Bivalve mollusks as tools for monitoring antibiotic resistance in the marine environment

Didrik Hjertaker Grevskott

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The work described in this PhD thesis was accomplished in the research group Contaminants and Biohazards at the Institute of Marine Research (IMR), and through the Department of Biological Sciences at the University of Bergen (UiB), Norway. Parts of the work were also conducted at the Norwegian Institute of Public Health (FHI), the Norwegian Veterinary Institute (NVI), and the Culture Collection University of Gothenburg (CCUG). The PhD position was funded by the IMR.

The work was performed under supervision of Prof. Bjørn Tore Lunestad (IMR, UiB) and co-supervision of Dr. Cecilie Smith Svanevik (IMR), Senior researcher Marianne Sunde (NVI) and Dr. Nachiket Prakash Marathe (IMR).

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Didrik Hjertaker Grevskott

Bergen

Abstract

Antibiotic resistance (AR) is a major global health concern, especially in clinical and veterinary settings. Environmental niches, including the aquatic environment, serve as a source of and/or a dissemination route for antibiotic resistance genes (ARGs) and resistant bacteria. Bivalves are suspension feeders that actively filter, retain and concentrates particles from their surrounding water, including free living or particle-bound bacteria.

The main aim of this thesis was to evaluate bivalve mollusks as tools for monitoring *Escherichia coli* and associated AR, in the marine environment in Norway. Sampling of bivalves were conducted from several sites along the Norwegian coast and the samples were examined for the presence of *E. coli*, according to the most probable number (MPN) EU reference method. More than half (61%) of the samples were positive for *E. coli*, and a selection of 200 *E. coli* isolates were further identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The majority (90%) were confirmed as *E. coli*, while the remaining isolates (10%) were identified as other species mostly belonging to the Enterobacteriaceae family. The isolates were antibiotic susceptibility tested (AST) using the disk diffusion method recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Seventy-five bacterial isolates (38%) showed phenotypic resistance to at least one antibiotic, while multidrug-resistance was observed in eight isolates (4%). Based on resistance phenotypes, selected *E. coli* isolates were subjected to whole-genome sequencing (WGS). Two isolates revealed to carry CTX-M-type extended-spectrum β-lactamases (ESBLs). Accordingly, the two *E. coli* isolates were subjected to long-read sequencing, and a hybrid *de novo* assembly using long-reads and short-reads to obtain complete and closed genome sequences. One isolate harbored four identical chromosomal copies of the $bla_{\text{CTX-M-14}}$ gene, while the other isolate carried the $bla_{\text{CTX-M-15}}$ gene on a conjugative plasmid.

Another aim of this thesis was to generate knowledge regarding the prevalence of antibiotic and heavy metal resistance, and associated resistance genes, among

environmental bacteria isolated from marine bivalves. Bivalves were collected from multiple sites along the Norwegian coast and the samples were subjected to quantitative and qualitative examinations. Quantitative examination involved growth of environmental bacteria on agar with and without antibiotics, while qualitative examination involved selective growth of bacteria in broths with antibiotics. A total of 205 bacterial isolates were identified by MALDI-TOF MS. Most of the bacterial species belonged to the genera *Pseudomonas* (36%) and *Vibrio* (11%). The bacterial isolates were AST by applying the EUCAST disk diffusion method. Accordingly, majority of the isolates revealed to be intrinsic resistant to a wide range of the antibiotics tested for. In addition, phenotypic susceptibility to the heavy metals copper, zinc and cadmium were examined by determining the minimum inhibitory concentration. Selected isolates were subjected to WGS. Among the isolates, clinically relevant ARGs, such as *qnrVC, aph(3')* and *catB*, were detected. Moreover, several heavy metal resistance genes, including *copA* and *copB,* were present.

Overall, the results presented in this thesis suggests that bivalves represent an important tool for the monitoring of clinically relevant ARGs and pathogens in the marine environment, especially in a low prevalence setting like Norway. It also strengthens the notion that the marine environment contributes to the dissemination of clinically important ARGs and pathogens.

List of publications

Paper I

Grevskott, D.H., Svanevik, C.S., Wester, A.L., Lunestad, B.T. 2016. The species accuracy of the Most Probable Number (MPN) European Union reference method for enumeration of *Escherichia coli* in marine bivalves. *Journal of Microbiological Methods* 131: 73-77.

Paper II

Grevskott, D.H., Svanevik, C.S., Sunde, M., Wester, A.L., Lunestad, B.T. 2017. Marine bivalve mollusks as possible indicators of multidrug-resistant *Escherichia coli* and other species of the Enterobacteriace*ae* family. *Frontiers in Microbiology* 8(24): 1-10.

Paper III

Grevskott, D.H., Francisco, S.-S., Moore, E.R.B., Marathe, N.P. 20XX. Nanopore sequencing reveals genomic map of CTX-M-type extended-spectrum β-lactamases carried by *Escherichia coli* strains isolated from blue mussels (*Mytilus edulis*) in Norway. (*Under review in Frontiers in Microbiology*).

Paper IV

Svanevik, C.S., Grevskott, D.H., Storesund, J.E., Bernssen, L.S., Marathe, N.P., Lunestad, B.T. 20XX. Antimicrobial and heavy metal resistance among environmental bacteria isolated from marine bivalves along the Norwegian coast. (*Manuscript*).

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Abbreviations

API	Analytic profile index
AR	Antibiotic resistance
ARG	Antibiotic resistance gene
CFU	Colony-forming units
ECDC	European Centre for Disease Prevention and Control
ECOR	*E. coli* Reference
ESBL	Extended-spectrum β-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal gene transfer
IMR	Institute of Marine Research
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MDR	Multidrug-resistant
MDR-region	Multidrug-resistance determining region
MGE	Mobile genetic element
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MLVA	Multiple-locus variable number tandem repeats analysis
MMGB	Minerals modified glutamate broth
MPN	Most probable number
NFSA	Norwegian Food Safety Authority
NORM/NORM-VET	Norwegian monitoring programme on antimicrobial resistance in bacteria from humans, food, feed and animals

TBX	Tryptone bile with x-glucuronide
WGS	Whole-genome sequencing
WHO	World Health Organization

1. Introduction

1.1 Antibiotics

Antibiotics are powerful drugs and have an essential role in treatment of previously untreatable infections, such as severe wound infections or sepsis (Aminov, 2010). Most of the antibacterial substances utilized are structural derivatives of natural compounds produced by soil bacteria and filamentous fungi (Lewis and Bush, 2015). Antibiotics are a group of agents that must be able to destroy or inhibit the growth of the bacteria, and simultaneously not be unacceptably harmful for the host. The term antibiotic means "against life" and these agents can be classified as broad- or narrow-spectrum. Broad-spectrum antibiotics are applied to treat a wide range of bacterial infections, while narrow-spectrum antibiotics are applied to treat a limited range of infections (van Saene et al., 1998). Antibiotics can either inhibit bacterial growth (bacteriostatic), or kill the bacterial cell (bactericidal) (Patel and Richter, 2015). Antibiotics acts on the bacterial cell by inhibiting various biochemical pathways, which are important for the biosynthesis of essential components, including cell wall synthesis, membrane structure, DNA replication, and protein or folate synthesis (Fig. 1) (Kohanski et al., 2010; Wright, 2011).

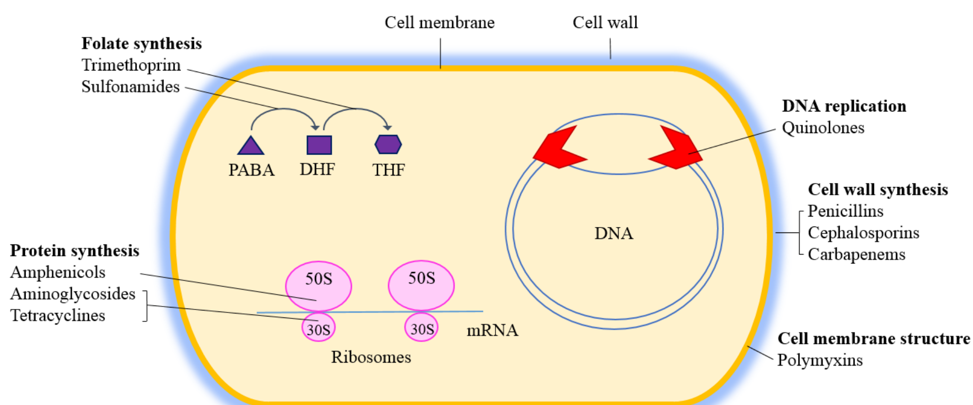

Figure 1. Antibiotics function by targeting various biochemical pathways, including cell wall synthesis, membrane structure, DNA replication, and protein or folate synthesis. PABA; para-aminobenzoic acid, DHF; dihydrofolate, THF; tetrahydrofolate

The effect of an antibiotic depends on the concentration, *i.e.* the lowest concentration of a specific drug needed to inhibit growth of a target bacterium under standard laboratory conditions (Mouton et al., 2012). In the course of antibacterial treatment the concentration needs to be higher than the minimum inhibitory concentration (MIC) of the given strain in the host for long enough time to clear the infection without having severe toxic effect (McKenzie, 2011; Asín-Prieto et al., 2015).

1.2 Antibiotic resistance

The development of antibiotic resistance (AR) is a natural process among bacteria (Davies and Davies, 2010). Genes conferring resistance to β-lactams, glycopeptides and tetracyclines have been isolated from 30,000-year-old Beringian permafrost sediments (D'Costa et al., 2011). In nature, certain bacteria have evolved to produce antibacterial substances to outcompete other bacteria for the same resource. Accordingly, the susceptible bacteria, as well as the producers, have developed or acquired antibiotic resistance genes (ARGs) in order to protect itself from the inhibitory effect (Martínez, 2008; Aminov, 2009). A bacterial strain can be defined as resistant when it is able to resist the effect of an antibiotic, which previously could successfully kill the strain (Martínez et al., 2015). At present, almost all employed antibacterial agents are becoming ineffective to certain bacteria due to rapidly evolving antibiotic resistance (Fig. 2) (Karaiskos and Giamarellou, 2014; Baker, 2015; Tagliabue and Rappuoli, 2018).

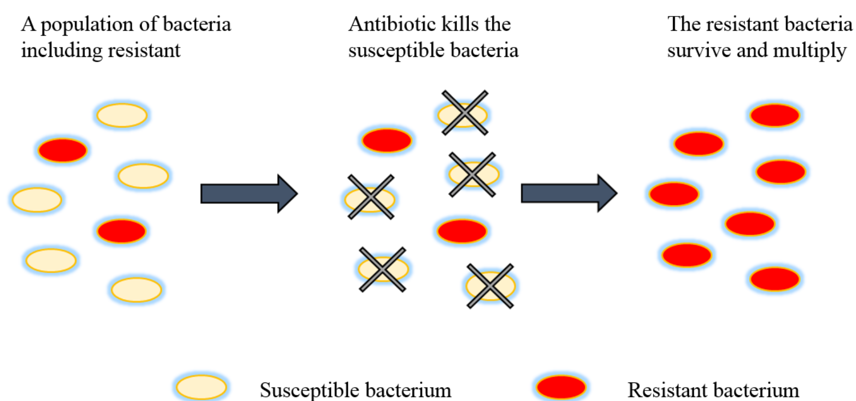

Figure 2. Selection of resistance on exposure to antibiotics.

Mechanisms of AR include; reduced permeability, enzymatic inactivation or degradation, altered target site, or upregulation of efflux pumps (Fig. 3) (Blair et al., 2014; Martinez, 2014). Certain bacteria can have an innate ability to resist different antibacterial agents, termed intrinsic resistance, due to amongst others cellular impermeability or active efflux pumps (Fajardo et al., 2008; Martinez et al., 2008). In addition, bacteria can have increased tolerance to antibiotics during formation of biofilm (Hoffman et al., 2005; Jones et al., 2013).

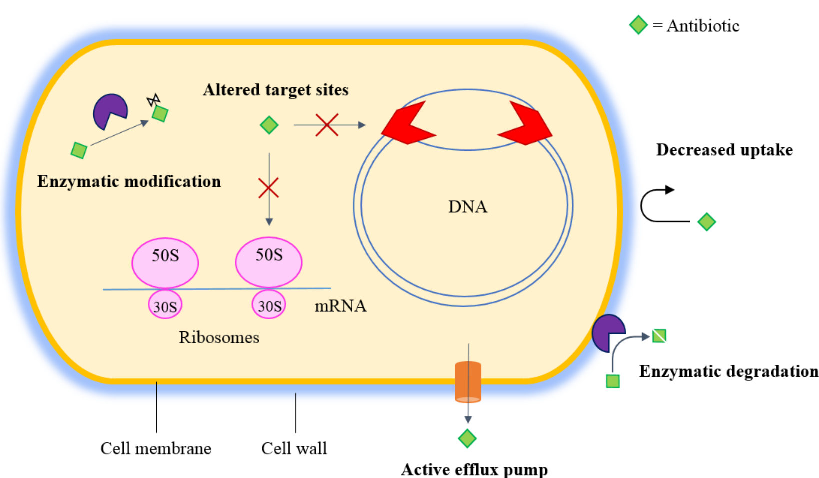

Figure 3. Mechanisms of antibiotic resistance includes decreased uptake, enzymatic inactivation or degradation, altered target site, or active efflux pump.

1.3 Acquisition and transmission of antibiotic resistance genes

Susceptible bacteria can acquire AR due to spontaneous mutations or through horizontal gene transfer (HGT) (Fig. 4). Mutation-mediated resistance depends on the mutation rate and the bacterial population size (Perron et al., 2015). The presence of an antibiotic triggers SOS responses in bacteria stimulating genetic changes, including recombination and mutation, and thereby inducing the potential for resistance (Fig. 4a) (Hastings et al., 2004; Michel, 2005; López et al., 2007; Blázquez et al., 2012). The SOS response function as a defense mechanism by temporarily inhibiting cell division during repair of DNA damage (Miller et al., 2004; Michel, 2005). Resistance properties due to mutations are usually transferred from one generation to the next by clonal

expansion, while gene exchange allow various bacterial species to grow in the presence of antibacterial substances.

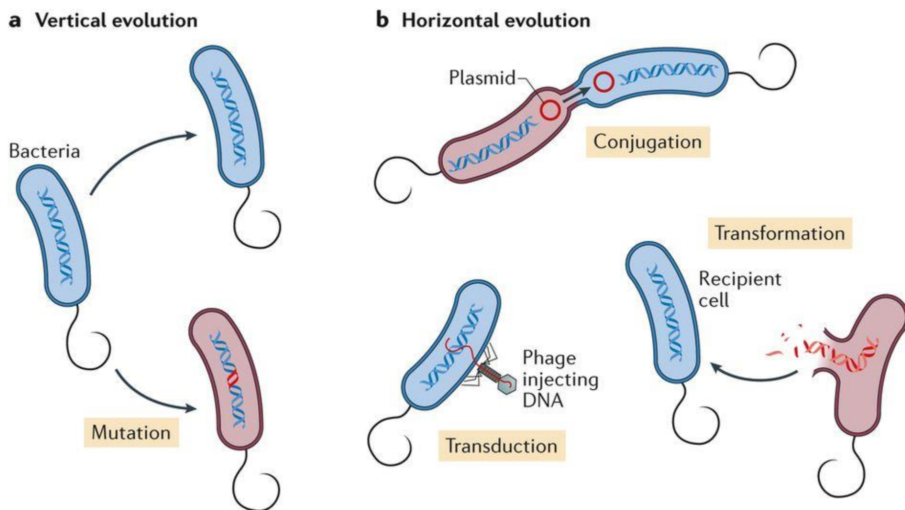

Figure 4. The mechanisms of development or acquisition of antibiotic resistance genes. Two mechanisms for development of antibiotic resistance: (a) mutation, or (b) horizontal gene transfer. Horizontal gene transfer includes uptake of free DNA (transformation), incorporation of genetic elements via bacteriophages (transduction), or exchange of plasmid between bacteria (conjugation). Reprinted with permission from Sommer et al. (2017). Prediction of antibiotic resistance: Time for a new preclinical paradigm? *Nat. Rev. Microbiol.* 15, 689-696. http://dx.doi.org/10.1038/nrmicro.2017.75

Transfer of resistance by HGT can occur through either transformation, conjugation or transduction (Fig. 4b) (Aminov, 2011). Transformation involves the uptake of naked DNA from the environment. Conjugation requires the exchange of mobile genetic elements (MGEs) between bacterial cells. Transduction refer to the incorporation of genetic elements by a bacteriophage vector. Clinically ARGs are commonly located on MGEs, such as conjugative plasmids (Sentchilo et al., 2013) and/or transposons (Harmer and Hall, 2016). Antibiotic resistance genes can spread across and between different species in a given habitat (Martínez et al., 2015). However, ARGs are generally associated with fitness cost unless there is a relatively strong selection pressure to maintain them (Martinez, 2012; Bengtsson-Palme et al., 2018). Fitness is measured by how much faster a bacterium is to reproduce compared to other competing bacteria, in which ARGs that present affordable fitness cost may successfully spread

(Martínez et al., 2015). This suggests that carrying ARGs is only an advantage in the presence of antibiotics or other antimicrobial compounds. Pathogens encountered in hospital and community settings are frequently exposed to antibiotics during treatment, thus the benefits of harboring resistance genes are crucial for their survival and maintenance of ARGs (Andersson and Hughes, 2010).

1.4 Antibiotic resistance is an emerging publich health threat

Antibiotic resistance is a major global health, social and economic concern in the 21st century. Currently, it has been estimated that more than 700,000 deaths annually are due to antibiotic resistant bacteria and this is estimated to increase to approximately 10 million deaths/year after 2050 (O'Neill et al., 2016). Antibiotics are essential for medical treatment and applied in all situations where infections can occur, such as complex surgery, cancer chemotherapy and organ transplants. The extensive misuse and overuse of antibiotics, both in human and veterinary medicine, has accelerated the spread of ARGs and emergence of resistant pathogens (FAO, 2016; WHO, 2017b). The consequence of AR in hospital- and community settings, as well as agriculture and aquaculture, is reduced effectiveness of antibiotics against infectious diseases leading to therapeutic failure. The World Health Organization (WHO) have published a list of priority pathogens, particularly the urgent threat concerning carbapenem-resistance *Acinetobacter baumannii* and extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (WHO, 2017b; CDC, 2019; ECDC, 2019). The Enterobacteriaceae family includes several important pathogens, such as strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella* spp., which are prevalent in the clinics (Forsythe et al., 2015; Strockbine et al., 2015). The prevalence of invasive *E. coli* and *K. pneumoniae* isolates resistant to 3rd gen. cephalosporins are lower in northern Europe compared to the south and east of Europe (Fig. 5 and Fig. 6) (WHO, 2017a; ECDC, 2019). In 2018, the prevalence of invasive *E. coli* isolates resistant to 3rd gen. cephalosporins was 28.7% and 38.7% in Italy and Bulgaria, respectively, compared to 6.8% and 7.3% in Norway and the Netherlands, respectively (ECDC, 2019). The usage of antibiotics in Norway is low compared to most other countries (EMA, 2017), and the total usage of antibiotics in humans have been reduced by 24% since 2012

(NORM/NORM-VET, 2018). Norway represents a low prevalence country in terms of AR. Factors that can influence this situation are increased travel and import/export of animals, food and feed on a global scale (Nawaz et al., 2012; Bengtsson-Palme et al., 2015; Han et al., 2017). Bacteria do not recognize borders and can be transferred with humans, animals and the environment even across continents. For instance, when travelling to countries with high prevalence of AR, such as the Indian subcontinent or Central Africa, humans can act as carriers of ARGs when returning home (Bengtsson-Palme et al., 2015; Espenhain et al., 2018). Therefore, monitoring AR in humans, animals and the environment is needed in order to gain knowledge about the current situation, as well as prepare for the future challenges.

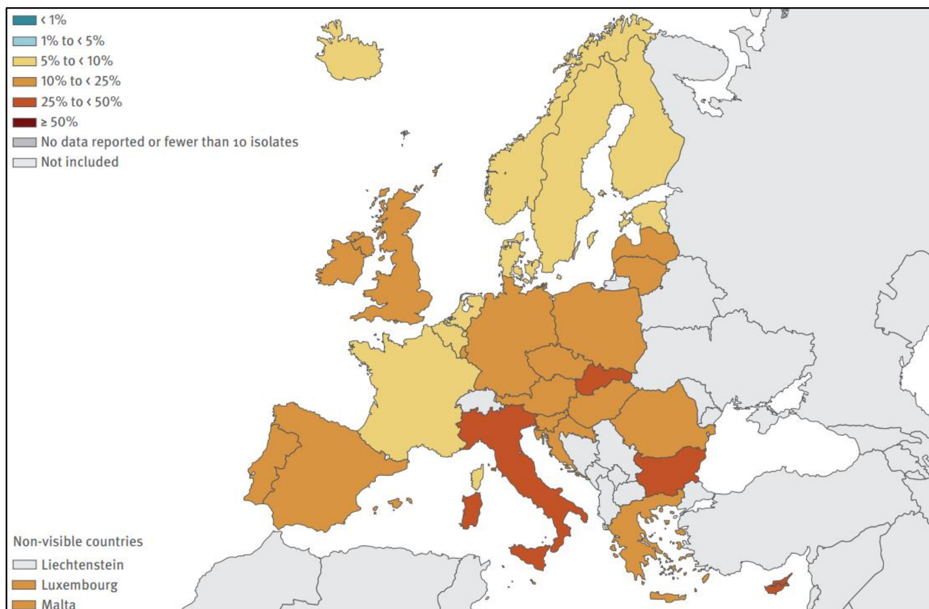

Figure 5. Prevalence of invasive *Escherichia coli* isolates resistant to 3rd generation cephalosporins in EU/EEA countries, 2018. Reprinted from "Surveillance of antimicrobial resistance in Europe 2018, www.ecdc.europa.eu (ECDC, 2018).

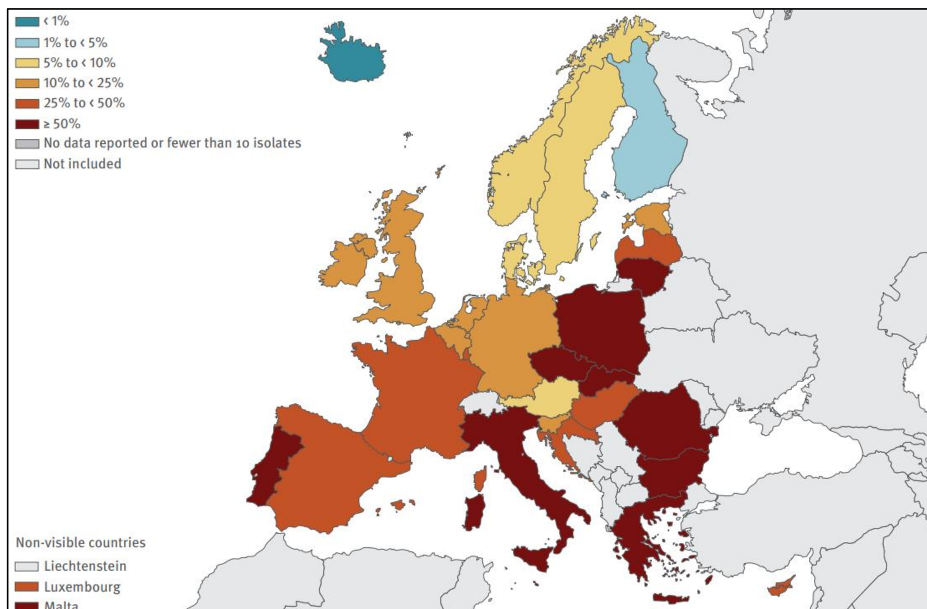

Figure 6. Prevalence of invasive *Klebsiella pneumoniae* isolates resistant to 3rd generation cephalosporins in EU/EEA countries, 2018. Reprinted from "Surveillance of antimicrobial resistance in Europe 2018, www.ecdc.europa.eu (ECDC, 2018).

1.5 Dissemination of resistance genes and resistant bacteria in the environment

Environmental niches, including the aquatic environments, have been acknowledged as a source of and/or a dissemination route for clinically important ARGs and pathogens (Wellington et al., 2013; Karkman et al., 2019). The microbial communities in coastal environments can be influenced by sewage contamination, waste from livestock farming and other runoff from land, concomitantly containing both ARGs and resistant bacteria, as well as antimicrobial substances (Taylor et al., 2011; Gillings, 2013; Michael et al., 2013; Wellington et al., 2013; Amos et al., 2014). A major proportion of the consumed antibiotics are still in a biologically active form when excreted through feces and urine (Gillings, 2013). Environmental pollution with antibiotics can lead to selection of ARGs and emergence of resistant bacteria (Marathe et al., 2013). Moreover, the presence of sub-lethal concentrations of antibiotics found in many natural environments are known to still select for resistance in bacterial

communities (Blázquez et al., 2012; Andersson and Hughes, 2014; Friman et al., 2015; Bengtsson-Palme and Larsson, 2016).

Even in the absence of a selective pressure exerted by an antibiotic, bacteria can still acquire or maintain ARGs due to co-selection with resistance to heavy metals (Baker-Austin et al., 2006; Seiler and Berendonk, 2012), biocides (Seier-Petersen et al., 2013; Jutkina et al., 2018) or disinfectants (Zhang et al., 2016). Environmental influences of metal ions, particular copper and zinc, on bacterial populations can lead to selection of genes conferring resistance to both metals and antibiotics (Poole, 2017; Zhou et al., 2019). Although, low concentrations of metals and antibiotics are important for normal bacterial cell function as metalloproteins (Foster et al., 2014) or signaling molecules (Linares et al., 2006), respectively, elevated levels of these compounds induce stress resulting in promotion of adaptive and protective responses (Lemire et al., 2013; Pal et al., 2017).

Hospital, municipal and industrial wastewater are important sources of resistant bacteria and ARGs, and considered hotspots for dissemination into the environment (Chagas et al., 2011; Berglund et al., 2015; Li et al., 2015; Xu et al., 2015; Ng et al., 2017). One of the most important point sources of clinically important ARGs and pathogens are effluent from wastewater treatment plants due to the large volumes released (Fig. 7) (Rizzo et al., 2013; Guo et al., 2017; Karkman et al., 2018). Discharge of treated sewage plays an important part in the dissemination of ARGs into the environment (Karkman et al., 2019). Moreover, untreated sewage from sanitary sewer overflow may reach the sea during periods of heavy rainfall.

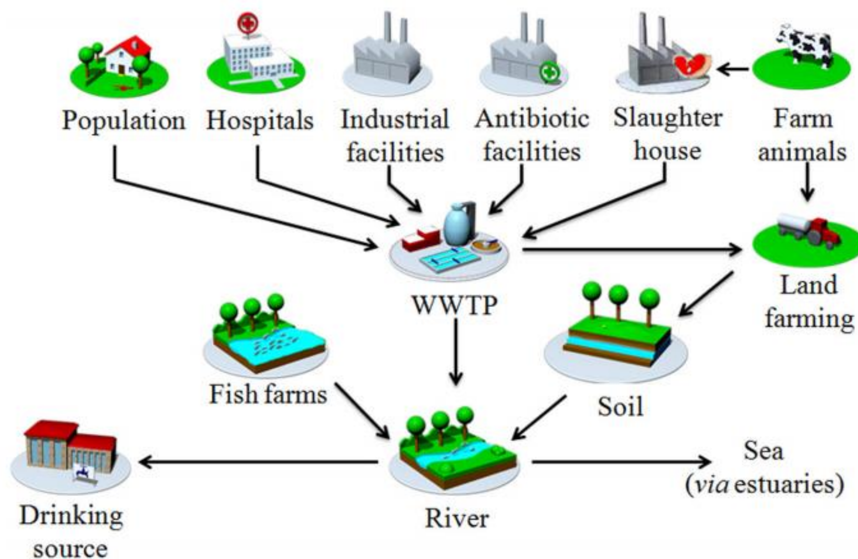

Figure 7. Dissemination of antibiotic resistant bacteria, resistance genes, and antibiotics or other resistance-promoting residues into the environment from various sources, such as from wastewater treatment plant (WWTP). Reprinted from Stalder et al. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* 3(119), 1-14. http://dx.doi.org/10.3389/fmicb.2012.00119.

Industrial agriculture, particularly livestock production, contributes to the development and dissemination of ARGs and resistant bacteria from fecal material through runoff from land (Marshall and Levy, 2011; Allen, 2014). Intensive production of animals, such as pigs and poultry, are prone to increase the burden of diseases due to the high density, and this niche represents a diverse and abundant reservoir of ARGs (Munk et al., 2018). In Norway, the use of antibiotics for food-producing animals, including horses, are very low compared to other European countries (EMA, 2019). In veterinary medicine, antibiotics are commonly used to treat bacterial infections individually or by herd therapy, and prophylactically. Even though the use of antibiotics as growth promotors are prohibited in Europe (1831/2003/EC, 2003), antibacterial agents are still used to increase growth and feed efficiency in many countries (van Boeckel et al., 2015; Woolhouse et al., 2015). Organic fertilizers made of sewage sludge or manure have large concentrations of organic substances, high bacterial density and sub-therapeutic concentrations of antibiotics and other antimicrobials (*e.g.* metals) (Tella et al., 2016),

which in combination can favor bacterial growth and induce gene exchange (Heuer et al., 2011; Calero-Cáceres et al., 2014; Jechalke et al., 2014; Su et al., 2015).

As under all other production of livestock, antibiotics are important to treat infections in aquaculture, but due to the different way of administration, the application may also have adverse effects on the surrounding environment (Heuer et al., 2009; Seyfried et al., 2010; Shah et al., 2014; Xiong et al., 2015; Cabello et al., 2016). A common practice for antibacterial therapy in aquaculture is metaphylaxis, in which both diseased and healthy fish in the population are affected (Sørum, 2006). Unconsumed food pellets and feces containing antibiotics or other antimicrobials contribute to the enrichment of ARGs in bacterial communities present in the sediments below the farm and in the proximity (Samuelsen et al., 1992; Cabello, 2006; Burridge et al., 2010; Buschmann et al., 2012; Han et al., 2017; Muziasari et al., 2017). Importantly, the use of antimicrobials in Atlantic salmon aquaculture industry in Norway is very low (Love et al., 2020), especially considering the production volume (EMA, 2019).

Wild animals, particularly birds (Poeta et al., 2008; Alves et al., 2014; Murugaiyan et al., 2015; Stedt et al., 2015), terrestrial (Gonçalves et al., 2013; Navarro-Gonzalez et al., 2013; Hansen et al., 2016; Mo et al., 2018) and aquatic mammals (Brownstein et al., 2011; Santestevan et al., 2015), can also act as potential carriers of ARGs and resistant bacteria. Particularly wild animals sharing the same habitats and water sources as humans and/or domestic animals can serve as potential reservoirs for resistance genes and subsequent transmission (Allen et al., 2010; Vittecoq et al., 2016; VKM, 2018).

Once fecal contamination from different sources are introduced into the aquatic environment, areas used for marine food production or recreational activities may serve as potential hotspots for exposure of resistant bacteria and ARGs (Blaak et al., 2014; Vignaroli et al., 2016; Leonard et al., 2018). Seafood represents a risk of infection in case of insufficient heat treatment or handling, or through products intended for raw- or light preserved consumption (Nawaz et al., 2012; Ryu et al., 2012; Roschanski et al., 2017; Yang et al., 2017). This have led to the need to identify sources contributing

to the dissemination of resistant bacteria and ARGs into aquatic environments. One possible candidate may be bivalve mollusks, which could function as tools for assessing the presence, as well as the abundance, of resistant bacteria and ARGs in coastal environments.

1.6 Bivalve mollusks as tools

Mollusca is the second largest phylum of invertebrates within the kingdom Animalia, and the class Bivalvia constitute numerous species, including mussels, clams, oysters, scallops and cockles (Gosling, 2003a). Bivalve mollusks are characterized by an external two-part hinged shell that enclose the soft parts of the animal (Gosling, 2003a). Bivalves are suspension feeders that actively filter, retain and concentrates particles from their surrounding water, including free living or particle-bound microorganisms (Fig. 8A) (Lees, 2000; Potasman et al., 2002). The gills have evolved into a specialized organ for both respiration and feeding, in which the captured particles are transported from the gills to the digestive tract by cilia in a selective process (Fig. 8B) (Gosling, 2003b; Rosa et al., 2018). A large number of species belonging to different genera, including *Vibrio, Shewanella* and *Stentrophomonas*, constitute the microbiota of bivalves (Antunes et al., 2010; Romalde et al., 2014; Vezzulli et al., 2018). Bivalves located near a sewage discharge, or otherwise exposed to runoff from land, are thereby excellent tools for examining fecal contamination and will reflect the load of *E. coli* in the water column at time of sampling (Roslev et al., 2010; Lunestad et al., 2016; Bighiu et al., 2019).

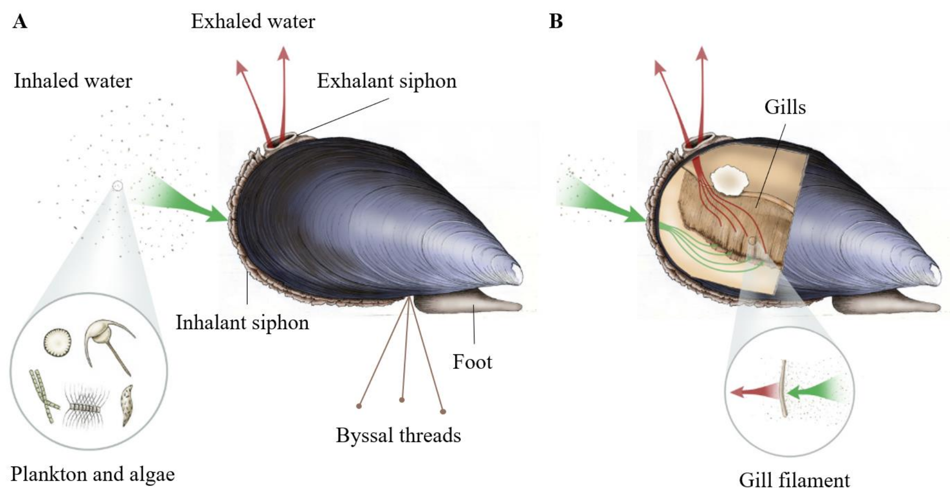

Figure 8. The mechanism of filter-feeding in a blue mussel (*Mytilus edulis*). (A) The mussel actively filters particles from the surrounding water. (B) Captured particles on the gills are transported to the digestive tract by cilia. Source: Kimberly Andrews, Connected, Are you Sure? Ministry of Education, Lift Education, Crown 2013.

Blue mussels (*Mytilus edulis*) frequently appear in robust aggregated structures. They attach themselves to a hard bottom substrate using strong byssal threads (Christensen et al., 2015). An adult blue mussel is able to filter approximately 70 liters of seawater daily (Cranford et al., 2011), and blue mussels living in the tidal zone are very tolerant against environmental fluctuations, such as temperature and salinity, and harbor a dense and diverse community of bacterial species of multiple origins (Utermann et al., 2018; Serra-Compte et al., 2019). Shortly after exposure to fecal contamination, blue mussels show high concentrations of *E. coli* in the digestive tract, while lower concentrations were found in the gills, muscles and hemolymph (Power and Collins, 1990). Ingested bacteria can be degraded by bacteriolytic enzymes in the stomach, or rejected and passed through the digestive tract while remaining viable (Bernard, 1989). Moreover, concentrations of land derived bacteria including *E. coli* do not impair immune competency in blue mussels, and does not represent a hazard on its survival in coastal areas subjected to sewage discharges (Gauthier-Clerc et al., 2013).

1.7 Cultivation and consumption of bivalves

Bivalve mollusks represents a sustainable food source of high quality animal protein content (Wright et al., 2018), and generally requires minimal input, in which no formulated feed or medication are needed. The global production of marine bivalves is more than 15 million tons per year (average period 2010-2015), *i.e.* 14% of the total marine production for human consumption (Wijsman et al., 2019). Asia, especially China, is the largest producer, consumer and exporter of marine bivalves, and account for 85% of the global production; with an annual marine bivalve production of 12.4 million tons (Wijsman et al., 2019). In Europe, the production has remained relatively constant during the last years, with the production volume of about 598,000 tons annually (Wijsman et al., 2019). Bivalve mollusks represents important species in Norwegian aquaculture, in which blue mussels represents the third most important species with a production volume of around 1,649 tons in 2018, and a gross sale of approximately 28.5 million NOK (Directorate of Fisheries, 2019).

As a requirement from the European Union (854/2004/EC, 2004), a national monitoring program for production areas for bivalves was initiated by the Norwegian Food Safety Authority (NFSA) in 2006. On behalf of the NFSA, the Institute of Marine Research (IMR) conducts annual surveillance of bivalve mollusks, by repeatedly sampling of harvested bivalves, as well as random sampling of retail products, to ensure that the bivalves fulfils the requirements for acceptable levels of *E. coli*. Depending on the content of *E. coli* in the soft parts and mantle water of harvested bivalves, the production areas are classified as A, B, C or prohibited areas according to the EU Directives 854/2004/EC (2004) (Table 1). This is performed in order to assess whether the bivalves are suitable for consumption. Bivalves from a class A area can go directly for human consumption, while bivalves from class B or C must be purified until meeting the limit of 230 *E. coli*/100 g or heat treated. According to 2015/2285/EC (2015), 20% of the harvested bivalves from a class A area can contain *E. coli* between 230 and 700/100 g sample material, while the remaining 80% of the samples must not exceed the class A limit, in order to remain a Class A area. In addition, 10% of the bivalves from a class B area can contain *E. coli* between 4,600 and 46,000/100 g, while

the remaining 90% of the samples must be within the class B limit (2008/1021/EC, 2008).

Table 1. Production areas for bivalves according to the EU Directive 854/2004 and 2015/2285. Areas are classified as A, B or C depending on the content of *Escherichia coli* in the soft parts and mantle water of harvested bivalves.

Class	Amount of *E. coli* per 100 g sample material measured as fresh weight	Treatment after harvesting
A	80% of the bivalves must not exceed 230 *E. coli* per 100 g, while the remaining 20% must not exceed 700 *E. coli*/100 g	None, go directly for human consumption
B	90% of the bivalves must not exceed 4,600 *E. coli* per 100 g, while the remaining 10% must not exceed 46,000 *E. coli*/100 g	Purification by resuspension at a Class A area*, or sufficient heat treatment by approved procedure
C	$\leq$ 46,000	Purification by resuspension at a Class A area for a long period of time*, or sufficient heat treatment by approved procedure

*Re-sampling after at least two weeks.

1.8 *Escherichia coli* as indicator for fecal contamination

E. coli is a Gram- and oxidase-negative, facultative anaerobic, rod-shaped, coliform bacterium belonging to the Enterobacteriaceae family (Welch, 2006), occurring naturally in the gut microbiota of humans, birds, and terrestrial and marine mammals (Kaper et al., 2004; Tenaillon et al., 2010). As ubiquitous in feces of humans and other warm-blooded animals, *E. coli* is considered an indicator for fecal contamination and improper hygiene in food and water, representing a possible risk for the consumer (Buttiaux and Mossel, 1961; Welch, 2006; Strockbine et al., 2015). Human feces normally harbors *E. coli* in concentrations ranging from 10^6 to 10^7 cells per gram (Forsythe, 2010). The gut microbiota of healthy humans are colonized by commensal *E. coli* strains, while some opportunistic, pathogenic strains are capable of causing serious diseases (Tenaillon et al., 2010; Richter et al., 2018). Infections with *E. coli* are

among the most frequent foodborne diseases worldwide, causing morbidity such as diarrhea and extra-intestinal infections that in some cases could result in mortality (Kaper et al., 2004; Croxen et al., 2013). Contaminated food and water, person-to-person contact, and contact with animals or the environment are the main transmission routs for such infections (Strockbine et al., 2015).

During antibacterial therapy, susceptible pathogens responsible for the infection will normally be eliminated, as well as other commensal and protecting gut microbes (Dethlefsen and Relman, 2011). Antibacterial treatments are known to substantially affect the gut microbiota favoring the survival and growth of resistant bacterial strains (Sommer and Dantas, 2011; Palleja et al., 2018). This may result in complications during subsequent infections, in which non-resistant pathogens could acquire ARGs from commensal gut bacteria. Once the gut microbiota is colonized with resistant bacteria, such as *E. coli*, individuals may contribute to subsequent dissemination via feces transported through sewage contamination, waste from livestock production or other runoff from land into the environment. Due to its genetic flexibility, *E. coli* has the ability to persist in terrestrial and aquatic environments for days to months depending on the conditions (Fremaux et al., 2010; van Elsas et al., 2011; Schang et al., 2016). Hence, *E. coli* can be considered a good indicator organism for fecal contamination.

2. Objectives

The main aim of this thesis was to evaluate bivalve mollusks as potential tools for the monitoring of *E. coli* and associated AR, in the marine environment in Norway. In addition, this thesis aimed to generate knowledge regarding the prevalence of antibiotic and heavy metal resistance, and associated resistance genes, among environmental bacteria isolated from marine bivalves.

The following three objectives were defined for this work:

1. Isolation and characterization of antibiotic resistant *E. coli* from marine bivalves
2. In-depth characterization based on data from whole-genome sequencing of ESBL-producing *E. coli* isolates
3. Isolation and characterization of antibiotic resistant environmental bacteria and associated heavy metal resistance from marine bivalves

3. Methodological Approach

3.1 Sampling of bivalve mollusks

To address Objective 1 & 3, batch samples of bivalve were obtained from several sites along the Norwegian coast. The IMR conducts annual surveillance of bivalves on behalf of the NFSA, in which the sampling and submission of samples were coordinated with inspectors at the District Offices of NFSA, as well as producers from several locations along the coast. Together, this comprised bivalves from both commercial active sites and reference monitoring positions (Fig. 9), sampled at multiple occasions. For Objective 3, two additional bivalve samples were collected from the city harbor (Bergen, Norway), and included as representatives of contaminated areas.

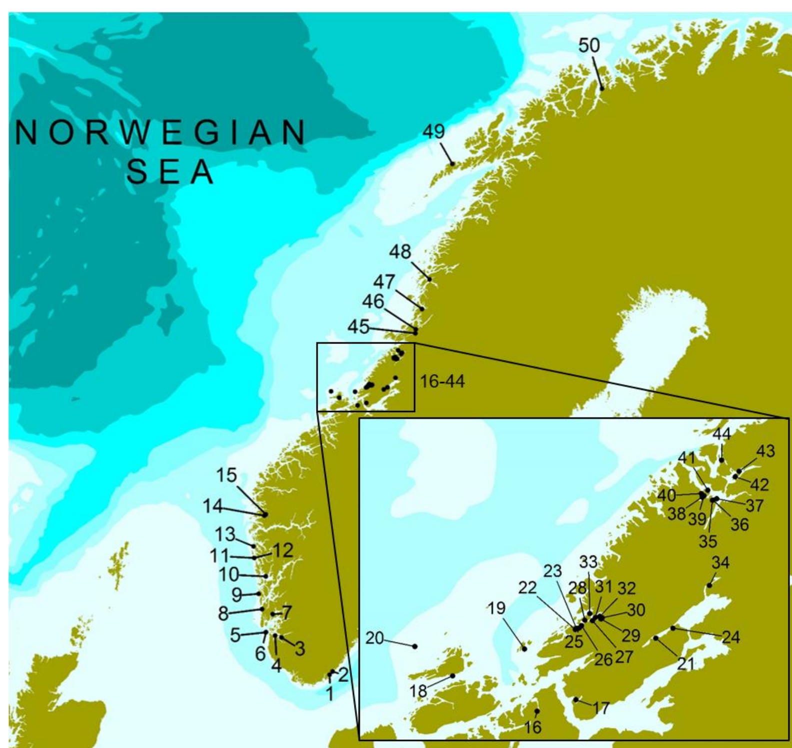

Figure 9. Sampling sites of bivalve mollusk along the Norwegian coast, from both commercial active sites and reference monitoring positions. Reprinted from Martin et al. (2019). Isolation and characterisation of Shiga toxin-producing *Escherichia coli* from Norwegian bivalves. *Food Microbiol.* 84, 1-5. http://dx.doi.org/10.1016/j.fm.2019.103268.

The samples were transported under chilled conditions (at around 4°C) to the laboratory within 24 hours for microbiological analysis. One bivalve sample constituted ten individuals or more, if necessary to obtain 50 g, and soft parts and mantle water were homogenized. Bivalves harvested from the same area at the same time most likely contain comparable amounts of *E. coli* present in the surrounding water at time of sampling. The bivalve samples, comprising blue mussels (*M. edulis*), great scallops (*Pecten maximus*), horse mussels (*Modiolus modiolus*), flat oysters (*Ostrea edulis*), pacific oysters (*Crassostrea gigas*), common cockles (*Cerastoderma edule*), soft-shell clams (*Mya arenaria*) and ocean quahog (*Arctica islandica*), were included. Detection and enumeration of *E. coli* in bivalves are specified in EU Council Directive 91/492/EEC (1991), and the method is based on a most probable number (MPN) principal with a five tubes each in three dilutions (Oblinger and Koburger, 1975). In accordance with the EU reference method ISO 16649-3 (ISO, 2005), the MPN method was performed in combination with verification on chromogenic agar, in which the number of *E. coli* in the bivalve samples were calculated (Donovan et al., 1998). From positive bivalve samples, one *E. coli* isolate was picked from a random selective plate and grown into pure culture before further analysis (Objective 1 & 2).

Objective 3 examined for a broad range of marine and allochthonous bacteria in bivalves, and the homogenized samples were examined by quantitative and qualitative analysis on non-selective media and selective media (containing antibiotics). The quantitative method was based on 10-fold dilution series followed by growth of bacteria on Mueller-Hinton (MH) agar plates. The total plate count was estimated by counting the number of colony-forming units (CFU) per gram and had lower and upper limit of quantification (LOQ) of 100 and 2.5×10^{11} CFU/g, respectively. In addition, sample homogenate was transferred directly to MH agar plates containing clinically relevant antibiotics (Table 2). All plates were aerobically incubated at 25°C for 72 hours. The reduced temperature where selected to retrieve more marine bacterial isolates. The qualitative method was based on growth of resistant bacteria in MH broth supplemented with antibacterial agents (Table 2), and further growth on MH agar containing the same agents for verification. The MH broths and agar plates were

incubated aerobically at 25°C for 48 hours. Morphological different colonies were collected, if present, from both the qualitative and quantitative method and grown to pure cultures by three-time transfer on solid media. A maximum of 20 bacterial isolates were retrieved from each sample, ten isolates from non-selective plates and ten isolates from media containing antibiotics.

Table 2. Antibiotics and concentrations applied in the Mueller-Hinton (MH) agar plates and MH broths.

Antibacterial class	Agent	Concentration (mg/l)
Penicillins	Ampicillin	50
Cephalosporins	Ceftazidime	2
Carbapenems	Imipenem	10
Fluoroquinolones	Ciprofloxacin	0.06

3.2 Characterization of the bacterial isolates

The presumptive *E. coli* isolates were tested for Gram character according to the method of Buck (1982), as well as tested for oxidase activity (Kovacs, 1956), before further identifications steps were performed (Objective 1). For the environmental bacteria (Objective 3), the isolates were divided based on Gram character, and subsequently tested for oxidase activity and catalase production with hydrogen peroxide.

For Objective 1, the *E. coli* isolates were characterized by the analytical profile index (API) 20E test kit (BioMérieux, France). The API 20E function by identifying members of the Enterobacteriaceae family based on biochemical reactions conducted in 20 miniature wells. The bacterial isolates were tested for the ability to utilize certain carbon sources and to produce specific enzymes. A scheme was used to compare positive or negative reactions in the different wells, *i.e.* to interpret the color, and the results was noted in a form and a seven-digit code was calculated. A comprehensive

numerical, identification database (APIweb™) was used to interpret the code and the identification (%).

Identification with matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was applied to verify the results obtained from API 20E (Objective 1), as well as to identify the environmental bacteria obtained from the quantitative and qualitative analysis (Objective 3). The MALDI-TOF MS instrument identify bacteria into genus and specie levels, by generating small molecules from ribosomal proteins that gives a characteristic spectrum called peptide mass fingerprint (PMF) (Bourassa and Butler-Wu, 2015; Singhal et al., 2015). Due to their mucoid appearance, some bacteria were not completely lysed by the matrix solution and formic acid was added to ensure complete lysis prior to applying the matrix. During PMF matching, the spectra of the unknown bacterial isolate was compared with the spectra of known bacterial species included in the database of reference spectra (MALDI Biotyper Library). The data was interpreted and the program provided an overview of the results as best score/match of a bacterium.

3.3 Antibiotic susceptibility testing

To determine the susceptibility of the obtained *E. coli* isolates (Objective 1), and for the environmental isolates (Objective 3), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method was applied (Matuschek et al., 2014). This method for antimicrobial susceptibility testing is based on measurements of the inhibition zones, which depends on the concentration of the antibacterial agent in the disk, its ability to diffuse into the medium, and the susceptibility of the bacterium (Bauer et al., 1966). The bacterium can be interpreted as susceptible, intermediate or resistant based on the inhibition zone within a defined incubation temperature and time period (Bauer et al., 1966). When defining a bacterium as susceptible or resistant, clinical breakpoints or epidemiological cut-off values are commonly used (Martínez et al., 2015). In this experiment, clinical breakpoints were applied when defining the bacteria as susceptible or resistant (EUCAST, 2016a). Accordingly, the inhibition zones are only applicable when employing the EUCAST protocol, included the approved quality control strains.

The *E. coli* isolates were tested against 24 antibacterial agents belonging to ten classes (Table 3) (Objective 1). According to the EUCAST clinical breakpoint tables v.6.0 available at time of analysis (EUCAST, 2016a), the isolates were interpreted as susceptible or resistant. The Clinical and Laboratory Standards Institute (2014) and Indian Council of Medical Research (2009) were used as clinical breakpoint tables for antibiotics not included in the EUCAST tables.

For environmental isolates not able to grow at 35°C, the same protocol was performed at 25°C including for quality control strains (Objective 3). The isolates were tested against 18 antibacterial agents, and different test panels were applied on Gram-positive and Gram-negative bacteria (Table 3). The inhibition zones were interpreted as susceptible or resistant based on the EUCAST clinical breakpoint tables v.8.0 available at time of analysis (EUCAST, 2016b). For isolates with no established breakpoints, only complete absence of inhibition zone around the antibacterial disks were considered resistant or non-susceptible (*i.e.* intrinsic resistance) (EUCAST, 2016c).

Table 3. Antibiotics and amounts (µg) applied (marked as X) in the disk diffusion testing for *Escherichia coli* and environmental bacteria (G+; Gram-positives, G-; Gram-negatives).

Antibacterial class	Agent	Disk potency (µg)	*E. coli*	Environmental bacteria G+	G-
Penicillins	Ampicillin	10	X	X	X
	Amoxicillin	10	X	X	X
	Amox./ Clavulanic acid	3 (2/1)	X		
	Mecillinam	10	X	X	X
	Piperacillin/ Tazobactam	36 (30/6)	X		
Cephalosporins	Cefotaxime	5	X	X	X
	Ceftazidime	10	X	X	X
Carbapenems	Imipenem	10	X	X	X
	Meropenem	10	X	X	X
Aminoglycosides	Gentamicin	10	X	X	X
	Tobramycin	10	X	X	
	Streptomycin	25	X		
	Kanamycin	30	X	X	X
Amphenicols	Chloramphenicol	30	X	X	X
Tetracyclines	Tetracycline	30	X	X	
	Doxycycline	30	X	X	X
Trimethoprim and sulfonamides	Trimethoprim	5	X	X	X
	Trim./ Sulfamethoxazole	25 (1.25/23.75)	X	X	X
Quinolones, incl. fluoroquinolones	Nalidixic acid	30	X	X	
	Ciprofloxacin	5	X	X	X
	Levofloxacin	5	X		X
	Norfloxacin	10	X		
Nitrofurans	Nitrofurantoin	100	X	X	X
Polymyxins	Colistin sulfate	25	X		
Macrolids	Erythromycin	15			X
Glycopeptides	Vancomycin	5			X

3.4 Phenotypic susceptibility to heavy metals

Phenotypic susceptibility to heavy metals was examined by a MIC test (Objective 3). The isolates were tested two times by point inoculation on solid agar containing increasing concentrations of copper, zinc and cadmium. The metal concentrations used were 0.095 mM, 0.188 mM, 0.375 mM, 0.75 mM, 1.5 mM, 3.0 mM, 6.0 mM and 12.0 mM as the corresponding cations. The different metal concentrations in the media were verified by inductive coupled plasma-mass spectrometry (ICP-MS) (Julshamn et al., 2007). Solid media without copper, zinc and cadmium were included as growth controls. Existing literature were applied when interpreting the breakpoints for metal resistance. The MIC were 3.0 mM for copper and zinc, and 0.75 mM for cadmium (Resende et al., 2012). For some bacterial taxa, the MIC_{50} was calculated. In addition, the concentrations of copper, zinc and cadmium were examined in batches of blue mussels by ICP-MS analysis (Julshamn et al., 2007).

3.5 Conjugation assay

For Objective 1, a conjugation experiment was performed in broth on a selection of resistant *E. coli* isolates to investigate the ability to transfer ARGs. The isolates were used as donors, and laboratory strains were used as recipients. The lactose-negative recipient strains used were *E. coli* DH5α (Culture Collection University Gothenburg, Sweden), resistant to nalidixic acid and One Shot *E. coli* (Invitrogen, USA), resistant to kanamycin. To be able to differentiate between donor and recipient, the applied recipient strains were resistant to a certain antibiotic, *i.e.* nalidixic acid and kanamycin, respectively, that inhibits growth of the donor strains. Conjugation is a process that involves cell-to-cell contact, in which the donor cell transfer MGEs directly into the recipient cell, termed transconjugant (Aminov, 2011). After mating (*i.e.* conjugation period), a 10-fold dilution series was made and aliquots from the mating solutions were spread onto media containing nalidixic acid or kanamycin and antibiotic disks, corresponding to the resistance profile of the donor, were applied on the agar surface. Growth of single colonies within the inhibition zone of the antibacterial disks were presumed to be transconjugants. Presumptive transconjugants were cultured on blood agar and subsequently tested against the antibiotics corresponding to the resistance

profiles of the donors when examined by the EUCAST method (Matuschek et al., 2014). Subsequently, the transconjugants were subcultured on lactose-saccharose-bromthymol blue agar to verify that the transconjugants were indeed recipients and not the donor strain with mutations, or possible contamination. The colony morphology of the recipients used were different, in which the colonies were notably smaller than wild-type *E. coli* strains (donors) and did not ferment lactose (Sunde and Norström, 2006; Sunde et al., 2015).

3.6 Whole-genome sequencing

Whole-genome sequencing (WGS) was performed on selected *E. coli* and environmental isolates based on phenotypes expressing resistance to clinically important agents, such as 3rd gen. cephalosporins and/or showing resistance towards multiple antibacterial agents (Objective 1 & 3).

For Objective 1, genomic DNA was extracted and quantified using Nanodrop™ 2000 Spectrophotometer (Thermo Fisher, USA) and Qubit™ 2.0 Fluorometer (Thermo Fisher, USA). A Kapa Hyper Plus Library preparation kit (Kapa Biosystems, USA) was used to prepare sequencing libraries. The libraries were sequenced on an Illumina MiSeq platform (Illumina, USA) (Bentley et al., 2008), using 2 x 250 bp chemistry at the Public Health Institute, Oslo, Norway. The raw data were quality trimmed and assembled using Trimmomatic (Bolger et al., 2014) and SPAdes (Bankevich et al., 2012), respectively. The processed sequences were analyzed for ARGs, serotype and multi-locus sequence types (MLSTs) using available databases. This includes ResFinder (Zankari et al., 2012), SerotypeFinder (Joensen et al., 2015), and MLSTs tool (Larsen et al., 2012) with *E. coli #1* profile (Wirth et al., 2006), from Centre for Genomic Epidemiology, at the Technical University of Denmark.

For Objective 3, genomic DNA was extracted and quantified using Nanodrop™ 2000 Spectrophotometer (Thermo Fisher, USA) and Qubit™ 2.0 Fluorometer (Thermo Fisher, USA). Sequencing libraries were prepared using Nextera DNA Flex Library Prep kit (Illumina, USA) and sequencing was performed on an Illumina MiSeq platform (Illumina, USA) (Bentley et al., 2008), using 2 x 300 bp chemistry, at the

Norwegian Sequencing center Oslo, Norway. The raw data were quality trimmed with BBMap v.81.31 (Bushnell, 2014) and assembled using SPAdes v.3.13.0 (Bankevich et al., 2012) or Unicycler v.0.4.7 (Wick et al., 2017). The processed sequences were annotated using Prokaryotic Genomes Annotation Pipeline (PGAP) v.4.8 at the National Center for Biotechnology Information (NCBI) (Tatusova et al., 2016). The presence of ARGs was examined, using AMRFinder v.3.1.1b (Feldgarden et al., 2019), and biocide- and heavy metal-resistance genes were analyzed, using the BacMet database v.2.0 (Pal et al., 2014), Diamond v.0.9.29 (Buchfink et al., 2015) and the Rapid Annotation using Subsystem Technology (RAST) v.2.0 database (Aziz et al., 2008).

3.7 Hybrid *de novo* assembly

Short-read Illumina-based sequencing only allow fragmented genome assembly (*i.e.* draft genome), which is useful for detecting genes present in a given strain (Bentley et al., 2008). However, the complete metabolic potential of the given strain is not revealed by the draft genome. Long-read sequencing technology like Oxford Nanopore (Oxford Nanopore Technologies Ltd., UK) allow assembly of complete genomes, but have higher sequencing error rates compared to Illumina (Loman et al., 2015). To address Objective 2, a combination of low error short-reads and long-reads were applied, to obtain high-quality complete and closed genome sequences of the selected ESBL-producing *E. coli* isolates to determine the genomic map of the resistance genes and associated mobile DNA elements. Genomic DNA was extracted, following the protocol described by Salvà-Serra et al. (2018). A sequencing library was prepared using a Rapid Barcoding kit (Oxford Nanopore Technologies Ltd., UK), and the library was sequenced using a MinION sequencer instrument (Oxford Nanopore Technologies Ltd., UK). Subsequently, a hybrid *de novo* assembly was performed by combining long-read Nanopore and short-read Illumina-based sequencing using Unicycler v.0.4.7 (Wick et al., 2017). The bacterial genome sequences were annotated using PGAP v.4.8 at the NCBI (Tatusova et al., 2016). Complete overview of the genome sequences and genomic maps were performed using GView Server v.1.7 (Petkau et al., 2010) and SnapGene® software v.4.3.8.2 (GSL Biotech, USA), respectively. Plasmid replicons

were typed using PlasmidFinder v.2.0 (Carattoli et al., 2014) as well as BLASTP analysis of the replication initiation (Rep) sequence against the NCBI database. The presence of ARGs were detected, using ResFinder v.3.2 (Zankari et al., 2012) and CARD v.3.0.7 (Alcock et al., 2019). Moreover, the VFDB database (Liu et al., 2019) was used to detect virulence genes, while biocide- and heavy metal-resistance genes were examined using the BacMet database v.2.0 (Pal et al., 2014).

3.8 Phylogenetic analysis

For Objective 1, rapid genotyping of the *E. coli* strains were performed using the multiple-locus variable number tandem repeats analysis (MLVA) method (Løbersli et al., 2012). The MLVA method measures the bp sizes for the variable number of tandem repeats (VNTR)-regions for each bacterial cell where a selected number of loci are present (Lindstedt et al., 2007). Genomic DNA was extracted, and the VNTR-regions were amplified by a PCR, and the amplicons were separated by a capillary electrophoresis (CE). During CE, the amplicons were run through a gel matrix in an electric field, in which the instrument measures the amplified amount of VNTR-regions by a fluorescence bound to the product to determine the size. According to size and color, each peak was identified and each multiple of repeat was assigned to a distinct allele number, and the results were interpreted using a library with an overview of *E. coli* strains. From the MLVA-profiles of the selected *E. coli* isolates, a minimal spanning tree (MST) was constructed. As markers for genetic relationships, we included 38 *E. coli* Reference (ECOR) strains obtained from the Microbial Evolutionary Laboratory (State University of Michigan, USA), 212 community-acquired *E. coli* strains causing blood stream infection (Wester et al., 2013), and four enterohemorrhagic *E. coli* strains associated with hemorrhagic uremic syndrome collection at the Norwegian Institute of Public Health (Wester et al., 2013; Wester et al., 2014).

For Objective 2, a single nucleotide polymorphism (SNP)-based comparative analysis of pathogenic ESBL-producing *E. coli* strains with other strains from different sources and countries was performed, as described by Sabat et al. (2017). The assembled genome sequences were analysed using the CSI Phylogeny tool 1.4 (Kaas et al., 2014)

and the SNP-based phylogenetic tree was displayed on-line with the Interactive Tree Of Life (iTOL) (Letunic and Bork, 2016).

4. Results and Discussion

4.1 Detection and characterization of *E. coli*

Sampling of marine bivalves were performed, between October 2014 and November 2015, from 57 sites along the Norwegian coast, including samples from class A and B area, to assess the AR situation in the environment. A total of 549 samples were examined for presence of *E. coli* by applying the MPN EU reference method. The material comprised 447 samples of blue mussels (*M. edulis*), 40 flat oysters (*O. edulis*), 39 great scallops (*P. maximus*), 12 soft-shell clams (*M. arenaria*) and 11 horse mussels (*M. modiolus*). Among the 549 bivalves examined, 335 (61%) contained *E. coli* at different concentrations, ranging from 20 to 3,500 *E. coli* per 100 g sample material. It is reasonable to assume that bivalves with a high concentration of *E. coli* had been exposed to fecal contamination recently, or that the sample sites were located closer to a sewage efflux point (Buttiaux and Mossel, 1961; Welch, 2006; Strockbine et al., 2015). A total of 200 *E. coli* isolates from different bivalve samples, originating from both class A and B areas, were selected for further analysis.

All isolates were Gram-negative and oxidase-negative. Totally 180 (90%) were identified as *E. coli* while 20 (10%) were identified as other species mostly belonging to the Enterobacteriaceae family (Paper I). Thirteen of these isolates were identified within the genera *Klebsiella*, *Citrobacter* and *Enterobacter*, all within the Enterobacteriaceae family. One isolate was identified as *Acinetobacter* spp. belonging to the Moraxellaceae family. The remaining six isolates were identified as different genus/species in the family Enterobacteriaceae with API 20E compared to MALDI-TOF MS (Paper I). As specified in the European Council Directive 91/492/EEC (1991), the standardized MPN method ISO 16649-3 (ISO, 2005) is currently applied for the detection and enumeration of *E. coli* in bivalves intended for human consumption. Thus, it was expected to detect *E. coli*, although other Gram-negative bacteria were identified as well. However, most of the isolates were shown to belong to the Enterobacteriaceae family, except for one isolate. As stated in the method, Donovan et al. (1998), the number of *E. coli* in bivalves are calculated based on growth of blue-green colonies on chromogenic agar, *i.e.* presence of β-glucuronidase activity. Hence,

the results were not quite in line with Donovan et al. (1998) who performed the MPN method on 204 isolates, in which all were *E. coli*. However, the genera *Klebsiella*, *Citrobacter* and *Enterobacter*, have shown to display β-glucuronidase production (Hofstra and Veld, 1988; Tryland and Fiksdal, 1998). This may explain why false-positives were detected during verification on chromogenic agar (Pearez et al., 1986; Leung et al., 2001). Presence of false-positives may therefore cause an overestimation of the number of *E. coli* resulting in incorrect values. In contrast, more than 95% of *E. coli* strains are β-glucuronidase positive, hence there might be a possibility that the MPN values are underestimated due to potential false-negatives on TBX agar (Feng and Hartman, 1982). Either way, the detection of species other than *E. coli*, still represents important findings since these includes several important opportunistic pathogens associated with fecal contamination, such as *K. pneumoniae, Citrobacter braakii* and *Enterobacter cloacae* (Forsythe et al., 2015). A possible solution could be to revise the reference method to include other Enterobacteriaceae species in addition to *E. coli*.

4.2 Marine bivalves as tools for the monitoring of antibiotic resistance

To get a better understanding of the prevalence of antibiotic resistant *E. coli* and other Enterobacteriaceae species, the selected isolates (n=200) were subjected to disk diffusion by the method recommended by EUCAST (Paper II) (EUCAST, 2016a). The isolate belonging to the *Moraxellaceae* family was removed from further analysis. In addition, amoxicillin/clavulanic acid and colistin sulfate were not included in the results due to experimental errors.

Seventy-five (38%) of 199 bacterial isolates showed phenotypic resistance to at least one agent, while multidrug-resistance was seen in eight (4%) isolates (Fig. 10), *i.e.* resistance to $\geq$3 antibacterial classes according to the definition given by Magiorakos et al. (2012). Phenotypic resistance was observed against penicillins (31%), aminoglycosides (6%), trimethoprim (5%), sulfonamides (4%), tetracyclines (3%) and cephalosporins (3%), among others. No phenotypic resistance was observed towards piperacillin/tazobactam, imipenem or meropenem. Three isolates B142, B117 and B184 obtained from blue mussels (*M. edulis*) were resistant against seven or more

antibacterial agents. *E. coli* isolate B184 was resistant toward 15 antibacterial agents, belonging to six classes. Moreover, *E. coli* isolate B177 showed resistance to nine antibacterial agents belonging to five classes, while *K. oxytoca* isolate B142 displayed resistance to seven agents in four classes.

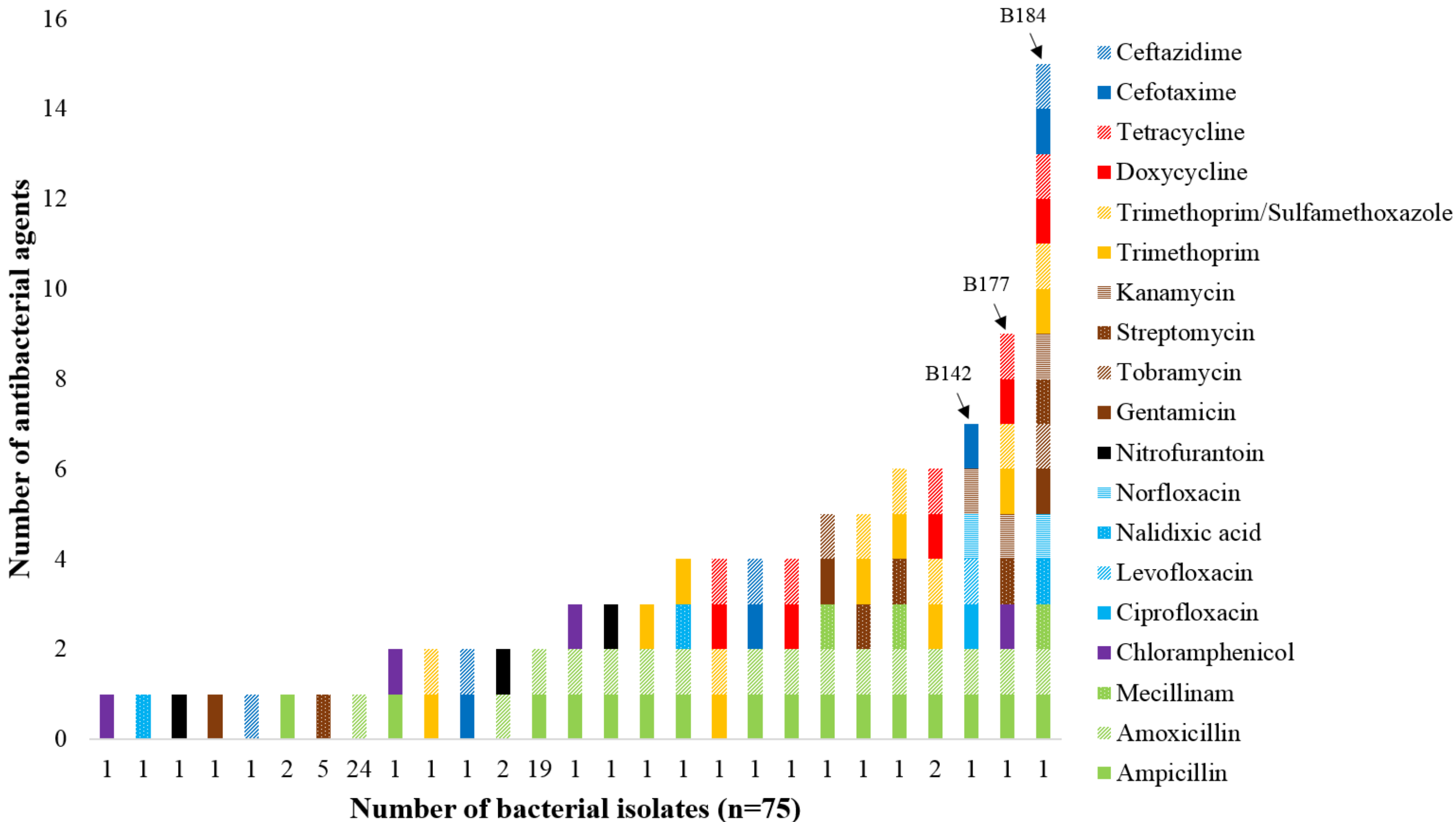

Figure 10. Number of *Escherichia coli* and other Enterobacteriaceae species showing phenotypic resistance towards antibacterial agents applied in accordance with the EUCAST, CLSI and ICMR clinical breakpoint tables. The three isolates B142, B117 and B184 (marked with arrows) conferred resistance against seven or more antibacterial agents.

Among the isolates (n=199) obtained from marine bivalves, resistance to extended-spectrum penicillins (31%) was observed. Thirteen isolates were identified within the genera *Klebsiella*, *Citrobacter* and *Enterobacter*, which are known to be intrinsic resistant to ampicillin and amoxicillin (Borenshtein and Schauer, 2006; Brisse et al., 2006; Grimont and Grimont, 2006). In Norway, the most commonly prescribed group of antibiotics in human and veterinary medicine (excluding farmed fish) are penicillins, including β-lactamase sensitive and extended-spectrum penicillins (NORM/NORM-VET, 2018). Suggesting that the high use of extended-spectrum penicillins in Norway may have been the reason for the prevalence of penicillin resistance observed in this study. A study performed by NORM/NORM-Vet (2016) reported that 4.2% of *E. coli* isolates obtained from bivalve mollusks (n=261) in Norway were resistant to at least one antibiotic, while prevalence of resistance to three antibiotics was 0.4%. Accordingly, phenotypic resistance was most frequently seen towards tetracycline (5.7%), ampicillin (4.6 %) and sulfamethoxazole (3.1%). In the present study, resistance to tetracycline, ampicillin and sulfamethoxazole was found in 3%, 11% and 4% of the *E. coli* isolates, respectively. Comparison between the results, however, is difficult due to methodological differences in classification and the application of epidemiological cut-off values instead of clinical breakpoints. Regardless, the results from both studies indicate that the prevalence of antibiotic resistant *E. coli* in the marine environment in Norway is low. In contrast, 33.3% of *E. coli* strains isolated from venus clams (*Chamelea gallina*) in Italy were resistant to at least one antibiotic, while multidrug-resistance were seen in 11% of the strains (Vignaroli et al., 2016). Among these, resistance to tetracycline, ampicillin and trimethoprim/sulfamethoxazole were 25.5%, 17% and 8.5%, respectively.

Based on resistance phenotypes, ten *E. coli* isolates were subjected to WGS (Table 4). Three isolates belonged to sequence type (ST) 69, ST95 and ST95, respectively, which have been associated with bloodstream infections (Adams-Sapper et al., 2012). The multidrug-resistant (MDR) *E. coli* isolate B184 belonging to ST38 is a prevalent clinical pathogen, predominantly associated with urinary tract infections (Chattaway et al., 2014). In Norway, clinical isolates of *E. coli* ST10, ST38, ST69 and ST95 have

previously been detected (Naseer et al., 2009; Naseer et al., 2010). Moreover, *E. coli* ST10, ST38 and ST69 have also been detected from recreational, wastewater and urine samples in Norway (Jørgensen et al., 2017). Based on molecular epidemiological analysis (Paper II), the ECOR strains of different phylogroups and *E. coli* strains causing blood stream infection were evenly distributed throughout the MST, together with both 30 isolates from bivalves and the enterohemorrhagic *E. coli* strains associated with hemorrhagic uremic syndrome. Suggesting that the *E. coli* isolates from the bivalves may have derived from humans through dissemination of contaminated sewage or from other sources. Accordingly, the detection of pathogenic STs (Table 4) further supports a human origin of the *E. coli* strains.

Table 4. Distribution of serotype, sequence type (ST) and antibiotic resistance genes (ARGs) among ten *Escherichia coli* isolates by whole-genome sequencing.

Isolate	Serotype	ST	ARGs*
B2	O8:H25	ST58	$bla_{\text{TEM-1B}}$, *strA-strB, dfrA5, sul2*
B53	No O type:H4	ST10	$bla_{\text{TEM-1B}}$
B117	O48:H20	ST191	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$
B158	O1:H7	ST95	$bla_{\text{TEM-1B}}$, *strA-strB, dfrA5, sul2*
B160	O8:H30	ST58	$bla_{\text{TEM-1B}}$, *qnrS1, tet(A)*
B161	O17/O44:H18	ST69	$bla_{\text{TEM-1B}}$, *aac(3)-IId*
B165	O1:H7	ST95	$bla_{\text{TEM-1C}}$, *strA-strB, dfrA14, sul2, tet(A)*
B167	O8:H17	ST88	$bla_{\text{TEM-1C}}$, *tet(A)*
B177	O89:H9	ST3572	$bla_{\text{TEM-1B}}$, *strA-strB, dfrA17, sul1, sul2, catA1, aadA5, aph(3')-Ia, tet(B)*
B184	O102:H6	ST38	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-14}}$, *strA-strB, dfrA17, sul1, sul2, catA1, aadA5, aac(3)-IId, tet(D), mph(A)*

***Genes**: **penicillins** ($bla_{\text{TEM-1}}$), **cephalosporins** ($bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$), **aminoglycosides** (*strA-strB, aadA5, aac(3)-IId, aph(3)-Ia*), **trimethoprim** (*dfrA5, dfrA14, dfrA17*), **sulfonamides** (*sul1, sul2*), **tetracyclines** (*tet(A), tet(B), tet(D)*), **amphenicols** (*catA1*), **quinolones** (*qnrS1*) and **macrolides** (*mphA*).

Multiple resistance genes were detected in the genome sequences of the ten *E. coli* isolates when performing WGS. Among the ten isolates examined, six transferred ARGs by conjugation (Paper II). All ten isolates harbored the bla_{TEM-1} gene, while isolate B117 and B184 carried the $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ genes, respectively. The TEM enzymes confer resistance to penicillins, while CTX-M enzymes confer resistance to penicillins and cephalosporins (Palzkill, 2018). These enzymes function by hydrolysis of the β-lactam ring causing inactivation of β-lactams (Pfeifer et al., 2010). A study has shown that among penicillin-resistant *E. coli* (n=13) isolated from bivalves along the Norwegian coast, four harbored bla_{TEM-1} (NORM/NORM-VET, 2016). In addition, soil samples from Norway have previously been shown to contain low levels of bla_{TEM-1} (Brusetti et al., 2008). Phenotypic resistance to 3rd gen. cephalosporins (*i.e.* cefotaxime and/or ceftazidime) was detected in five isolates (3%). Based on the WGS results and a conjugation assay, isolate B117 carried the $bla_{CTX-M-15}$ gene on a conjugative plasmid (Paper II). Although, the knowledge about the prevalence of bla_{CTX-M} in the Norwegian environment is limited, cephalosporin-resistant *E. coli* harboring $bla_{CTX-M-15}$ have been isolated from bivalves in Norway in another study (NORM/NORM-VET, 2016). CTX-M-carrying *E. coli* have also been detected from other niches in Norway, including healthy humans (Ulstad et al., 2016; Espenhain et al., 2018), wild red foxes (*Vulpes vulpes*) (Mo et al., 2018), water (Jørgensen et al., 2017) and wastewater (Paulshus et al., 2019b). Suggesting that humans and warm-blooded animals may act as sources of bla_{CTX-M}, which could disseminate into the environment. A Norwegian study revealed that ESBL-producing *E. coli* strains from recreational fresh- and saltwater were clonally related to strains isolated from human urine and wastewater (Jørgensen et al., 2017).

Fecal contamination plays an important part in the dissemination of ARGs in the environment (Karkman et al., 2019). Marine bivalves could be potential tools for monitoring of resistant *E. coli* as well as other Enterobacteriaceae species present in the marine environment at time of sampling (Paper I and II). Several studies have shown the presence of antibiotic resistant *E. coli* strains obtained from bivalves in other countries, such as Sweden (Bighiu et al., 2019), France (Balière et al., 2015), Italy

(Vignaroli et al., 2016) and Tunisia (Mani et al., 2018). This suggests that bivalves containing antibiotic resistant *E. coli* could represent a possible risk of transmission. This can occur through spillage of contaminated mantle water during handling of bivalves, or due to poor heat treatment of bivalves prior to consumption. However, most of the isolates in this study were obtained from blue mussels, which are usually heat treated for sufficient time before consumption. In contrast, contaminated bivalves intended for raw- or light preserved consumption, such as flat oysters (*O. edulis*) and the muscle of great scallops (*P. maximus*), could pose a possible risk of exposure to resistant *E. coli*. Infections with *E. coli*, particularly Shiga toxin-producing *E. coli* (STEC), are among the most frequent foodborne diseases worldwide (WHO, 2018). In Norway, the prevalence of STEC associated with marine bivalves has shown to be low (1.1%) (Martin et al., 2019). In addition to being important for food safety, monitoring of marine bivalves for the presence of resistant *E. coli* is also relevant for areas used for recreational activities (Leonard et al., 2018). Thus, our study highlights the importance for monitoring of bivalves for the presence of resistant *E. coli* strains in the marine environment.

4.3 The presence of CTX-M-producing *E. coli* strains

As defined by the WHO (2017c), the emergence of ESBL-producing Enterobacteriaceae are of great concern. Among these, plasmid-mediated class A β-lactamases, belonging to CTX-M type, are prevalent and globally disseminated in the clinics, especially in Europe (Canton et al., 2012; Bevan et al., 2017).

The MDR *E. coli* isolate B184 belongs to ST38 (Paper II), which is a pathogenic strain of clinical importance and associated with AR (Greig et al., 2018; Rafaque et al., 2018; Abril et al., 2019). Based on a comparative analysis (SNP-based) of isolate B184 with other strains of ST38 from various sources and countries, isolate B184 clustered closer to human isolates compared to ST38 isolates from animals (Paper III). Suggesting a human origin of isolate B184. In addition, isolate B184 revealed to harbor several virulence factors on the chromosome, including hemorrhagic *E. coli* pilus, invasive brain endothelial cells, hemolysin/cytolysin A, hemin uptake and yersiniabactin siderophore.

Complete genome sequence of isolate B184 (GenBank accession number: CP040263-CP040268) was assembled into a circular chromosome of 5.19 Mb and five plasmids, ranging from 98 kb to 5 kb. Despite the number of plasmids carried by this isolate, all the ARGs were located on the chromosome. The majority of ARGs were clustered together on the chromosome at two separate multidrug-resistance determining regions (MDR-regions), each flanked by IS*26* transposases (Fig. 11). MDR-region 1 (25.2 kb) harbored genes conferring resistance to penicillins, tunicamycin, aminoglycosides, macrolides, sulfonamides and trimethoprim (Fig. 11A). In addition, this region carried a gene conferring resistance to chromate (Aguilar-Barajas et al., 2008). The MDR-region 1 had two DNA fragments (17,687 bp and 3,094 bp, respectively) that were identical (>99.9%) to fragments of a conjugative IncFII plasmid pE2855-3 (92.7 kb) reported in *E. coli* (GenBank accession number: AP018799) (Fig. 11A). This suggest the possibility that the MDR-1 in isolate B184 may have been transferred from a plasmid onto the chromosome by transposition (Rubio-Cosials et al., 2018). MDR-region 2 (19.8 kb) carried genes conferring resistance to aminoglycosides, penicillins, amphenicols, tetracycline and sulfonamides (Fig. 11B). The MDR-region 2 had three DNA fragments (13,222 bp, 4,188 bp and 1,176 bp, respectively) that were identical (>99.9%) to fragments of a plasmid pKPN5 (88.6 kb) reported in *K. pneumoniae* (GenBank accession number: CP000650) (Fig. 11B). Four identical copies of *bla*$_{CTX-M-14}$ gene were detected outside these two regions, located on the chromosome at separate positions, each flanked by IS*5* and IS*Ec9* transposases. Interestingly, isolate B184 showed elevated MIC of 0.094 µg/ml for ertapenem during antibiotic susceptibility testing. This could partly be explained by the high copy numbers of the CTX-M-14 gene detected in isolate B184. *E. coli* ST38 carrying chromosomal CTX-M genes have previously been reported (Rodríguez et al., 2014; Greig et al., 2018). Although *bla*$_{CTX-M-14}$ was detected on the chromosome, the DNA fragment carrying *bla*$_{CTX-M-14}$ and the flanking IS*5* and IS*Ec9* transposases was identical (100%) to fragment of plasmids carried by different Enterobacteriaceae species, including *K. pneumoniae* (GenBank accession number: CP041102), *E. cloacae* (GenBank accession number: CP035635) and *Salmonella enterica* (GenBank accession number: MH522424), suggesting that *bla*$_{CTX-M-14}$ is mobile. Lastly, a chromosomal mutation in

the *gyrA* gene (S83L) was detected in isolate B184. This mutation is considered to have the strongest effect on quinolone resistance (Bagel et al., 1999).

Figure 11. Genomic map of multidrug-resistance determining regions (MDR-regions) in *Escherichia coli* isolate B184 (GenBank accession number: CP040263) (A) MDR-region 1 located on the chromosome between positions 1,184,422 bp - 1,209,571 bp, flanked by IS*26* transposase, encoding *bla*$_{TEM-1}$, *aac(3)-IId, tmrB, mph(A), mrx, sul1, qacE*Δ1, *aadA5* and *dfrA17*. (B) MDR-region 2 located on the chromosome between positions 4,406,649 bp - 4,426,421 bp, flanked by IS*26* transposase, encoding *aph(3'')-Ib, aph(6)-Id, sul2, tet(D), bla*$_{TEM-1}$ and *catA1*. Arrows indicate the size of the ORFs and their orientation in the genome. Antibiotic resistance genes are highlighted in red, transposases in blue, transcriptional regulators in green, heavy metal resistance genes in orange, and other genes are highlighted in dark grey, respectively. Δ represents truncated genes. Grey shaded regions represent >99.9% nucleotide identity.

Isolate B117, belonging to ST191, (GenBank accession number: CP040269-CP040271) had one circular chromosome of 4.73 Mb and two plasmids (91 kb and 4 kb, respectively). This isolate carried the *bla*$_{\text{CTX-M-15}}$ gene on the 91 kb IncI1 plasmid pEc1500_CTX (GenBank accession number: CP040270), as well as a *bla*$_{\text{TEM-1}}$ gene (Fig. 12). The *bla*$_{\text{CTX-M-15}}$ gene was flanked by a Tn*3* and an IS*Ec9* transposases. The IS*Ec9* transposase flanking the *bla*$_{\text{CTX-M-15}}$ gene in isolate B117 revealed to be identical (100%) to the IS*Ec9* transposase flanking *bla*$_{\text{CTX-M-14}}$ in isolate B184. Suggesting that the IS*Ec9* transposase play a role in dissemination of CTX-M type ESBLs. The pEc1500_CTX had high sequence identity (>99.9%) with two different CTX-M-carrying plasmids (91,109 bp and 93,732 bp, respectively) reported in *Shigella sonnei* (GenBank accession number: KJ406378) and *E. coli* (GenBank accession number: EU935740), respectively. Plasmid pEc1500_CTX have demonstrated to transfer *bla*$_{\text{CTX-M-15}}$ (Paper II), which highlights the potential for transfer of this plasmid from isolate B117 to other environmental bacteria.

Figure 12. Structure of plasmid pEc1500_CTX carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1}}$ gene (GenBank accession number: CP040270). Arrows indicate the size of the ORFs and their orientation in the genome. Antibiotic resistance genes are highlighted in red, transposases in blue, conjugal transfer proteins in green, replication initiation proteins in black, and other genes are highlighted in grey, respectively.

Our study highlights the importance of combining low error short-reads and long-reads for obtaining complete bacterial genome sequences, to understand the genomic structure and mobility of the ARGs. It demonstrates the presence of clinically important CTX-M-type ESBLs carried by two *E. coli* strains isolated from blue mussels (Paper II), representing a risk for further dissemination of such genes into the environment (Paper III). This is of particular concern in Norway which is a low prevalence country in terms of infections caused by ESBL-producing Enterobacteriaceae (NORM/NORM-VET, 2018). Although, ESBL-producing *E. coli* strains have shown to be present in hospitals (11.5%), community (6.9%) and urban wastewater (3.7%) in Norway (Paulshus et al., 2019a). In addition, strains of *E. coli* and *K. pneumoniae* carrying CTX-M-encoding plasmids have been reported from the clinics (Naseer et al., 2009; Löhr et al., 2015; Knudsen et al., 2018). In addition, isolates B184 and B117 harbored multiple heavy metal resistance genes, as well as several biocide resistance genes, indicating the potential for co-selection (Pal et al., 2017; Jutkina et al., 2018). Thus, we have provided a comprehensive account of the presence of clinically relevant mobile ARGs in two *E. coli* strains isolated from blue mussels from the marine environment in Norway (Paper III). Our study provides an approach to generate information on resistance genes and associated mobile DNA elements present in the marine environment in low prevalence settings.

4.4 Environmental bacteria with resistance to antibiotics and heavy metals

Sampling of marine bivalves were performed, during June, July and August 2017, from 18 sites along the Norwegian coast to assess the antibiotic and heavy metal resistance, as well as associated resistance genes, among environmental bacteria (Paper IV). A total of 26 samples were examined, comprised of 18 samples of blue mussels (*M. edulis*), two flat oysters (*O. edulis*), two great scallops (*P. maximus*), one soft-shell clams (*M. arenaria*), one pacific oyster (*C. gigas*), one common cockle (*C. edule*) and one sample of ocean quahog (*A. islandica*). Among the 25 bivalve samples examined by MPN method, 22 (88%) samples contained *E. coli* at different concentrations, ranging from 20 to 1,600,000 *E. coli* per 100 g sample material. One bivalve sample did not give any MPN results due to experimental errors. Moreover, the concentrations

of copper, zinc and cadmium were analyzed by ICP-MS in 25 individual blue mussels from 13 sites. The results were compared with provisional high reference concentrations (PROREF) of heavy metals (NIVA, 2016), and all samples were within the environmental status classification 1, *i.e.* no toxic levels or signs of contamination.

From non-selective agar plates, totally 59 bacterial isolates from 26 bivalve samples were collected and identified. Among the Gram-negative isolates, phenotypic resistance was observed against ampicillin (80%), cefotaxime (60%), nitrofurantoin (55%), trimethoprim (50%), kanamycine (48%) and chloramphenicol (45%). Only one Gram-positive isolate detected and was resistant to mecillinam, trimethoprim, ceftazidime and imipenem. Totally 146 isolates from agar plates with antibiotics, including 36 isolates were retrieved from MH with ampicillin, 49 from MH with ceftazidime, 33 from MH with ciprofloxacin, and 28 isolates from MH with imipenem. Among the Gram-negative isolates, resistance was observed against ampicillin (86%), cefotaxime (69%), trimethoprim (69%), nitrofurantoin (66%) and kanamycin (56%). Among the Gram-positive isolates, resistance was seen towards mecillinam (27%), cefotaxime (27%), trimethoprim (23%), nitrofurantoin (18%) and sulfonamide (13%).

The combined resistance to antibiotics and heavy metals among a selection of 195 bacterial isolates from both non-selective and selective methods are shown in Figure 13. Many of the environmental bacteria retrieved in this study are intrinsic resistant to a wide range of the antibiotics tested for, such as species belonging to the *Pseudomonas* spp. and *Stenotrophomonas* spp. (Breidenstein et al., 2011; Sánchez, 2015).

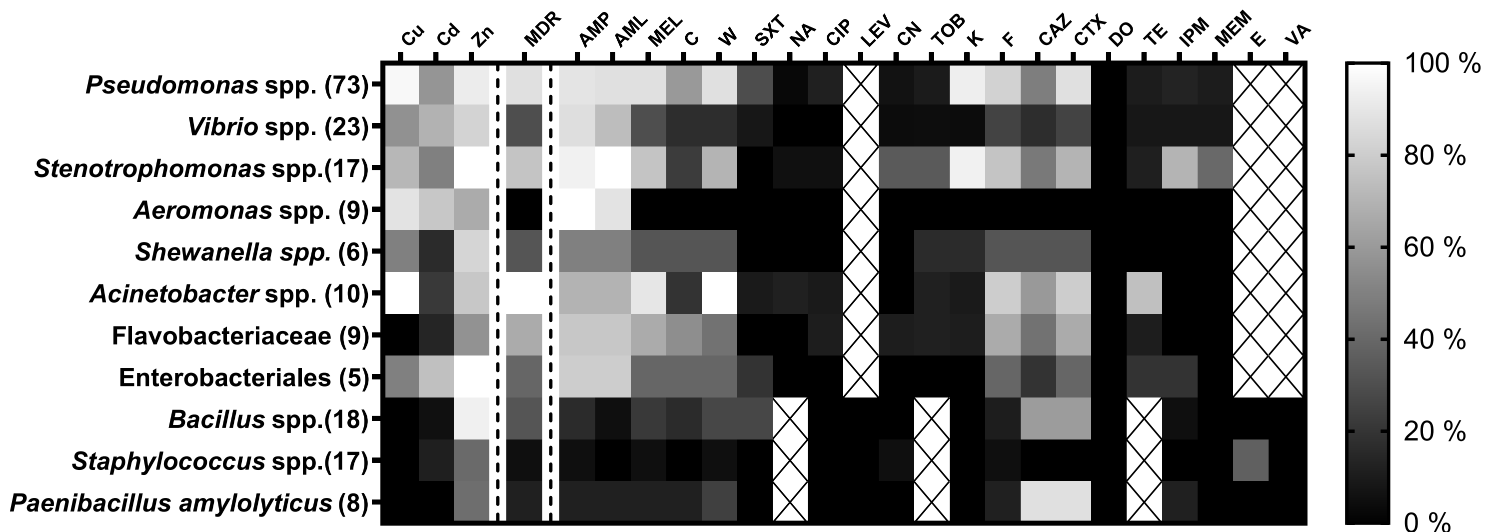

Figure 13. Heat map of the distribution of heavy metal resistance, multidrug-resistance (MDR) and antibiotic resistance among genus/species of bacteria isolated (n=195) from marine bivalves. Antibiotics not tested are marked as X. Abbreviations: Cu; Copper, Cd; Cadmium, Zn; Zinc, AMP; Ampicillin, AML; Amoxicillin, MEL; Mecillinam, C; Chloramphenicol, W; Trimethoprim, SXT; Trim./Sulfamethoxazole, NA; Nalidixic acid, CIP; Ciprofloxacin, LEV; Levofloxacin, CN; Gentamicin, TOB; Tobramycin, K; Kanamycin, F; Nitrofurantoin, CAZ; Ceftazidime, CTX; Cefotaxime, DO; Doxycycline, TET; Tetracycline, IPM; Imipenem, MEM; Meropenem, E; Erythromycin, VA; Vancomycin.

Among the *Pseudomonas* spp., phenotypic resistance was frequently observed towards kanamycine (93%), ampicillin (89%), amoxicillin (88%), mecillinam (88%), cephalosporins (88%), trimethoprim (88%) and nitrofurans (82%) (Fig. 13). No resistance was detected against levofloxacin or doxycycline. Most of the isolates showed high tolerance for copper (97%), zinc (93%) and cadmium (58%). Based on the WGS results of 27 isolates (Table 5), all *Pseudomonas* isolates harbored multiple ARGs, including genes belonging to the efflux transporter families MATE, MFS, SMR and RND, providing resistance to a wide range of antibiotics (Delmar et al., 2014; Greene et al., 2018). The *ampC* gene providing resistance to cephalosporins were present in all isolates, while the macrolide efflux pump *macA/macB* gene was found in eight isolates. Four isolates harbored the *aph* gene conferring resistance to aminoglycosides, while two isolates carried the *catB*-related gene encoding resistance to chloramphenicol. In addition, the *Pseudomonas* isolates harbored genes conferring heavy metal resistance and these genes have been shown to be linked to the tolerance of heavy metals in this genus (Pitondo-Silva et al., 2016). Among the *Vibrio* isolates resistance was mostly seen towards ampicillin (87%). No resistance was seen to nalidixic acid, ciprofloxacin and doxycycline. High tolerance to zinc (83%), cadmium (70%) and copper (57%) were detected. All *Vibrio* spp. subjected to WGS carried the multidrug transporter gene *emrD* (Table 5), and *tet(34)* gene conferring resistance to tetracycline. Six *V. anguillarum* isolates carried the *varG* gene encoding resistance against β-lactams (Lin et al., 2017). Four isolates harbored *catB* or *catB*-related genes, while two isolates carried the *aph* gene. One isolate carried the *qnrVC* gene conferring resistance to quinolones. Several heavy metal resistance genes, including *cusA/cusB* and *czcA*, were observed among the *Vibrio* isolates. In the bacterial species belonging to the *Stenotrophomonas* spp. resistance was frequently seen to amoxicillin (100%), ampicillin (89%) and kanamycin (94%). No resistance was observed towards doxycycline and sulfonamide. Most of the isolates showed high tolerance for zinc (100%), copper (71%) and cadmium (50%). Based on the WGS results (Table 5), all *Stenotrophomonas* isolates harbored bla_{L1} and bla_{L2} genes. Several efflux-encoding genes, including MATE and RND, were detected in the isolates and explains the high prevalence of resistance observed (Sánchez, 2015). The *aph* gene was present in all

isolates, while the *qnr* gene was detected in the six *S. maltophilia* isolates. The *S. rhizophilia* isolates carried a $bla_{\text{SUBCLASS B3}}$ gene. The heavy metal resistance genes *copB* and *cuzA/czcA* were detected in all isolates. Among the *Acinetobacter* spp. resistance was frequently seen towards trimethoprim (100%), mecillinam (90%), cefotaxime (80%) and nitrofurantoin (80%). No resistance was observed for gentamicin, doxycycline, imipenem and meropenem. High tolerance to heavy metals were seen for copper (100%) and zinc (78%), and low tolerance was seen for cadmium (22%). Based on WGS results (Table 5), the bla_{OXA}, *macA/macB* and *catB*-related genes were detected in all isolates, as well as genes conferring efflux transporter families RND, MATE and MFS, providing resistance to multiple antibiotics. Two isolate carried the *ampC* and *aph(3')* genes, respectively. All isolates harbored genes encoding heavy metal resistance.

Table 5. Distribution of antibiotic resistance genes (ARGs) and heavy metal resistance genes (HMRGs) among the examined bacterial isolates by whole-genome sequencing.

Isolate	Species	ARGs	HMRGs
1152/12	*Pseudomonas brassicacearum*	*ampC, bla*$_{OXA}$, *catB*-related, *fos*, *bcr/cflA, adeC/adeK/oprM, macA/macB*, *mdtA/muxA*, *mdtB/muxB, norM,* MATE, MFS, SMR, RND	*cusA/czcA*
0784/11	*P. fluorescens*	*ampC*, *aph*, *vanW*, *fos*, *macA/macB*, *mdtA/muxA*, *norM,* MATE, PACE, MFS, SMR, RND	*copA, copB, cusA/czcA, cueA*
1121/15	*P. koreensis*	*ampC*, *fos*, *vanW*, *bcr/cflA, macA/macB*, *mdtA/muxA*, *norM,* MATE, PACE, MFS (*emrBsm*), *smrA*, RND	*copA, copB, cusA/czcA*
1122/18	*P. koreensis*	*ampC*, *aph*, *fos*, *vanW, macA/macB*, *mdtA/muxA*, *norM*, MATE, PACE, MFS, SMR, RND	*copA, copB, cusA/czcA, cueA*
1208/15	*P. koreensis*	*ampC*, *aph*, *fos, vanW*, *macA/macB*, *mdtA/muxA*, *norM*, MATE, PACE, MFS, SMR, RND	*copA, cusA/czcA, cueA*
1208/03	*Pseudomonas* spp.	*ampC*, *fos*, *vanW*, *macA/macB*, *mdtA/muxA*, *norM*, MATE, PACE, MFS, SMR, RND	*copA, copB, cusA/czcA, cueA*
0910/21	*Pseudomonas* spp.	*ampC*, *aph(3')*, *catB*-related, *vanW*, *bcr/cflA, adeC/adeK/oprM, macA/macB*, *mdtA/muxA, mdtB/muxB, norM*, MATE, PACE, SMR, MFS, RND	*cusA/czcA*
0910/23	*Pseudomonas* spp.	*ampC*, *fos, adeC/adeK/oprM, bcr/cflA*, *macA/macB*, *mdtA/muxA*, *mdtB/muxB*, *norM*, MATE, SMR, MFS, RND	*cusA/czcA*
1121/17	*Pseudomonas* spp.	*ampC*, *adeC/adeK/oprM, macB*, *mdtA/muxA*, *mdtB/muxB*, *norM*, MATE (*mepB/mepC*), PACE, SMR, MFS, RND (*ttgB, ttgC*)	*copB, cusA/czcA*
1180/03	*Vibrio aestuarianus*	*tet(34), catB, aph, bcr/clfA, emrD*	
1151/11	*V. anguillarum*	*tet(34), qnrVC, varG, bcr/clfA, emrD*	*cusA, czcA, czcC, czcD*
1218/11B	*V. anguillarum*	*tet(34), varG, aph, catB*-related, *bcr/clfA, emrD*	*cusA, czcA, czcD*
1219/08	*V. anguillarum*	*tet(34), varG, bcr/clfA, bcr/clfA, emrD*	*cusA, czcA, czcC*
1219/11	*V. anguillarum*	*tet(34), varG, catB*-related, *bcr/clfA, emrD*	-
1219/16	*V. anguillarum*	*tet(34), varG, bcr/clfA, emrD*	*cusA, czcA, czcD*
1219/23B	*V. anguillarum*	*tet(34), varG, catB, bcr/clfA, emrD*	*cusA, czcA, czcC*

Table 5. Continued.

Isolate	Species	ARGs	HMRGs
0860/19	*Stenotrophomonas maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-IIc, aph(6), qnr, cmlA/floR, bcr/cflA, macB, smrA, emrABCR, mexH, smeDEF*, MATE, RND	*copA, copB, cusA/czcA*
0860/20	*S. maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-IIc, aph(6), qnr, cmlA/floR, bcr/cflA, macB,, smrA, emrABCR, mexH, smeDEF,* MATE, RND	*copA, copB, cusA/czcA*
0861/20	*S. maltophilia*	bla_{L1}, bla_{L2}, *aac(6')-Iz, aph(3')-II, aph(6), qnr, macB, smrA, emrABCR, mexH, smeDEF*, MATE, RND	*copA, copB, cusA/czcA*
1125/12	*S. maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-II, aph(6), qnr, macB, smrA, emrABCR, smeDEF, mdtA/muxA,* MATE, RND	*copB, cusA/czcA*
1125/20	*S. maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-II, aph(6), cmlA/floR, bcr/cflA, macB, smrA, emrABCR, smeDEF, mdtA/muxA*, MATE, RND	*copB, cusA/czcA*
1151/23	*S. maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-II, aph(6), qnr, macB, smrA, emrABCR, smeDEF, mdtA/muxA*, MATE, RND	*copB, cusA/czcA*
1180/23B	*S. maltophilia*	bla_{L1}, bla_{L2}, *aph(3')-IIc, aph(6), qnr, macB, smrA, emrABCR, mexH, smeDEF*, MATE, RND	*copA, copB, cusA/czcA*
1121/13	*S. rhizophila*	bla_{L1}, bla_{L2}, $bla_{subclass\ B3}$, *aph, macB, smrA, emrA, smeDEF, mdtA/muxA,* MATE, RND, PACE	*copB, cusA/czcA*
1124/18	*Acinetobacter calcoaceticus*	$bla_{ADC\text{-}129}$, $bla_{OXA\text{-}213}$, *catB*-related, *macA/macB, emrA/emrK, adeIJK*, RND, MATE, MFS	*copB, cusA/czcA, nlpE*
1125/18A	*A. guillouiae*	$bla_{OXA\text{-}274}$, *aph(3')-Vib, catB*-related, *vanW*-like, *macA/macB*, *adeIJK*, TCR/Tet, RND, MATE, MFS	*copB, cusA/czcA, nlpE*
1207/04	*Acinetobacter* sp.	*ampC*, $bla_{Class\ A}$, bla_{MCD}, bla_{OXA}, *catB*-related, *macA/macB, cusA/czcA*, RND, MATE, MFS, SMR	*copB, cusA/czcA, nlpE*

Clinically relevant ARGs associated with pathogenic *E. coli* strains can be introduced into the marine environment (Paper II & III), thereby interact with the environmental bacteria providing opportunities for acquisition of ARGs via horizontal transfer (Aminov, 2011; Martinez, 2012). Hence, opportunistic pathogens thriving in soil and water, such as *Stenotrophomonas* or *Pseudomonas* spp., may function as intermediate that could transfer ARGs back into human-associated bacteria (Berg et al., 2005).

Many of the bacterial species examined belonged to the genera *Pseudomonas* (36%), *Vibrio* (11%) and *Stenotrophomonas* (8%). Species belonging to the *Pseudomonas* spp. and *Stenotrophomonas* spp. are intrinsic resistant to a wide range of antibiotics (Breidenstein et al., 2011; Sánchez, 2015), and not surprisingly high rate of resistance was observed. Hence, the large degree of intrinsic resistance among several of the environmental bacteria isolated makes it difficult to interpret the data (EUCAST, 2016c).

However, clinically relevant ARGs towards aminoglycosides (*aph(3'))*, phenicols (*catB*) and/or fluoroquinolones (*qnrVC*), were detected. The *S. rhizophilia* isolates carried a new variant of the $bla_{\text{SUBCLASS B3}}$ gene that are highly similar (86.27% nucleotide identity) to a $bla_{\text{SUBCLASS B3}}$ gene reported in a *Stenotrophomonas* sp. LM091 (GenBank accession number: WP_070426224). Interestingly, increased phenotypic resistance were seen for *Vibrio* spp. isolated from bivalves from high vs. low exposure to fecal contamination. One *Vibrio* isolate detected harbored a *qnrVC* gene (Zhang et al., 2018). The *qnrVC* gene was highly similar (>99.9% nucleotide identity) to a plasmid-borne *qnrVC6* reported in *V. parahaemolyticus* (GenBank accession number: AGH08253.1), suggesting that the *qnrVC* gene in *V. anguillarum* is mobile.

This study has described the antibiotic and heavy metal resistance patterns, and associated resistance genes, among several environmental bacteria isolated from marine bivalves in Norway. Most of the environmental bacteria carried multiple genes belonging to the efflux transporter families (Table 5), and hence were intrinsic resistant to several antibiotics. The presence of clinically relevant ARGs were detected, however the genomic map and associated DNA elements should be investigated further.

5. Conclusion

Environmental surveillance of AR can contribute towards better understanding and management of human and ecosystem health. A major cause for the spread of clinically relevant ARGs and pathogens is the partial lack of proper infrastructure for wastewater treatment and uncontrolled discharge of untreated urban waste. The results of the work performed in this thesis, suggests that marine bivalves represent an important tool for monitoring antibiotic resistant *E. coli* and other Enterobacteriaceae present in the marine environment. This study also highlights the presence of clinically important CTX-M-type ESBLs in the environment in Norway, with the potential for further dissemination. Moreover, clinically relevant ARGs, such as *qnrVC, aph(3')* and/or *catB*-related, were detected in environmental bacteria isolated from marine bivalves. Thus, highlighting the importance for surveillance of clinically relevant ARGs in the environment, especially in a low prevalence setting like Norway. In addition, several heavy metal resistance genes were detected, suggesting the possibility for co-selection of ARGs in the absence of antibiotic exposure. The presence of *E. coli* in bivalves are indicators for fecal contamination, concomitantly containing both clinically relevant ARGs and pathogens. Dissemination of resistant *E. coli* strains into the marine environment represents a possible health concern, especially in areas used for marine food production or recreational activities. Thus, the results presented in this study strengthens the notion that the marine environment plays an important role in the dissemination of clinically important ARGs and pathogens (Bengtsson-Palme et al., 2018). It also emphasizes the need for environmental surveillance of AR in countries with low burden of resistance, in order to be better prepared for managing future challenges.

6. Future perspective

By this thesis, we have demonstrated that marine bivalves can function as tools for assessing antibiotic resistant *E. coli* present in the marine environment. However, only qualitative detection of antibiotic resistant *E. coli* isolates was performed. Future monitoring of bivalve mollusks should include quantitative detection of resistant *E. coli* and other bacteria derived from fecal contamination, such as *Klebsiella* spp. *Enterobacter* spp., *Citrobacter* spp. and *Enterococcus* spp. These genera includes several important human pathogens and are included in the priority list published by the WHO (2017c). Future samples should also include bivalves from areas with known high and low influence of anthropogenic activity, such as bivalves from densely populated and from more pristine areas. Not only would this provide knowledge about the differences in the prevalence of AR, but also provide a rough overview of fecal carriage of AR in humans and/or warm-blooded animals in Norway. Moreover, interactions between enteric and environmental bacteria should be assessed, since fecal contamination has been shown to contribute to the abundance of ARGs in the environment (Karkman et al., 2019). This would be of interest since environmental bacteria, like *Pseudomonas* spp. or *Vibrio* spp., could function as intermediates for ARGs, thereby contributing to the persistence of such genes in the environment. In addition, the resistome and virulome of the biota of bivalves should be examined to provide knowledge regarding the presence, as well as the abundance, of resistance and virulence genes in the environment. Another important aspect should be to determine if bivalves could be used exclusively for surveillance of AR in the environment. This includes obtaining samples of water and sediment, as well as bivalves, from the same location (at the same time), to evaluate if bivalves are sufficient enough for monitoring of ARGs and pathogens present in the marine environment. Interestingly, a Swedish study performed by Bighiu et al. (2019) included both bivalves and water samples, and detected higher concentrations of fecal bacteria, as well as AR, in the bivalve. Suggesting that bivalves represents good tools for monitoring of fecal-derived bacteria and associated AR present in the surrounding environment.

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The species accuracy of the Most Probable Number (MPN) European Union reference method for enumeration of *Escherichia coli* in marine bivalves

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ABSTRACT

Continuous European Union programmes with specified methods for enumeration of *Escherichia coli* in bivalves for human consumption are currently running. The objective of this research was to examine the species accuracy of the five times three tube Most Probable Number (MPN) EU reference method used for detection of *E. coli* in marine bivalves. Among 549 samples of bivalves harvested from Norwegian localities during 2014 and 2015, a total number of 200 bacterial isolates were prepared from randomly selected culture-positive bivalves. These presumptive *E. coli* isolates were characterized biochemically by the Analytical Profile Index (API) 20E, as well as by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The majority of isolates (90%) were identified as *E. coli*, by both API 20E and MALDI-TOF MS. Ten isolates (5%) were identified as *Klebsiella pneumoniae*, while one isolate was identified as *K. oxytoca* by both methods, whereas three isolates were identified as *Acinetobacter baumannii*, *Citrobacter braakii*, and *Enterobacter cloacae*, respectively. The identification of the remaining six isolates were not in compliance between the two methods.

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1. Introduction

Bivalve molluscs, including blue mussels (*Mytilus edulis*), great scallops (*Pecten maximus*), flat oysters (*Ostrea edulis*), horse mussels (*Modiolus modiolus*), and carpet shells (*Mya arenaria*), are suspension feeders and actively filter and retain particles from their surrounding water, including free living or particle bound bacteria. Bivalves are thereby excellent bio-samplers that reflects the load of *Escherichia coli* and other microorganisms of faecal origin, such as enteric viruses (e.g., Norovirus), in the water column at a given location (Lunestad et al., 2016). These microorganisms may originate from humans and other homeothermic animals either via sewage, by runoff from land, or from the wild fauna. *E. coli* is a well-established indicator of faecal contamination, and its absence in food products indicates a manufacturing process under appropriate sanitary conditions (Baylis et al., 2011; Buttiaux and Mossel, 1961).

According to EU Directive 854/2004/EC (2004), national food safety authorities, in this case the Norwegian Food Safety Authority (NFSA), has the responsibility of monitoring and classifying production areas for bivalve molluscs (NFSA, 2013). The production areas are classified as A, B, C or prohibited areas depending on the content of *E. coli* in the soft parts and mantle water of harvested bivalves. A Class A area have an upper limit of 230 *E. coli*/100 g sample material measured as fresh weight, and such bivalves may go directly for human consumption. A Class B area has an upper limit of 4,600 *E. coli*/100 g, whereas a class C area has an upper limit of 46,000 *E. coli*/100 g. Bivalves from B and C area must be purified by resuspension at Class A area until meeting the limit of 230 *E. coli*/100 g or heat treated. Areas with samples exceeding the upper limit of a Class C area are prohibited for harvesting. According to 2015/2285 (2015) concerning bivalve product to be placed on the market, 20% of the samples may contain *E. coli* between 230 and 700/100 g sample material, while the remaining 80% of the samples must be below 230/100 g sample material.

The quantitative method for detection and enumeration of *E. coli* in bivalve molluscs are specified in EU Council Directive 91/492/EEC (1991). This method is based on a Most Probable Number (MPN) principal (Oblinger and Koburger, 1975) with five tubes, each in three dilutions. The MPN principal is based on the number of positive tubes at increasing dilutions of a sample, and further calculations are necessary to convert the results into a MPN value, with a probable range. This MPN technique is commonly used in combination with verification on chromogenic agar to calculate the number of *E. coli* in bivalves (Donovan et al., 1998). The applied MPN method utilise Minerals Modified Glutamate Broth (MMGB) as growth medium, and material from positive tubes, i.e., tubes with colour change from acid production, are confirmed on Tryptone Bile with X-glucuronide (TBX) agar for the determination of β-glucuronidase production, a common feature of *E. coli* (Donovan et al., 1998). According to the EU method, bacterial growth with colour change in MMGB and presence of β-glucuronidase

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production is considered to be *E. coli*. It is known that other members of the Enterobacteriaceae may possess the β-glucuronidase enzyme and it could be assumed/suspected that they may also give false-positive blue-green colonies on the TBX agar. To examine the species accuracy of the standardised EU MPN method, we performed further characterisation of the presumptive *E. coli* isolates by both the Analytical Profile Index (API) 20E test kit and a Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bourassa and Butler-Wu, 2015). The API 20E test kit is designed to identify members of the Enterobacteriaceae family and to differentiate between closely related, and morphologically similar, bacterial strains based on enzymatic degradation of carbohydrates, amino acids and some other reactions in 20 miniature wells, resulting in a biochemical profile specific for each species. In MALDI-TOF MS small molecules from lyophilized bacteria gives distinct spectra allowing identification into genus and specie levels. A target plate with pre-treated samples is exposed to a nitrogen laser applying short pulses of high-energy, causing desorption and ionization of each sample (Bourassa and Butler-Wu, 2015). To identify a particular bacterium a characteristic Peptide Mass Fingerprint (PMF) pattern of highly abundant peptides derived from ribosomal proteins are matched, which ionize readily and represent about 60–70% of the dry weight of a bacterial cell (Singhal et al., 2015). During PMF matching, the spectra of known bacterial species included in the database of reference spectra (MALDI Biotyper Library) are compared with the spectra of the unknown bacterial isolate (Bourassa and Butler-Wu, 2015; Singhal et al., 2015).

The objective of this research was to examine the species accuracy of the five times three tube MPN EU reference method used for detection of *E. coli* in marine bivalves assessed by API 20E and MALDI-TOF MS.

2. Materials and methods

2.1. Samples

From October 2014 to November 2015, a total of 549 samples were collected and examined, comprising 447 samples of blue mussels (*M. edulis*), 40 samples of flat oysters (*O. edulis*), 39 samples of great scallops (*P. maximus*), 12 samples of carpet shells (*M. arenaria*), and 11 samples of horse mussels (*M. modiolus*). The samples were collected at rearing localities along the coast of Norway (Fig. 1), and transported to the laboratory under chilled conditions close to 4 °C. The microbiological analyses were initiated within 24 h of sampling.

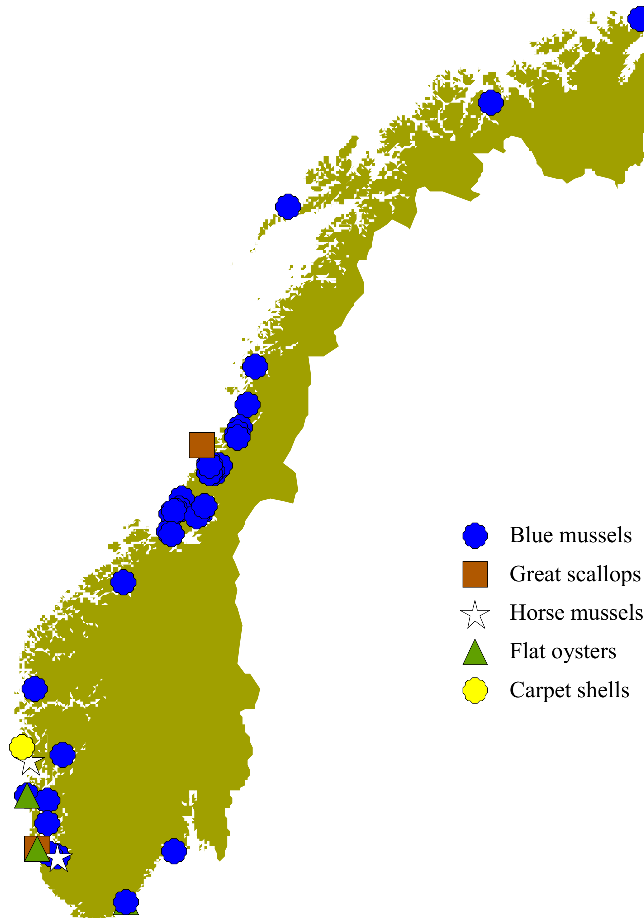

Fig. 1. Sampling sites of bivalve molluscs along the Norwegian coast in the period from 2014 to 2015.

2.2. MPN method for enumeration of E. coli

The 549 bivalve samples were examined applying the MPN method for enumeration of *E. coli* in accordance with the EU reference method ISO 16649-3 (ISO, 2005). Ten individuals, or more if necessary, were selected to obtain 50 g sample material. The flesh and intravalvular liquid were collected in a sterile stomacher bag with 100 ml of Peptone water (BioMérieux, France) and homogenised using a Stomacher for 2.5 min. The homogenate was added additionally 350 ml of Peptone water (BioMérieux, France) resulting in a final 1:10 dilution. Aliquots from this 1:10 homogenate was transferred to tubes with MMGB (Oxoid, UK). An effective 1:1 dilution of the sample material was obtained by transferring 10 ml of the homogenate into five tubes with 10 ml of MMGB with double strength, resulting in tubes containing 1 g of the original sample material. Furthermore, aliquots of 1.0 ml and 0.1 ml of the 1:10 homogenate were transferred to five tubes each with 10 ml single strength MMGB, resulting in tubes containing 0.1 g and tubes 0.01 g sample material, respectively. The tubes were incubated aerobically at 37 ± 1 °C for 24 ± 2 h. The MMGB tubes that changed colour from purple to yellow, were assumed positive, and material from these tubes were plated on chromogenic TBX agar (Oxoid, UK) to confirm β-glucuronidase activity (Donovan et al., 1998). Each TBX plate were subdivided into five sections and 10 μl from positive MMGB tubes were inoculated by a loop, and incubated aerobically at 44 ± 1 °C for 22 ± 2 h. The growth of blue-green colonies were recognised as presumptive *E. coli* (Donovan et al., 1998). By counting the number of positive tubes that gave growth of blue-green colonies on TBX agar, the level of *E. coli*/100 g was estimated using the MPN table (Donovan et al., 1998). From 200 selected positive bivalve samples (n = 335), one single colony was isolated and cultivated on standard Plate count agar (Oxoid, UK) for further examination. The selection of samples to be included were done on a random basis by laboratory personnel not involved in further analysis. *E. coli* strain CCUG 17620 was used as positive control for MMGB and for TBX agar. Negative control strains included *Pseudomonas aeruginosa* CCUG 22801 for MMGB and *Klebsiella pneumoniae* CCUG 10785 for TBX agar.

2.3. API 20E

The bacterial isolates were Gram tested (Buck, 1982) and examined for oxidase production on BBL™ DrySlide™ Oxidase (BD, USA). The isolates were biochemically characterized by the API 20E test kit as described in the instructions from the supplier (BioMérieux, France). Positive and negative reactions in the different wells were noted, resulting in a seven-digit code, which was interpreted with a numerical identification database (APIweb™). The identification strength (% ID and T-value) of each bacterium was noted.

2.4. MALDI-TOF MS

To obtain a fresh bacterial culture the isolates were cultivated on Columbia blood agar with 5% horse blood (Oxoid, UK) incubated aerobically at 35 ± 1 °C for 18 ± 4 h. A sterile loop was used to transfer some bacterial material from an isolated colony onto two spots on a target plate, making a thin layer. The target plate was air dried at room temperature for 2–3 min. To improve lysis of mucoid bacteria, 1 μl of 70% formic acid was added prior to applying the Matrix (Bourassa and Butler-Wu, 2015; Singhal et al., 2015). The bacterial isolates were prepared by coating with α-cyano-4-hydroxycinnamic acid (HCCA), an energy-absorbent, organic matrix solution composed of soluble acid molecules (Bourassa and Butler-Wu, 2015; Singhal et al., 2015). Each spot was added 1 μl of Matrix HCCA (Bruker, Germany) that specifically absorbed the laser beam (Könönen et al., 2015), and the Matrix had to evaporate before conducting the analysis. The results were interpreted and registered on the MALDI Biotyper RTC and FlexControl. The programs provided an overview of the results as best match of the bacterial isolates. The MALDI-TOF MS instrument is weekly examined with a control bacterium (*E. coli* ATCC 25922). The ID must match and the score value have to be higher than 1.7. In addition, a monthly monitoring of the MALDI-TOF MS instrument is performed by a technician, on behalf of the company producing the instrument, with a defined bacterial test standard.

3. Results

3.1. Detection of E. coli

Among the 549 bivalve molluscs examined, 479 (87%) had presumptive *E. coli* (i.e., β-glucuronidase-positive) levels within the limits of a Class A area, and 70 (13%) had levels within a Class B area. The 200 selected bivalve samples positive for presumptive *E. coli*, originating from both Class A and B areas, comprised 177 (88.5%) blue mussels (*M. edulis*), ten (5%) flat oysters (*O. edulis*), seven (3.5%) great scallops (*P. maximus*), three (1.5%) carpet shells (*M. arenaria*), and three (1.5%) samples of horse mussels (*M. modiolus*).

3.2. Identification of the presumptive E. coli isolates

All presumptive *E. coli* isolates (n = 200) were Gram- and oxidase-negative. Of the isolates, 191 (95.5%) were identified using API 20E with an identification value above 80% and a T-value above 0.5, in which 175 isolates were identified as *E. coli*. When examined by the MALDI-TOF MS method, the presumptive *E. coli* isolates were identified within the four genera *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*, all within the Enterobacteriaceae family. One isolate were identified to the genus *Acinetobacter* within the Moraxellaceae family. When applying MALDI-TOF MS, all bacterial isolates had a score value above 2.0, in which 151 isolates (75.5%) conferred 'highly probable species identification' and 49 isolates (24.5%) conferred 'secure genus and probable species identification'. Totally 180 (90%) of the bacterial isolates were identified as *E. coli*, ten of the isolates were identified as *Klebsiella pneumoniae*, while one isolate was identified as *K. oxytoca*. Three isolates were identified as *Acinetobacter baumannii*, *Citrobacter braakii*, and *Enterobacter cloacae*, respectively. Totally 194 isolates (97%) were identified to the same species by API 20E and MALDI-TOF MS. The remaining six isolates were identified differently on API 20E as compared to MALDI-TOF MS (Table 1). Four of the six isolates were identified as 1) *K. pneumoniae* and *E. coli*, 2) *K. oxytoca* and *E. coli*, 3) *R. ornithinolytica* and *K. pneumoniae*, and, 4) *E. hermannii* and *E. coli*, on API 20E and MALDI-TOF MS, respectively. Two isolates had inconclusive results by API 20E, but were identified as *E. coli* by MALDI-TOF MS.

4. Discussion

Continuous programmes for enumeration of *E. coli* in bivalve molluscs for human consumption are currently running in the European Union (EU). The objective of this research was to examine the species accuracy of the five times three tube MPN EU reference method used for detection of *E. coli* in marine bivalves assessed by API 20E and MALDI-TOF MS. *E. coli* are found in stable concentrations in faeces of humans (between 10^6 and 10^7 cells g^{-1}) (Forsythe, 2010) and other homeothermic animals, including birds and marine mammals, and some strains are reported to survive from a few days to several months in the marine environment (Fremaux et al., 2010; Labelle et al., 1980; Young-Joo et al., 2002). The examination for *E. coli* is commonly applied to provide evidence of poor hygiene, inadequate processing or post-process contamination (Baylis et al., 2011). Marine bivalves may become contaminated by faecal bacteria transported to the marine environment through an overloaded sewage systems or through runoffs from land during periods with heavy rainfall (Lunestad et al., 2016). Alternatively, the rearing localities could be influenced by a sewage efflux point. Flat oysters (*O. edulis*) and the muscle of great scallops (*P. maximus*)

Table 1
Presumptive *E. coli* isolates from the MPN method where API 20E and MALDI-TOF MS either doubted or rejected them as being *E. coli*. The API 20E identifications are presented in % match to the suggested species. The T-value indicates the proximity (reliability) of the results. The MALDI-TOF MS Score value ≥ 2.300 indicates 'highly probable species identification' and 2.000–2.299 indicates a 'secure genus and probable species identification'.

	API 20E			MALDI-TOF MS	
Isolate no.	Bacterium	Identification (%)	T-value	Bacterium	Score value (log)
B69	*Klebsiella pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.364
B70	*K. pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.509
B102	*K. pneumoniae*	82.60% (++)	0.75	*K. pneumoniae*	2.511
B105	*K. pneumoniae*	97.30% (+++)	1	*K. pneumoniae*	2.508
B152	*K. pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.466
B162	*K. pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.325
B183	*K. pneumoniae*	81.80% (+++)	1	*K. pneumoniae*	2.538
B189	*K. pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.352
B192	*K. pneumoniae*	81.80% (+)	1	*K. pneumoniae*	2.457
B194	*K. pneumoniae*	98.00% (+++)	0.8	*K. pneumoniae*	2.521
B142	*Klebsiella oxytoca*	94.10% (+)	0.41	*K. oxytoca*	2.391
B174	*Acinetobacter baumannii*	92.80% (+)	0.79	*A. baumannii*	2.159
B80	*Citrobacter braakii*	87.60% (++)	0.43	*C. braakii*	2.354
B138	*Enterobacter cloacae*	98.80% (+++)	0.72	*E. cloacae*	2.438
B12	*K. pneumoniae*	93.00% (+++)	0.75	*E. coli*	2.327
B20	*K. oxytoca*	92.30% (+++)	0.48	*E. coli*	2.257
B47	*Raoultella ornithinolytica*	92.00% (+++)	0.88	*K. pneumoniae*	2.344
B172	*Escherichia hermannii*	80.40% (+++)	0.64	*E. coli*	2.378
B96	*Serratia odorifera*	– (+)	–	*E. coli*	2.49
B181	*E. coli*	– (+)	–	*E. coli*	2.168

(+++) indicates good identification. (++) indicates acceptable, while (+) indicates doubtful identification or if the bacterial isolate had unacceptable profile. "–" indicates that no value was obtained.

represent an increased risk for transmitting faecal microorganisms to the consumers, as they are commonly consumed raw.

According to the EU Council Directive (91/492/EEC, 1991), a MPN method with further verification on chromogenic agar is to be employed for the determination of *E. coli* in bivalve molluscs.

In the present trial, 10% of the presumptive *E. coli* were not confirmed as *E. coli* by API 20E and MALDI-TOF MS. However, with the exception of one *Acinetobacter* isolate, all presumptive *E. coli* isolates belonged to the four genera *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, all within the Enterobacteriaceae family. Our finding is not fully in line with Donovan et al. (1998) who performed the MPN method, followed by confirmation on TBX agar and found that all 204 isolates producing blue-green colonies were indeed *E. coli*. Furthermore, research performed by Rice et al. (1990) on 720 isolates from Enterobacteriaceae, showed that there were no non-*E. coli* isolates that were positive on a β-glucuronidase assay, including *Klebsiella*, *Enterobacter*, and *Citrobacter*. However, during our examinations two isolates identified as *K. pneumoniae*, displayed growth of blue-green colonies on TBX agar. According to Kilian and Bulow (1976), β-glucuronidase activity have shown to be mostly limited to *E. coli*. In other Enterobacteriaceae genera, such as *Salmonella*, *Shigella*, and *Yersinia*, β-glucuronidase activity is less common (Feng and Hartman, 1982; Frampton and Restaino, 1993; Kilian and Bulow, 1976; Massanti et al., 1981). While β-glucuronidase-positive *E. coli* have been observed in 94–96% of the isolates tested (Edberg and Kontnick, 1986; Feng and Hartman, 1982; Kaspar et al., 1987; Kilian and Bulow, 1976), a higher proportion of β-glucuronidase-negative *E. coli* (a median of 15%) have been described in Chang et al. (1989).

Apart from β-glucuronidase production in *E. coli* as seen in most strains, some other Enterobacteriaceae members as *Klebsiella*, *Citrobacter*, and *Enterobacter* may display this enzyme (Hofstra and Veld, 1988; Tryland and Fiksdal, 1998), and could therefore cause false-positives on TBX agar (Fiksdal et al., 1997; Frampton and Restaino, 1993; Leung et al., 2001; Pearez et al., 1986; Sarhan et al., 1991; Van Poucke and Nelis, 1997).

Ogden et al. (1998) made attempts to find alternatives to the MPN method for the enumeration of *E. coli* in bivalves. In their research, alternatives included the Merck Chromocult agar method, a Malthus conductance technique, and the 3M Petrifilm system. After statistical analysis, no significant differences were observed when employing the Merck Chromocult agar method. In addition, poor correlation with a Malthus conductance technique as alternative to the MPN method was reported. The method based on the 3M Petrifilm system was found to be unsuitable and therefore not included.

Our research is the first systematic examination of the species accuracy of the EU MPN method conducted on marine bivalves harvested from in temperate waters. In total, 180 (90%) of the isolates examined were confirmed as *E. coli*, whereas eleven isolates belonged to the genus *Klebsiella*, and three isolates belonged to *Acinetobacter* spp., *Citrobacter* spp., and *Enterobacter* spp., respectively. Bacteria belonging to the genus *Acinetobacter* are widely distributed in soil, manure and water, and are frequently isolated in nosocomial infections (Tower, 2006). *Klebsiella* spp., *Citrobacter* spp., and *Enterobacter* spp. includes several important opportunistic pathogenic bacteria causing infections such as enteritis, pneumonia, meningitis, bloodstream and urinary tract infections (Borenshtein and Schauer, 2006; Brisse et al., 2006; Grimont and Grimont, 2006). Further, six isolates were identified differently when applying API 20E or MALDI-TOF MS. Among these, the results were difficult to interpret due to unexpected positive or negative reactions in the 20 wells of the API 20E system, resulting in unacceptable profiles. In particular, two isolates were identified as *Klebsiella* spp. with the API 20E system, while being identified as *E. coli* with an acceptable genus and species profile by the MALDI-TOF MS instrument. When using both API 20E and MALDI-TOF MS, a more reliable identification were obtained and the liability of the results was enhanced, resulting in a total of 194 out of 200 the bacterial isolates identified sufficiently by API 20E and MALDI-TOF MS.

5. Conclusion

In this research, presumptive *E. coli* were isolated from marine bivalves harvested along the Norwegian coast. Other bacteria within the Enterobacteriaceae family besides *E. coli*, gave growth of false-positive blue-green colonies on TBX agar, indicating that this chromogenic medium may not solely be used to verify the presence of *E. coli*. When applying the API 20E system and the MALDI-TOF MS instrument, 90% were identified as *E. coli*. However, the 10% false-positives will lead to an overestimation of the number of *E. coli*. If a specific enumeration of *E. coli* are required, an alternative chromogenic medium to replace the

TBX agar should be considered, or further biochemical verification or DNA based methods should be included.

Conflicts of interest

The authors declare no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Paper II

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ORIGINAL RESEARCH
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Marine Bivalve Mollusks As Possible Indicators of Multidrug-Resistant *Escherichia coli* and Other Species of the Enterobacteriaceae Family

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The mechanisms for the development and spread of antibacterial resistance (ABR) in bacteria residing in environmental compartments, including the marine environment, are far from understood. The objective of this study was to examine the ABR rates in *Escherichia coli* and other Enterobacteriaceae isolates obtained from marine bivalve mollusks collected along the Norwegian coast during a period from October 2014 to November 2015. A total of 549 bivalve samples were examined by a five times three tube most probable number method for enumeration of *E. coli* in bivalves resulting in 199 isolates from the positive samples. These isolates were identified by biochemical reactions and matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry, showing that 90% were *E. coli*, while the remaining were species within the genera *Klebsiella*, *Citrobacter*, and *Enterobacter*. All 199 isolates recovered were susceptibility tested following the European Committee on Antimicrobial Susceptibility Testing disk diffusion method. In total, 75 of 199 (38%) isolates showed resistance to at least one antibacterial agent, while multidrug-resistance were seen in 9 (5%) isolates. One isolate conferred resistance toward 15 antibacterial agents. Among the 75 resistant isolates, resistance toward extended-spectrum penicillins (83%), aminoglycosides (16%), trimethoprim (13%), sulfonamides (11%), tetracyclines (8%), third-generation cephalosporins (7%), amphenicols (5%), nitrofurans (5%), and quinolones (5%), were observed. Whole-genome sequencing on a selection of 10 *E. coli* isolates identified the genes responsible for resistance, including $bla_{\text{CTX-M}}$ genes. To indicate the potential for horizontal gene transfer, conjugation experiments were performed on the same selected isolates. Conjugative transfer of resistance was observed for six of the 10 *E. coli* isolates. In order to compare *E. coli* isolates from bivalves with clinical strains, multiple-locus variable number tandem repeats analysis (MLVA) was applied on a selection of 30 resistant *E. coli* isolates. The MLVA-profiles were associated with community-acquired *E. coli* strains causing bacteremia. Our study indicates that bivalves represent an important tool for monitoring antibacterial resistant *E. coli* and other members of the Enterobacteriaceae family in the coastal environment.

Keywords: bivalve mollusks, Enterobacteriaceae, *Escherichia coli*, antibacterial resistance, horizontal gene transfer

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INTRODUCTION

The development of antibacterial resistance (ABR) is a natural process and ancient among bacteria (Aminov and Mackie, 2007; D'Costa et al., 2011). However, the current global use of antibacterial agents in human and veterinary medicine, as well as in agriculture, are a driving force for ABR development and also increase the release of these substances to the environment (Davies and Davies, 2010).

The intestines of humans and other homeothermic animals are colonized by a dense and diverse microbiota belonging to, among others, the Enterobacteriaceae family (Tancrède, 1992; Dethlefsen et al., 2006). The predominant genus within this family is *Escherichia*, with *Escherichia coli* being the main species. *E. coli* occurs naturally in the large intestine of humans, birds, and terrestrial and marine mammals (Welch, 2006). Most *E. coli* of the large intestine of humans and other homeothermic animals are commensal strains, however opportunistic and pathogenic strains may be present (Strockbine et al., 2015). *E. coli* cause morbidity and mortality as a result of common infections, including enteritis, meningitis, urinary tract, or bloodstream infections (Strockbine et al., 2015). The main sources of infections with pathogenic *E. coli* are consumption of contaminated water and food, as well as through animal contact (ILSI, 2011).

Antibacterial treatments are known to substantially affect the normal intestinal microbiota favoring resistant strains (Sommer and Dantas, 2011). The prevalence of resistant *E. coli* and other bacteria in the intestinal microbiota of humans are shown to be strongly correlated with the use of antibacterial agents (Murray et al., 1982; Bruinsma et al., 2003; van der Veen et al., 2009).

The microbiological communities in coastal environments can be influenced by sewage and runoff from land, concomitantly containing both fecal bacteria as well as residues of antibacterial substances (Martinez, 2009; Alves et al., 2014; Balière et al., 2015). A significant proportion of the antimicrobial agents are excreted unchanged and in a biologically active form (Dolliver and Gupta, 2008; Gillings, 2013; Michael et al., 2013). During periods with heavy rainfall, increased amount of fecal material from land living animals will reach the sea. In addition, high precipitation could cause an overload and possible leakage from sewage systems. Sewage and manure harbor bacteria of high diversity, have a high concentration of organic substances, as well as anthropogenic pollution as heavy metals and antimicrobial agents, which in combination can favor bacterial growth and promote spread of genetic elements through horizontal gene transfer (Moura et al., 2010; Heuer et al., 2011). Bacteria conferring ABR colonizing the intestines of humans and other homeothermic animals, may contribute to the dissemination of antibiotic resistant bacteria (ABR-B) via sewage to the marine environment (Poeta et al., 2005; Penders et al., 2013). The survival of these bacteria in aquatic environments are affected by both abiotic and biotic factors, e.g., nutrient availability, osmotic stress, variations in temperature and pH, and predation (Barcina et al., 1997; Rozen and Belkin, 2001; Campos et al., 2013). Importantly, *E. coli* have the ability to persist in the aquatic habitat due to its genetic flexibility (van Elsas et al., 2011).

The presence of Enterobacteriaceae conferring resistance to antibacterial agents in coastal waters may represent a human health issue, especially in areas used for marine food production or recreational activities (Murugaiyan et al., 2015). Multidrug-resistant (MDR) bacteria have been detected in coastal waters, and could result in the transmission of resistance among marine and contaminating bacteria via exchange of genetic elements, such as plasmids (Wright, 2010; Alves et al., 2014; Moura et al., 2014).

Bivalve mollusks are invertebrates that have an external two-part hinged shell that contains the soft parts. Typical bivalve mollusks comprise among others clams, oysters, mussels, and scallops. As these mollusks are suspension feeders, they actively filter, retain, and concentrates particles from their surrounding water, including free living or particle-bound bacteria (Bernard, 1989; Leff et al., 1992; Maugeri et al., 2004). Bivalve associated members of the Enterobacteriaceae family, may originate from humans and other homeothermic animals either via sewage, by runoff from land, or from representatives of the wild fauna such as birds or marine mammals (Bogomolni et al., 2008). These bivalves are therefore excellent indicators for fecal contamination and will reflect the load of *E. coli* and other bacteria in the Enterobacteriaceae family present in the water column at a given location. However, different environmental conditions, e.g., temperature, water flow rate, and food availability, can affect the filtration rate, consequently also the accumulation of fecal bacteria (Šolić et al., 1999; Strohmeier et al., 2012; Campos et al., 2013).

Bivalve mollusks are good candidate for studies on resistance in bacteria originating from several sources including humans and animals, and gives the possibility of comparing temporal and spatial changes and the potential for exposure to humans by consumption of marine bivalves. The main objective of this study was to examine the ABR rates in Enterobacteriaceae isolates obtained from marine bivalve mollusks collected along the Norwegian coast. In addition, an assessment of the transferability of certain resistance genes, as well as comparing bivalve isolates with clinical isolates of human origin, was performed.

MATERIALS AND METHODS

Sampling and Identification of Bacterial Isolates

As part of the mandatory EU surveillance program (854/2004/EC, 2004) conducted by the Norwegian Food Safety Authority (NFSA), sampling of bivalve mollusks were performed from 57 localities covering the Norwegian coast on several occasions from October 2014 to November 2015. A standardized most probable number (MPN) reference method for enumeration of *E. coli* in bivalves (Oblinger and Koburger, 1975), with Minerals Modified Glutamate Broth (MMGB) (Oxoid, UK) as growth media in combination with verification on Tryptone Bile with X-glucuronide (TBX) agar (Oxoid, UK) (Donovan et al., 1998), was performed as described in Grevskott et al. (2016). A total of 549 bivalves were collected and examined at the National Institute of Nutrition and Seafood

Research and the Norwegian Institute of Public Health, as presented in Grevskott et al. (2016). More than a half of the bivalve samples (51%) was harvested from commercially active rearing localities, while the rest were collected from positions established by NFSA for long time reference monitoring purposes of shellfish safety. A total number of 199 bacterial isolates, one from each randomly selected culture-positive bivalve sample ($n = 335$), was grown into pure culture for further analysis.

Antibacterial Susceptibility Testing

The bacterial isolates were susceptibility tested by disk diffusion on Mueller-Hinton (MH) agar (Oxoid, UK) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al., 2014). Each bacterial isolate was tested for 24 antibacterial agents, representing 10 drug classes (WHOCC Server, 2016). The following disks (Oxoid, UK) were applied: ampicillin (10 μg), amoxicillin (10 μg), amoxicillin/clavulanic acid (2/1 μg), mecillinam (10 μg), piperacillin/tazobactam (30/6 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), nitrofurantoin (100 μg), gentamicin (10 μg), tobramycin (10 μg), streptomycin (25 μg), kanamycin (30 μg), trimethoprim (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), cefotaxime (5 μg), ceftazidime (10 μg), doxycycline (30 μg), tetracycline (30 μg), colistin sulfate (25 μg), imipenem (10 μg), and meropenem (10 μg). To monitor the quality for each new batch of MH agar, and antibacterial disks, *E. coli* CCUG 17620 was included on a regular basis. The inhibition zones were interpreted according to the EUCAST clinical breakpoint tables v.6.0 (EUCAST, 2016). For some substances breakpoints were not available and for these substances clinical breakpoints given by Clinical and Laboratory Standards Institute (CLSI, 2014) or Indian Council of Medical Research (ICMR, 2009), were used.

Whole-Genome Sequencing

A selection of 10 isolates was subjected to whole-genome sequencing (WGS). The isolates were selected on the basis of phenotypes showing resistance toward multiple antibacterial agents and/or expressing resistance to critically important agents, such as to third-generation cephalosporins. DNA was isolated by the use of the MagNA Pure 96 DNA and Viral NA Small Volume Kit and a MagNApure 96 instrument (Roche Diagnostics, Germany). The sequencing libraries were prepared using the Kapa HyperPLus Library Preparation Kit (Kapa Biosystems, USA). The isolates were sequenced on an Illumina MiSeq platform (Illumina, USA), producing (2 bp × 250 bp) paired-end reads. The data were adaptor and quality trimmed using Trimmomatic (Bolger et al., 2014), and assembled using SPAdes (Bankevich et al., 2012). The processed sequence data were analyzed for genes encoding resistance to antimicrobial resistance using the web-based ResFinder tool (Zankari et al., 2012), for serotype using the SerotypeFinder tool (Joensen et al., 2015) and for multi-locus sequence types (MLSTs) using the MLSTs tool (Larsen et al., 2012) from Centre for Genomic Epidemiology[1], at the Technical University of Denmark.

Conjugation Experiments

The whole-genome sequenced strains were subjected to conjugation experiments in order to investigate the ability of self-transfer of resistance properties to susceptible recipient strains. The 10 donor isolates were mated with one of the two sensitive recipient strains, *E. coli* DH5α (Culture Collection, University of Göteborg, Sweden) and One Shot *E. coli* (Thermo Fisher, USA). Eight of the donor *E. coli* isolates were susceptible to quinolones, and were conjugated with *E. coli* DH5α resistant to nalidixic acid, as recipient. Two of the donor *E. coli* isolates were resistant to quinolones, but susceptible to kanamycin, and were therefore conjugated with One Shot *E. coli* resistant to kanamycin, as recipient. The conjugal transfer was conducted in a Luria-Bertani (LB) broth (Sigma-Aldrich, USA) and the mating was prepared as previously described by Sunde and Sørum (2001). The transconjugant was selected as described by Sunde and Norström (2006), by applying antibacterial disks corresponding to the resistance profile of the donors (Oxoid, UK; Rosco, Denmark) onto the surface of the MH agar plates (BD, USA), with 20 μg/ml nalidixic acid (N-8878 Sigma-Aldrich, USA) or 50 μg/ml kanamycin (K4000 Sigma-Aldrich, USA). The obtained transconjugants were subcultured for inspection of colony morphology as previously described (Sunde and Norström, 2006) and subsequently subjected to susceptibility testing by disk diffusion.

Multiple-Locus Variable Number Tandem Repeats Analysis

Based on resistance profile, 30 of the 199 isolates were selected for multiple-locus variable number tandem repeats analysis (MLVA). Extraction of DNA was done by dissolving bacterial cells in 350 μl sterile, distilled water (Fresenius Kabi, Germany) and boiling at 100°C for approximately 15 min. Extracted DNA was mixed with reagents from Qiagen Multiplex PCR kit (Qiagen, Germany). The PCR mixture consisted of 12.5 μl of 2x Master mix, 0.5 μl of primer mix and 11 μl of sterile water. Four different primer mixes were used for each DNA sample: EC-5, EC-6, CVN002 and EC-12, where 1 μl extracted DNA was added to the PCR mixtures, to a total volume of 25 μl. The PCR mixtures were placed in the GeneAmp® PCR System 9700 machine (Applied-Biosystems, USA) followed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied-Biosystems, USA), as described by Løbersli et al. (2012). A control DNA sample (GJ57) was measured along with the unknown DNA samples for quality assurance.

Molecular Epidemiologic Analysis of the *E. coli* Isolates by BioNumerics

From the MLVA-profiles of the 30 bivalve *E. coli* isolates, the allele numbers generated were entered into BioNumerics database version 7.6 (Applied Maths, Belgium) as character values, and

[1] https://cge.cbs.dtu.dk/services/

an analysis based minimal spanning tree (MST) clustering was constructed. As markers of genetic relationships, we included 212 community-acquired *E. coli* bacteremia isolates, 38 other human strains from the *E. coli* Reference (ECOR)-collection obtained from the Microbial Evolutionary Laboratory (State University of Michigan, USA), four Enterohemorrhagic *E. coli* (EHEC) strains associated with hemorrhagic uremic syndrome (HUS) from the strain collection at the Norwegian Institute of Public Health, as described (Wester et al., 2013, 2014). The community-acquired *E. coli* isolates causing blood stream infection (BSI) were classified as non-severe, early organ failure (≥organs affected within 1 day of admittance to hospital), or in-hospital death within 14 days of admission (Wester et al., 2013). We applied MST for categorical data, with one-locus difference as first priority rule (weight 10,000), and two-loci difference as second priority rule (weight 10).

RESULTS

Sampling and Identification

The majority of the bacterial isolates (90%) were identified as *E. coli*, both by Analytical Profile Index 20E (Oxoid, UK) and by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (Bruker, Germany). The remaining isolates (10%) belonged to the three genera *Klebsiella*, *Citrobacter*, and *Enterobacter*.

Prevalence of Antibacterial Resistance

A total of 75 (38%) of the 199 isolates showed resistance to at least one antibacterial agent, while multidrug-resistance was seen in nine (5%) of the isolates (**Figure 1**), using the definition by Magiorakos et al. (2012). Among the 75 resistant isolates, resistance toward extended-spectrum penicillins (83%), aminoglycosides (16%), trimethoprim (13%), sulfonamides (11%), tetracyclines (8%), third-generation cephalosporins (7%), amphenicols (5%), nitrofurans (5%), and quinolones (5%), were observed. Amoxicillin-resistance was found in 59 (79%) isolates, while ampicillin-resistance was found in 36 (48%) isolates. The two *E. coli* isolates B177 and B184 showed phenotypic resistance against nine and 15 antibacterial agents, respectively.

Genetic Characterization of Selected Resistant *E. coli* Isolates

Among the 10 bacterial isolates subjected to WGS, eight sequence types (STs) were identified. Two isolates belonged to ST-95, and two isolates belonged to ST-58, the remaining six isolates belonged to ST-10, ST-38, ST-69, ST-88, ST-191, or ST-3572, respectively.

Multiple resistance genes were present as examined by ResFinder (**Table 1**). Resistance toward extended-spectrum penicillins was observed in all 10 *E. coli* isolates and they all harbored the $bla_{\text{TEM-1}}$ gene. Isolate B117 and B184 were resistant to third-generation cephalosporins, and carried the

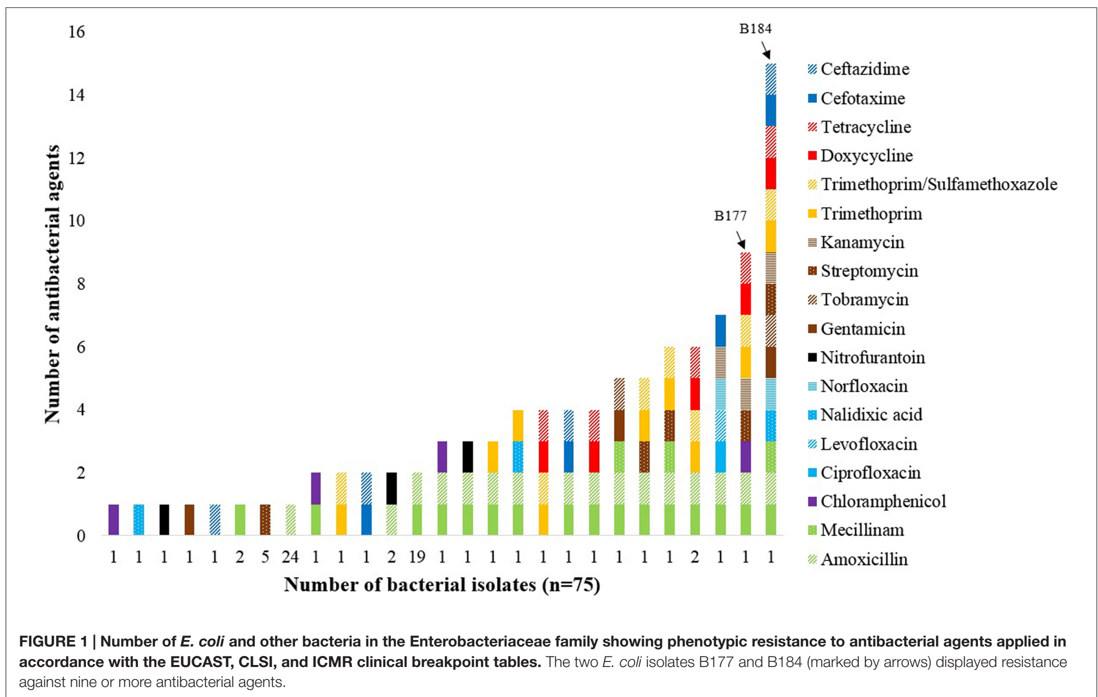

FIGURE 1 | Number of *E. coli* and other bacteria in the Enterobacteriaceae family showing phenotypic resistance to antibacterial agents applied in accordance with the EUCAST, CLSI, and ICMR clinical breakpoint tables. The two *E. coli* isolates B177 and B184 (marked by arrows) displayed resistance against nine or more antibacterial agents.

TABLE 1 | Distribution of sequence type (ST), resistance genes, and serotype among 10 *Escherichia coli* isolates by WGS.

Isolate no.	MLST	ResFinder	Serotype
B2	ST-58	*bla_{TEM-1B}, strA-strB, dfrA5, sul2*	O8:H25
B53	ST-10	*bla_{TEM-1B}*	No O type:H4
B117	ST-191	*bla_{TEM-1B}, $bla_{CTX-M-15}$*	O48:H20
B158	ST-95	*bla_{TEM-1B}, strA-strB, dfrA5, sul2*	O1:H7
B160	ST-58	*bla_{TEM-1B}, qnrS1, tet(A)*	O8:H30
B161	ST-69	*bla_{TEM-1B}, aac(3)-IId*	O17/O44:H18
B165	ST-95	*bla_{TEM-1C}, strA-strB, dfrA14, sul2, tet(A)*	O1:H7
B167	ST-88	*bla_{TEM-1C}, tet(A)*	O8:H17
B177	ST-3572	*bla_{TEM-1B}, strA-strB, dfrA17, sul1, sul2, catA1, aadA5, aph(3′)-Ia, tet(B)*	O89:H9
B184	ST-38	*bla_{TEM-1B}, $bla_{CTX-M-14}$, strA-strB, dfrA17, sul1, sul2, catA1, aac(3)-IId, aadA5, tet(D), mph(A)*	O102:H6

Genes conferring resistance toward: extended-spectrum penicillins (bla_{TEM-1}), third-generation cephalosporins ($bla_{CTX-M-14}$, $bla_{CTX-M-15}$), aminoglycosides [strA-strB, aadA5, aac(3)-IId, aph(3)-Ia], trimethoprim (dfrA17, dfrA5, dfrA14), sulfonamides (sul1, sul2), tetracyclines [tet(A), tet(B), tet(D)], amphenicols (catA1), quinolones (qnrS1), and macrolides (mphA).

TABLE 2 | Conjugative transfer and antibacterial resistance (ABR) profile in transconjugants.

Donor	Resistance profile	Conjugation*	Resistance profile transconjugants
B2	AMP-AML-TRI-SXT-S	+	TRI-SUL
B117	AMP-AML-CTX-CAZ	+	AMP-CTX-CAZ
B158	AMP-AML-MEL-TRI-SXT-S	+	TRI-SUL
B160	AMP-AML-TRI-SXT-DO-TE	+	TE
B165	AMP-AML-TRI-SXT-DO-TE	+	TRI-SUL
B167	AMP-AML-DO-TE	+	TE
B53	AMP-AML-NA-TRI	−	
B161	AMP-AML-MEL-GEN-TOB	−	
B177	AMP-AML-C-S-K-TRI-SXT-DO-TE	−	
B184	AMP-AML-MEL-NA-NOR-GEN-TOB-S-K-TRI-SXT-DO-TE-CTX-CAZ	−	

**Transferability of resistance plasmids; "+" transconjugants were obtained, "−" no transconjugants were obtained. AMP, ampicillin; AML, amoxicillin; MEL, mecillinam; C, chloramphenicol; NA, nalidixic acid; NOR, norfloxacin; GEN, gentamicin; TOB, tobramycin; S, streptomycin; K, kanamycin; TRI, trimethoprim; SUL, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; DO, doxycycline; TE, tetracycline; CTX, cefotaxime; CAZ, ceftazidime.*

$bla_{CTX-M-15}$ and *$bla_{CTX-M-14}$* genes, respectively. Six isolates possessed genes conferring resistance to aminoglycosides, while five isolates carried genes for resistance against trimethoprim, sulfonamides, and tetracyclines. A gene conferring resistance against amphenicols was observed in two isolates. Two isolates had genes conferring resistance toward quinolones and macrolides, respectively. Notably, three isolates harbored resistance genes (*strA-strB, catA1,* and *qnrS1*, respectively) which did not correspond to the phenotypic resistance pattern.

Conjugal Transfer of Antibacterial Resistance Determinants

Six of 10 *E. coli* isolates transferred resistance genes by conjugation (**Table 2**). The three bacterial isolates B2, B158, and B165 transferred trimethoprim- and sulfamethoxazole-resistance, two isolates (B160 and B167) transferred tetracycline-resistance, while one isolate (B117) transferred resistance to cefotaxime and ceftazidime. The resistance patterns of transconjugants were examined by the EUCAST disk diffusion method, in which only a selection of antibacterial agents were employed as determined by the resistance profile of the donor.

Phylogenetic Diversity of the *E. coli* Isolates

A total of 284 strains were included and MLVA-profiles matching nine specific loci were regarded as phylogenetic related (**Figure 2**). The ECOR strains of different phylogroups and *E. coli* isolates causing BSI did not cluster, nor showed to be located in any specific branch of the MST, except from strains belonging to phylogroup A. The 30 *E. coli* isolates from bivalves seemed to be evenly distributed throughout the MST, together with both the bacteremia *E. coli* and the ECOR strains and the HUS-associated EHEC strains.

DISCUSSION

Antibacterial resistant fecal bacteria from animals or humans may spread among the human population by direct contact, or via water and food. The transfer of ABR-B in the food production chain may affect the development and spread of resistance among the foodborne pathogens (Sørum and L′Abée-Lund, 2002; VKM, 2015). This could also apply for seafood. Contaminated seafood as fish, bivalves, and crustaceans may cause ABR-B from both marine and fecal origins to reach humans during handling and consumption. A possible risk of transmission of ABR-B may occur from unintentional improper heat treatment, or through bivalves intended for raw- or light preserved consumption. Especially, flat oysters (*Ostrea edulis*) and great scallops (*Pecten maximus*) represents a risk, as they are commonly consumed raw. If these food products are consumed without proper heat treatment, resistant bacteria may enter the consumer and subsequently interact with the intestinal microbiota (Sullivan et al., 2001).

In this study, two *E. coli* isolates displayed phenotypic resistance toward as many as nine or more antibacterial agents, indicating a potential risk of exposure to MDR Enterobacteriaceae during consumption or handling of marine bivalves. In addition, extended spectrum beta-lactamase (ESBL)-producing *E. coli* isolates were identified from this food source (**Table 1**). Among the European countries, Norway has the lowest production corrected use of antimicrobial agents in animals (EMA, 2016). Furthermore, as reported in the

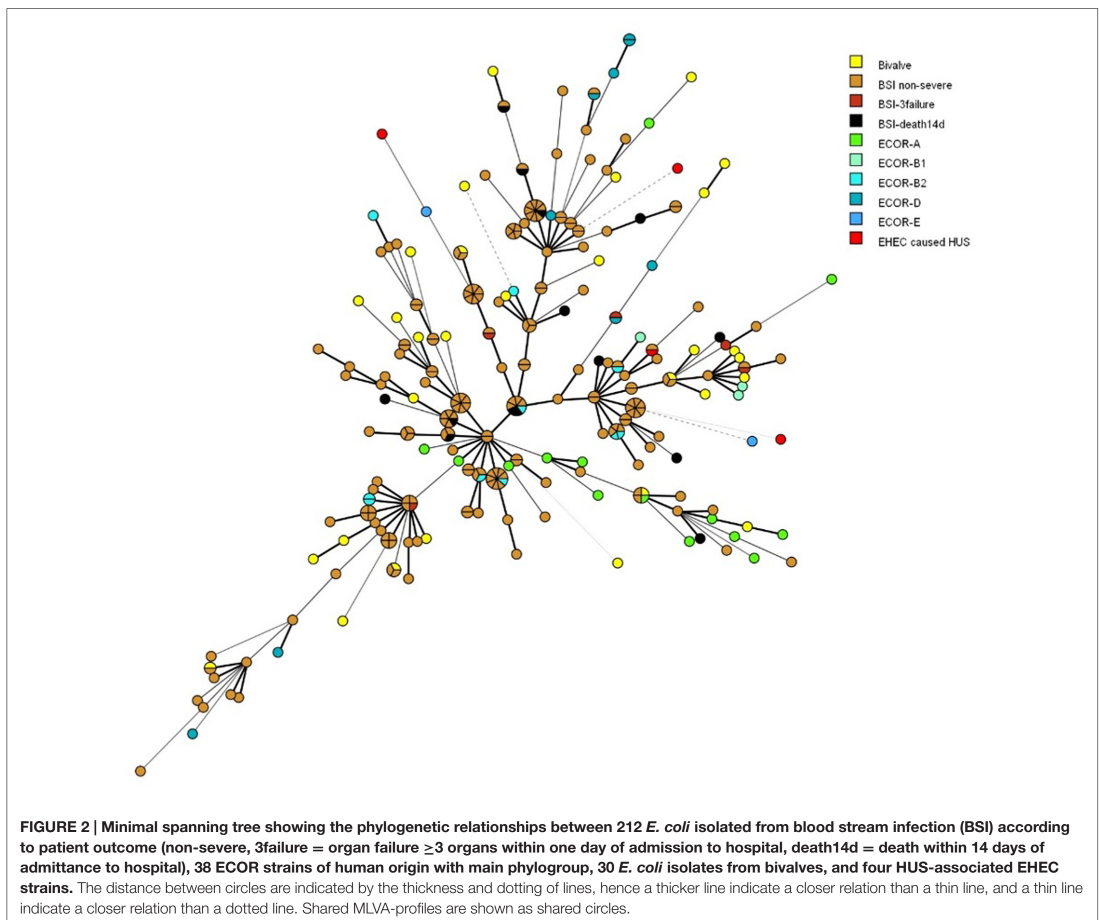

FIGURE 2 | Minimal spanning tree showing the phylogenetic relationships between 212 *E. coli* isolated from blood stream infection (BSI) according to patient outcome (non-severe, 3failure = organ failure ≥3 organs within one day of admission to hospital, death14d = death within 14 days of admittance to hospital), 38 ECOR strains of human origin with main phylogroup, 30 *E. coli* isolates from bivalves, and four HUS-associated EHEC strains. The distance between circles are indicated by the thickness and dotting of lines, hence a thicker line indicate a closer relation than a thin line, and a thin line indicate a closer relation than a dotted line. Shared MLVA-profiles are shown as shared circles.

Norwegian monitoring program for antimicrobial resistance in human pathogens, and in bacteria from food, feed and animals (NORM/NORM-VET, 2015), Norway is a low prevalence country in terms of antimicrobial resistance and it is therefore surprising to detect a high rate of resistant Enterobacteriaceae in marine bivalves, including the ESBL-producing *E. coli* strains. Notably, this should be taken into account in order to determine if bivalves should be included in annual monitoring of ABR in the coastal environment.

The majority of resistant isolates ($n = 75$) examined in the current work were resistant to the extended-spectrum penicillins ampicillin and/or amoxicillin (83%) (**Figure 1**), which is interesting since the use of antimicrobial agents in Norway is dominated by narrow-spectrum penicillins (NORM/NORM-VET, 2015). However, an increase in the use of penicillins with extended spectrum have been reported lately (NORM/NORM-VET, 2015). The increased use of ampicillin and amoxicillin in humans and/or food-producing animals may have led to the development of resistance within the bacterial species observed in this study. Moreover, it is well-known that the $bla_{\text{TEM-1}}$ gene conferring resistance against extended-spectrum penicillins has been widely distributed in bacterial populations for decades (Hedges et al., 1974). All 10 *E. coli* isolates subjected to in-depth characterization by WGS harbored the $bla_{\text{TEM-1}}$ gene, whereas two isolates had $bla_{\text{CTX-M}}$ genes, the latter conferring ESBL-production (**Table 1**). The various TEM enzymes are mutant derivatives of plasmid-mediated beta-lactamases conferring resistance to penicillins, while the CTX-M enzymes confer resistance to penicillins and cephalosporins and have their origin in environmental bacteria (Cantón et al., 2012). The CTX-M enzymes have become the most prevalent ESBLs in bacteria causing human infection, both in hospital and in community settings (Cantón and Coque, 2006; Cantón et al., 2008). The presence of ESBL-positive *E. coli* is of great concern due to possible lack of therapeutic success in the treatment of serious infections, hence defined as critically important by

the World Health Organization [WHO] (2014). ESBL-positive *E. coli* have also been recovered from food products for human consumption, as well as from wildlife (Li et al., 2007; Smet et al., 2010; Guenther et al., 2011). A fraction of the bacterial isolates were resistant to aminoglycosides (16%), and six of the 10 sequenced *E. coli* isolates harbored resistance genes. Resistance toward trimethoprim and sulfonamides were seen in 13 and 11% of the isolates, respectively, and five of the 10 sequenced *E. coli* isolates harbored genes conferring resistance toward trimethoprim and sulfonamides. All isolates expressing resistance to trimethoprim and sulfonamides contained genes responsible for the resistance phenotype, except isolates B53 and B160. This indicates that resistance among the bacterial isolates could be a result of selection by increased use, since these agents are synthetic and thus not commonly found in the natural environment. However, observations of resistance toward quinolones and sulfonamides have been seen in the intestinal microbiota of an 11th Century pre-Columbian Andean mummy, showing that resistance even to some synthetic agents may date back to Ancient times (Santiago-Rodriguez et al., 2015).

Among the 10 *E. coli* isolates subjected to conjugation experiments, transferable resistance was detected in six isolates (**Table 2**). The transfer of genes conferring resistance toward third-generation cephalosporins (cefotaxime and ceftazidime) are especially alarming, since the spread of these genes to clinically relevant *E. coli* strains will dramatically reduce the possible choice of antibacterial agents for medical treatment. Moreover, transfer of multiple resistance genes may occur with a higher frequency when the bacteria are exposed to antibacterial agents. ABR among, e.g., enteric bacteria may form reservoirs, in which resistance determinants could transfer to non-resistant bacteria, including those responsible for diseases (Salyers et al., 2004; Stecher et al., 2012). Intestinal bacteria from the human microbiota may, in addition to sharing resistance genes among themselves, also exchange resistance genes to other bacteria that are temporary passing through the intestine (Teuber et al., 1999; Salyers et al., 2004). Thus, commensal bacteria may function as a vector in transferring resistance genes between environmental and pathogenic bacteria.

Whole-genome sequencing and subsequent analysis showed that two isolates belonged to ST-95, while two isolates belonged to ST-38 and to ST-69, respectively (**Table 1**). These STs are associated with bacteremia and urinary tract infection in humans (Adams-Sapper et al., 2012; Alghoribi et al., 2015; Hertz et al., 2016). The MLVA-profiles of the bivalve *E. coli* isolates displayed a seemingly high degree of diversity (**Figure 2**). Furthermore, they scattered among BSI-causing, including those leading to death within 14 days of admission to hospital, as well as among representatives of all *E. coli* main phylogroups. Both instances indicate no common source, but also that the bacteria have the potential for causing serious infection in humans. Consequently, the presence of pathogenic *E. coli* isolates in the coastal environment represent a risk to human health, especially in areas use for aquaculture or recreational activities. This is supported by the findings of Balière et al. (2015) who reported that a few *E. coli* strains of EHEC and Enteropathogenic *E. coli* (EPEC) isolated from bivalve mollusks harbored resistance toward amoxicillin, cefotaxime, and imipenem. The World Health Organization [WHO] (2014) have stated that infections with *E. coli* strains, e.g., EHEC and EPEC, are among the most frequent foodborne causative agents worldwide.

Allochthonous bacteria from different sources (e.g., urban, industrial, and agriculture waste), and residues of antimicrobial agents, will ultimately be transported to the marine environment through waste water effluents, rivers, or streams, and mixed with the indigenous bacterial population (Baquero et al., 2008; Wellington et al., 2013). This can result in the rise of resistance due to selection pressure, and/or genetic exchange between environmental and intestinal bacteria. Bivalves may promote gene transfer among bacteria in the marine environment, by collecting bacteria from various sources and concentrate them within a stable micro-environment at a high density (Taylor et al., 2011). The increasing pressure exerted by antimicrobial agents affects the acquisition, selection, and transmission of resistance determinants among a wide range of bacteria.

CONCLUSION

Our study indicates that marine bivalves may represent an important tool for monitoring antibacterial resistant *E. coli* and other members of the Enterobacteriaceae family in coastal environments. Bivalves may furthermore act as a “hot spot” for resistance transfer between Enterobacteriaceae and indigenous bacteria, as the conditions they offer may facilitate the conjugational frequency. As continuous EU programs for the detection of *E. coli* from bivalves are currently implemented, an additional characterization of their ABR profile would represent a good cross-compartment added value indicator of spatial and temporal trends in resistance rates.

AUTHOR CONTRIBUTIONS

BL, CS, and DG designed the experimental set up and DG, MS, and AW performed the experiments. DG, CS, MS, AW, and BL wrote the manuscript. All authors agree to be accountable for the content of the work and gave final approval to the manuscript.

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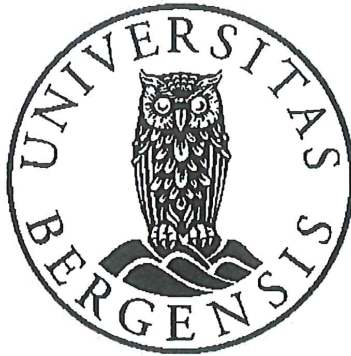
Errata

Errata for

Bivalve mollusks as tools for monitoring antibiotic resistance in the marine environment

Didrik Hjertaker Grevskott



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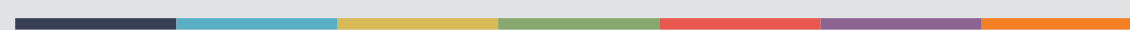

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