*V.alginolyticus*	92.1	0.66
T.3 A5	*S. putrefaciens* group	99.7	0.9
T.3 A7	*S. putrefaciens* group	99.7	0.9
T.3 A9	*S. putrefaciens* group	99.7	0.9
T.3 A14	*V.alginolyticus*	92.1	0.66
T.3 A15	*S. putrefaciens* group	99.9	0.42
T.3 A19	*V.alginolyticus*	97.8	0.74
T.3 A20	*V.alginolyticus*	97.8	0.74
B1-2	*Aeromonas hydrophila/caviae/sobria* 1	54.2	0.36
B1-4	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-1	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-3	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-4	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-5	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-6	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-7	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-8	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-9	*A. hydrophila/caviae/sobria* 2	61.2	0.28
B4-10	*A. hydrophila/caviae/sobria* 2	87.3	0.56
B4-11	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-12	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-13	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-14	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-15	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4-16	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B4	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B7	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B8-1	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B8-2	*A. hydrophila/caviae/sobria* 2	70.5	0.56
B9-1	*V. alginolyticus*	95.8	0.76
B9-2	*V. alginolyticus*	92.1	0.66
B9-3	*V. alginolyticus*	85.9	0.81

8.3 Appendix C. Identification by MALDI-TOF MS

MALDI-TOF		
Isolate	**Bacterium**	**Score**
1-1 (4)	*V. alginolyticus*	2,24
1-1 (4-a)	*V. alginolyticus*	2,29
1-1 (7)	*V. anguillarum*	2,32
1-1 (8)	*V. alginolyticus*	2,22
1-2 (2)	*V. aestuarianus*	2,46
1-2 (3)	*V. alginolyticus*	2,19
1-2 (3-a)	*V. alginolyticus*	2,19
1-2 (4)	*V. alginolyticus*	2,19
1-2 (5)	*S. hominis*	2,32
1-2 (6)	*V. aestuarianus*	2,35
1-2 (7)	*V. alginolyticus*	2,17
1-2 (7-a)	*V. alginolyticus*	2,21
1-2 (8)	*V. aestuarianus*	2,43
1-2 (9)	*S. hominis*	2,3
1-3 (1)	*V. alginolyticus*	2,12
1-3 (1-a)	*V. alginolyticus*	2,21
1-3 (2)	*V. alginolyticus*	2,05
1-3 (2-a)	*V. alginolyticus*	2,17
1-3 (3)	*V. alginolyticus*	2,1
1-3 (4)	*V. alginolyticus*	2,48
1-3 (4-a)	*V. alginolyticus*	2,21
1-3 (4-b)	*V. alginolyticus*	2,15
1-3 (5)	*V. alginolyticus*	2,18
1-3 (6)	*V. alginolyticus*	2,11
1-3 (6-a)	*V. alginolyticus*	2
1-3 (7)	*V. alginolyticus*	2
1-3 (8)	*V. aestuarianus*	2,29
1-3 (10)	*V. alginolyticus*	1,93
1-4 (1)	*V. alginolyticus*	2,18
1-4 (2)	*V. alginolyticus*	2,1
2-1 (2)	*V. alginolyticus*	2,21
2-1 (2-a)	*V. alginolyticus*	2,13
2-1 (5)	*V. alginolyticus*	2,21
2-1 (6)	*V. alginolyticus*	2,14
2-1 (6-a)	*V. alginolyticus*	2,19
2-1 (7)	*V. alginolyticus*	2,2
2-1 (7-a)	*V. alginolyticus*	2,23
2-1 (9)	*V. alginolyticus*	1,84
2-2 (2)	*V. alginolyticus*	2,09
2-2 (2-a)	*V. alginolyticus*	2
2-2 (3)	*V. alginolyticus*	2,11
2-2 (3-a)	*V. alginolyticus*	2,13

2-2 (7)	*V. alginolyticus*	2,21
2-2 (8)	*V. anguillarum*	2,37
2-2 (9)	*V. alginolyticus*	2,19
2-3 (1)	*V. alginolyticus*	2,2
2-3 (3)	*V. aestuarianus*	2,37
2-3 (3-a)	*S. warneri*	2,22
2-3 (5)	*V. alginolyticus*	2,14
2-3 (5-a)	*V. alginolyticus*	2,13
2-3 (6)	*V. alginolyticus*	2,17
2-3 (6-a)	*V. alginolyticus*	2,19
2-3 (9)	*V. alginolyticus*	2,12
2-3 (9-a)	*V. alginolyticus*	2,29
3-1 (1)	*V. alginolyticus*	2,14
3-1 (1-a)	*V. alginolyticus*	2,23
3-2 (1)	*V. anguillarum*	2,37
4-1 (2)	*V. alginolyticus*	2,16
4-2 (2)	*V. alginolyticus*	2,11
4-2 (5)	*V. aestuarianus*	2,35
4-3 (1)	*V. alginolyticus*	2,12
4-3 (1-a)	*V. aestuarianus*	2,43
4-3 (2)	*V. alginolyticus*	2,14
4-5 (1)	*V. alginolyticus*	2,16
4-5 (1-a)	*V. alginolyticus*	2,23
4-5 (2)	*V. alginolyticus*	2,17
5-1 (1)	*P.vulgaris*	2,49
5-1 (2)	*P.vulgaris*	2,41
5-1 (4)	*V. metschnikovii*	1,82
5-2 (1)	*V. metschnikovii*	1,75
5-2 (2)	*V. metschnikovii*	1,75
5-2 (3)	*V. metschnikovii*	1,7
5-3 (1)	*P. vulgaris*	2,53
6-2 (1)	*V. metschnikovii*	1,73
6-3 (1)	*P. vulgaris*	2,55
7-5 (1)	*V. metschnikovii*	1,85
7-5 (1-a)	*V. alginolyticus*	2,11
8-1 (1)	*V. alginolyticus*	2,12
8-1 (1-a)	*V. alginolyticus*	2,17
11-4 (1)	*V. alginolyticus*	2,13
11-4 (1-a)	*P. vulgaris*	2,4
12-2 (3)	No organism identification possible	
12-2 (3-a)	No organism identification possible	
A2-1	*V. metschnikovii*	1,87
A2-2	*V. metschnikovii*	1,77
A5-1	No organism identification possible	
A5-2	No organism identification possible	
A6	*V. metschnikovii*	1,75

A7	*V. metschnikovii*	1,74
A8-1	No organism identification possible	1,78
A8-2	*V. metschnikovii*	1,85
A9	*V. metschnikovii*	1,73
A10	*V. metschnikovii*	1,78
A11	*V. metschnikovii*	1,73
A12	*V. metschnikovii*	1,75
A13	No organism identification possible	
A14	No organism identification possible	
A15	*V. metschnikovii*	1,85
A17	*V. metschnikovii*	1,8
A19	No organism identification possible	
TA 2	No organism identification possible	
TA 4-1	*V. metschnikovii*	1,9
TA 4-2	*V. metschnikovii*	1,84
TA 5	*V. metschnikovii*	1,83
TA 6	No organism identification possible	
TA 7	No organism identification possible	
TA 8	No organism identification possible	
TA 11	No organism identification possible	
TA 12	No organism identification possible	
TA 13	*V. metschnikovii*	1,76
TA 14	No organism identification possible	
TA 16	*V. metschnikovii*	1,81
TA 17	No organism identification possible	
TA 20	No organism identification possible	
TS2	*V. metschnikovii*	1,72
TS 4	*V. metschnikovii*	1,75
TS 6	*V. metschnikovii*	1,83
TS 8	No organism identification possible	
TS 18-1	*P. vulgaris*	2,33
TS 18-2	*P. vulgaris*	2,23
TS 20	No organism identification possible	
GA 2	No organism identification possible	
GA 3	No organism identification possible	
GA 4	No organism identification possible	
GA 5	*V. metschnikovii*	1,84
GA 6	No organism identification possible	
GA 7	No organism identification possible	
GA 8	No organism identification possible	
GA 9	*V. metschnikovii*	1,87
GA 10	*V. metschnikovii*	1,85
GA 11	No organism identification possible	
GA 13	*V. metschnikovii*	1,73
GA 14	*V. metschnikovii*	1,82
GA 15	No organism identification possible	

GA 16	*V. metschnikovii*	1,77
GA 17	No organism identification possible	
GA 18-2	No organism identification possible	
GA 19	No organism identification possible	
GA 20	*V. metschnikovii*	1,78
GS 15	*V. metschnikovii*	1,87
T.2 A12	*V. metschnikovii*	1,73
T.2 A16	*V. metschnikovii*	1,79
T.2 A17	*V. metschnikovii*	1,77
T.2 T7	*V. metschnikovii*	1,81
T.3 A1	*S. putrefaciens*	2,34
T.3 A2	*V. alginolyticus*	2,15
T.3 A5	*S. putrefaciens*	2,27
T.3 A7	*P. hauseri*	2,39
T.3 A9	*S. putrefaciens*	2,42
T.3 A14	*V. alginolyticus*	2,24
T.3 A15	*S. putrefaciens*	2,35
T.3 A19	*V. alginolyticus*	2,09
T.3 A20	*V. alginolyticus*	2,06
B1-2	*V. anguillarum*	2,32
B1-4	*V. anguillarum*	2,36
B4-1	*V. anguillarum*	2,4
B4-3	*V. anguillarum*	2,36
B4-4	*V. anguillarum*	2,39
B4-5	*V. anguillarum*	2,4
B4-6	*V. anguillarum*	2,39
B4-7	*V. anguillarum*	2,32
B4-8	*V. anguillarum*	2,4
B4-9	*V. anguillarum*	2,47
B4-10	*V. anguillarum*	2,41
B4-11	*V. anguillarum*	2,42
B4-12	*V. anguillarum*	2,38
B4-13	*V. anguillarum*	2,42
B4-14	*V. anguillarum*	2,33
B4-15	*V. anguillarum*	2,38
B4-16	*V. anguillarum*	2,4
B4	*V. anguillarum*	2,37
B7	*V. anguillarum*	2,48
B8-1	*V. anguillarum*	2,38
B8-2	*V. anguillarum*	2,3
B9-1	*V. alginolyticus*	2,18
B9-2	*V. alginolyticus*	2,1
B9-3	*V. alginolyticus*	2,16

8.4 Appendix D. Quality control measurements for antimicrobial susceptibility testing at 28 °C

- : No measurement

	Quality control (*E. coli* CCUG 17620). Inhibition zone (mm)					
Date	**AMP**	**E**	**CN**	**OA**	**SXT**	**FFC**
05.12	17	10	20	30	31	22
07.12 #1	16	10	20	28	31	23
07.12 #2	19	-	20	27	-	22
09.12 #1	16	10	20	28	29	23
09.12 #2	18	11	21	29	31	22
11.12	19	10	21	28	32	22
14.12#1	16	10	19	27	32	23
14.12#2	17	9	20	27	32	24
18.12 #1	19	10	19	27	29	22
18.12#2	16	10	19	27	29	21

8.5 Appendix E. Quality control measurements for antimicrobial susceptibility testing at 22 °C

	Quality control (*E. coli* CCUG 17620). Inhibition zone (mm) after 24 h					
Date	**AMP**	**E**	**CN**	**OA**	**SXT**	**FFC**
7/1	15	15	22	32	36	26
15/1	16	15	22	31	34	23

	Quality control (*E. coli* CCUG 17620). Inhibition zone (mm) after 48 h					
Date	**AMP**	**E**	**CN**	**OA**	**SXT**	**FFC**
7/1	12	15	21	32	34	24
15/1	16	15	22	31	34	23

8.6 Appendix F. MIC for imipenem

MIC for Imipenem	
Isolate	**MIC (µg/ml)**
B1-2	4
B1-4	8
B4-1	4
B4-3	8
B4-4	4
B4-5	8
B4-6	8
B4-7	8
B4-8	8
B4-9	8
B4-10	4
B4-11	4
B4-12	4
B4-13	4
B4-14	4
B4-15	4
B4-16	2
B4	4
B7	4
B8-1	8
B8-2	4
1-1 (7)	4
2-2 (8)	2
3-2 (1)	2

8.7 Appendix G. Hemolytic activity of *V. metschnikovii* on blood agar

	Hemolysis			
	Sheep blood		**Human blood**	
Isolate	**α**	**β**	**α**	**β**
5-1 (4)	-	+	+	-
5-2 (1)	+	-	+	-
5-2 (2)	+	-	+	-
5-2 (3)	+	-	+	-
6-2 (1)	+	-	+	-
7-5 (1)	+	-	+	-
A2-1	+	-	+	-
A2-2	+	-	+	-
A6	+	-	+	-
A7	+	-	+	-
A8-1	+	-	+	-
A8-2	+	-	+	-
A9	+	-	+	-
A10	+	-	+	-
A11	+	-	+	-
A12	+	-	+	-
A15	-	+	+	-
A17	+	-	+	-
TA 4-1	+	-	+	-
TA 4-2	+	-	+	-
TA 5	+	-	+	-
TA 13	+	-	+	-
TA 16	+	-	+	-
TS2	+	-	+	-
TS 4	+	-	+	-
TS 6	+	-	+	-
GA 5	+	-	+	-
GA 9	+	-	+	-
GA 10	+	-	+	-
GA 13	+	-	+	-
GA 14	+	-	+	-
GA 16	-	+	+	-
GA 20	-	+	+	-
GS 15	+	-	+	-
A12 (T.2)	+	-	+	-
A16 (T.2)	+	-	+	-
A17 (T.2)	+	-	+	-
T7 u.f (T.2)	+	-	+	-
2017-1457/9	+	-	+	-
2017-1457/10	+	-	+	-
2017-1457/11	+	-	+	-

2017-1457/13	+	-	+	-
2017-1457/15	+	-	+	-
2017-1457/20	-	+	+	-

8.8 Appendix H. Hemolytic activity of *V. alginolyticus* on blood agar

	Hemolysis			
	Sheep blood		**Human blood**	
Isolate	**α**	**B**	**α**	**β**
1-1 (4)	-	-	-	-
1-1 (4-a)	-	-	-	-
1-1 (8)	-	-	-	-
1-2 (3)	-	-	-	-
1-2 (3-a)	-	-	-	-
1-2 (4)	-	-	-	-
1-2 (7)	-	-	-	-
1-2 (7-a)	-	-	-	-
1-3 (1)	-	-	-	-
1-3 (1-a)	-	-	-	-
1-3 (2)	-	-	-	-
1-3 (2-a)	-	-	-	-
1-3 (3)	-	-	-	-
1-3 (4)	-	-	-	-
1-3 (4-a)	-	-	-	-
1-3 (4-b)	-	-	-	-
1-3 (5)	-	-	-	-
1-3 (6)	-	-	-	-
1-3 (6-a)	-	-	-	-
1-3 (7)	-	-	-	-
1-3 (10)	-	-	-	-
1-4 (1)	-	-	-	-
1-4 (2)	-	-	-	-
2-1 (2)	-	-	-	-
2-1 (2-a)	-	-	-	-
2-1 (5)	-	-	-	-
2-1 (6)	-	-	-	-
2-1 (6-a)	-	-	-	-
2-1 (7)	-	-	-	-
2-1 (7-a)	-	-	-	-
2-1 (9)	-	-	-	-
2-2 (2)	-	-	-	-
2-2 (2-a)	-	-	-	-
2-2 (3)	-	-	-	-
2-2 (3-a)	-	-	-	-

2-2 (7)	-	-	-	-
2-2 (9)	-	-	-	-
2-3 (1)	-	-	-	-
2-3 (5)	-	-	-	-
2-3 (5-a)	-	-	-	-
2-3 (6)	-	-	-	-
2-3 (6-a)	-	-	-	-
2-3 (9)	-	-	-	-
2-3 (9-a)	-	-	-	-
3-1 (1)	-	-	-	-
3-1 (1-a)	-	-	-	-
4-1 (2)	-	-	-	-
4-2 (2)	-	-	-	-
4-3 (1)	-	-	-	-
4-3 (2)	-	-	-	-
4-5 (1)	-	-	-	-
4-5 (1-a)	-	-	-	-
4-5 (2)	-	-	-	-
7-5 (1-a)	-	-	-	-
8-1 (1)	-	-	-	-
8-1 (1-a)	-	-	-	-
11-4 (1)	-	-	-	-
B9-1	-	-	-	-
B9-2	-	-	-	-
B9-3	-	-	-	-
A2	-	-	-	-
A14	-	-	-	-
A19	-	-	-	-
A20	-	-	-	-