SCREENING FOR ANTIMICROBIAL- AND HEAVY METAL RESISTANT BACTERIA IN COPPER CONTAMINATED AREAS

Editor(s): Cecilie Smith Svanevik (IMR)

Title (English and Norwegian):

Screening for antimicrobial- and heavy metal resistant bacteria in copper contaminated areas

Kartlegging av antibiotika- og tungmetallresistente bakterier i kobberforurensede områder

Author(s):

Cecilie Smith Svanevik (IMR), Anne Margrete Urdahl (NVI), Bjørn Tore Lunestad (IMR), Madelaine Norström (NVI), Jannice Schau Slettemeås (NVI), Hanne Nilsen (NVI) and Julia Storesund (IMR)
Editor(s): Cecilie Smith Svanevik (IMR)

Approved by: Research Director(s): Gro-Ingunn Hemre Program leader(s): Livar Frøyland

Partners

Summary (English):

A One heath approach is essential to examine the role of the marine environment in the selection and spread of antimicrobial resistance. Norwegian aquaculture used high amounts of antimicrobials during the 80s and 90s, which facilitated widespread resistance among fish pathogens. Moreover, fish farms have since their beginning used copper containing coating as anti-fouling agents on the fish nets, in addition to copper supplements in the fish feed. Consequently, some fish farms have high concentrations of copper in det sediments beneath the pens. Several studies have reported that even sublethal concentrations of heavy metals can facilitate co-selection of antimicrobial resistance and heavy metal resistance. This study applied *Vibrio* spp. as indicator of antimicrobial- and heavy metal resistance in copper contaminated areas. Six different locations were sampled twice. These represented four different areas in regard of aquaculture activity; Active aquaculture, Non-active aquaculture, Non-aquaculture and Unpolluted. In total, 70 samples, including sediments, seawater, marine fauna and marine algae, were examined for the presence of *Vibrio* spp. and *Escherichia coli*. The sediments were examined for heavy metals, where sediments from Active aquaculture areas held exstensively toxic mean concentration with 272.3 mg/kg dry weight (d.w.) copper, significant higher than Non active (24.4 mg/kg d.w.), Non-aquaculture (34.8 mg/kg d.w.) and Unpolluted (2.28 mg/kg d.w.), and moderate toxic mean concentration with 424.6 mg/kg d.w. zinc, also significant higher than Non-active (70.7 mg/kg d.w.), Non-aquaculture (144.6 mg/kg d.w.) and Unpolluted (16.4 mg/kg d.w.). The majority of the retrieved 350 *Vibrio splendidus* and 72 *Vibrio anguillarum* isolates were susceptible to all the antimicrobials tested for. Increased minimum inhibition concentrations (MIC) to oxytetracycline and copper were seen for some isolates from Active aquaculture, however only one- to two-fold higher than from Non-active and Non-aquaculture areas. Several antimicrobial- and heavy metal resistance genes were found across the different examined areas, with exception of the *nlpE* gene encoding a copper sensing lipoprotein inducing efflux pumps, that were only found in one isolate from one location with aquaculture. The majority of the 336 *E. coli* isolates were susceptible to all antimicrobial tested for. The Active aquaculture areas conferred highest number of *E. coli*, and most of the resistant *E. coli*. Many factors, such as weather and topography, can influence the inflow of *E. coli* from sewage and run-offs from land. No association was found between the detected phenotypic and genotypic resistance traits in *V. splendidus* and in *E. coli* in any of the examined areas. Importantly, a higher sample size including more locations is needed to determine whether any possible indicated differences are significant. In conclusion, the high proportion of susceptible isolates found in this project, indicates that the prevalence of selectable resistance mechanisms in the examined marine vibrios from copper contaminated areas are low.

Summary (Norwegian):

Et «Én-Helse»-perspektiv er viktig for å bedre forstå hvordan det marine miljøet påvirker seleksjon og spredning av antibiotikaresistens. Norsk akvakultur brukte store mengder antibiotika gjennom 80- og 90-tallet, og antibiotikaresistens blant fiskepatogene bakterier ble den gang sett i utstrakt grad. I tillegg har akvakulturnæringen siden sin oppstart brukt kobber som antibegroingsmidler i merdnøter og som tilskudd i fiskefôr. Som et resultat av dette har noen akvakulturanlegg høye konsentrasjoner av kobber i sedimentene under merdene. Flere studier har rapportert at tungmetall, også i lave konsentrasjoner som ikke hemmer bakterier, kan selektere for både tungmetall- og antibiotikaresistens. I dette studiet ble *Vibrio* spp. benyttet som en marin indikatorbakterie for antibiotika- og tungmetallresistens i kobberforurensende områder. Seks ulike lokasjoner ble prøvetatt ved to separate tidspunkt. Områdene ble delt inn i fire kategorier etter påvirkning fra akvakultur: Aktiv akvakultur, Ikke-aktiv akvakultur, Ikke-akvakultur og Rene områder. Totalt 70 prøver, inkludert sedimenter, sjøvann, marin fauna og marine alger, ble undersøkt for forekomst av *Vibrio* spp. og indikatorbakterier for fekal forurensing (*Escherichia coli*). Sedimentene ble også undersøkt for tungmetaller, hvor akutt toksiske konsentrasjoner ble funnet ved de Aktive akvakulturområdene med et gjennomsnitt på 272,3 mg/kg tørrvekt (t.v.) for kobber, signifikant høyere sammenlignet med Ikke-aktive (24,4 mg/kg t.v.), Ikke-akvakultur (34,8 mg/kg t.v.) og Rene områder (2,28 mg/kg t.v.), samt moderat toksiske konsentrasjoner med et gjennomsnitt på 474,6 mg/kg t.v. for sink, også signifikant høyere sammenlignet med Ikke-aktiv (70,7 mg/kg t.v.), Ikke-akvakultur (144,6 mg/kg t.v.) og Rene områder (16,4 mg/kg t.v.). Blant de 350 undersøkte *Vibrio splendidus* isolatene og de 72 *Vibrio anguillarum* isolatene, var størsteparten følsomme for alle de antibiotika de ble testet mot. Høyere minste hemmende konsentrasjon ble sett for oksytetrasyklin og kobber blant noen isolater fra Aktive akvakulturområder, men dette var kun én-til-to ganger høyere enn blant isolater fra Ikke-aktiv akvakultur og Ikke-akvakulturområdene. Flere antibiotika- og tungmetallresistensgener ble funnet i alle de undersøkte områdene, med unntak av genet *nlpE*, som kun ble funnet ved et akvakulturområde. Dette genet koder for et kobbersensorprotein som induserer membranpumper som skiller ut ulike uønskede substanser. Størsteparten av de 336 undersøkte *E. coli* isolatene var følsomme for alle antibiotika de ble testet mot. De Aktive akvakulturområdene hadde høyeste antall *E. coli*, og også flest av de resistente *E. coli* isolatene. Det er likevel mange faktorer som påvirker tilførselen av *E. coli* fra kloakk og avrenning fra land, slik som værforhold og topografien til området. Det ble ikke funnet noe sammenheng mellom resistens hos *V. splendidus* og *E. coli* ved noen av de undersøkte områdene, hvor flere prøver fra flere lokaliteter trolig vil kunne gitt bedre nyanser rundt de observerte forskjellene. Basert på den høye forekomsten av sensitive bakterieisolater, konkluderes det med at det er

lav forekomst av resistensmekanismer blant vibrioer som det kan som det kan selekteres for i de undersøkte kobberforurensede områdene.

Content

1 - Abbreviation

AMP	Ampicillin
AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
AST	Antimicrobial Sensitivity Testing
AZM	Azithromycin
CAZ	Ceftazidime
CIP	Ciprofloxacin
CHL	Chloramphenicol
CST	Colistin
CTX	Cefotaxime
d.w.	dry weight
ECV	Epidemiological cut-off value
FLO	Florfenicol
HMRGs	Heavy metal resistance genes
GEN	Gentamicin
MEM	Meropenem
NAL	Nalidixic acid
NIT	Nitrofurantoin
OTE	Oxytetracycline
OXA	Oxolinic acid
SMZ	Sulfamethoxazole
TET	Tetracycline
TGC	Tigecycline
TMP	Trimethoprim
w.w.	wet weight

2 - Background

Norwegian fish farming in net pens impact the biotic and abiotic environment surrounding the facilities in multiple ways. In the 1980s and 90s, large quantities of antimicrobials were used to treat bacterial infections in fish and a significant increase in antimicrobial resistant bacteria in sediments near fish farms were detected [1]. The highest consumption was in 1987, with close to 50 tonnes measured as pure substance of antimicrobials, including 21 130 kg of oxytetracycline, 15 840 kg of nitrofurazolidone, 3 700 kg of oxolinic acid and 1 900 kg of trimethoprim/sulfadiazine (sales statistics from the Norwegian Medicinal Depot AS). Following the development of effective vaccines against vibriosis (*Vibrio anguillarum*), cold-water vibriosis (*V. salmonicida*) and furunculosis (*Aeromonas salmonicida*) in the 1990s [2, 3], there has been a sharp reduction in the use of antimicrobials in fish farming, and the five-year average 2015-2019 was 452 kg, comprising 321 kg florfenicol, 125 kg of oxolinic acid and 6 kg of oxytetracycline [4]. A significant proportion of these drugs were, however, for the treatment of farmed cleaner fish for salmon lice control, and not for the breeding of fish for human consumption [4, 5].

The use of antimicrobials is recognised as the strongest driver for antimicrobial resistance, but other substances also stimulate the selection of resistance in bacteria. This applies in particular to heavy metals, such as copper, zinc and cadmium [6, 7]. High concentrations of heavy metals are bactericidal, and exposed bacteria therefore develop resistance for protection. Among the heavy metals discharged into the sea from various sources in Norway, it is estimated that copper input is the greatest with 1 251 tonnes in 2016, followed by zinc with 576 tonnes [8]. Unlike many types of antimicrobials, metals do not break down and will therefore accumulate and probably have long lasting effects on the surrounding environment. Today's aquaculture activity does not use and discharge significant quantities of antimicrobials into the sea, but fish farming is still considered as the greatest contributor of copper due to the use of copper containing anti-fouling agents on fish nets, and direct discharge of copper-enriched fish feed. The risk report for fish farming from 2018, showed that sediments in some areas were classified as toxic (Table A1) e.g., above 84 mg/kg dry weight (d.w.) [9]. Furthermore, sublethal concentrations of antimicrobials [10] and heavy metals [11] have been shown to select for antimicrobial resistance.

The mechanisms of heavy metal resistance may be the ability to reduce the toxicity by binding and forming complexes, or by reducing the metal ions to less toxic forms. A third mechanism is excretion through membrane pump systems [6, 12]. The presence of such complex pumping systems in some bacteria can result in co-resistance to a variety of antimicrobials, and several links between heavy metal resistance and antimicrobial resistance have been found. However, it is the co-localization of heavy metal resistance genes (HMRGs) and antimicrobial resistance genes (ARGs) on mobile genetic elements, such as transposons and plasmids that allow selection of both genes, when exposed to one of the selective factors. For instance, when pigs were fed copper supplemented feed, the copper resistance gene *tcrB* and the gene encoding resistance to erythromycin *ermB* were simultaneously selected for in *Enterococcus faecalis* and *E. faecium* [13]. Such co-selection of resistance mechanisms could also happen in the environment and promote the spread of resistance genes within or between bacterial species. It is plausible to hypothesise that continuous discharge and exposure of heavy metals may sustain the selection pressure for some antimicrobials even though the drug is no longer discharged [14].

Antimicrobial resistant bacteria in the marine environment are a major collective term for all bacteria in the ocean that are able to grow at antimicrobial concentrations above the wild type levels. These bacteria represent large parts of the marine microbiota and cannot be easily described or classified. They include bacteria in water and sediments, on seaweed and kelp, and in and on larger organisms, such as bivalves, crustaceans and fish.

Some bacteria are restricted to one of these habitats, while others may be ubiquitous [15]. In addition, some non-marine bacteria such as *Escherichia coli* that are predominantly from homeothermic animals, including humans, may contaminate aquatic environments for instance through run-offs from agricultural areas or sewage facilities. Mobile resistance mechanisms that are of special concern in a One Health perspective may then be of importance, like extended spectrum beta-lactamases and plasmid mediated quinolones resistance. Several marine bacteria harbour intrinsic resistance properties to several antimicrobials, which are unlikely to be shared and thus not spread between species. Nevertheless, many marine bacteria are particularly prone to pick up foreign genes and collect different resistance properties. Some of these bacteria are also opportunistic pathogens, and since they often have a high degree of intrinsic resistance, there is a limited choice of antimicrobial for treatment. It is therefore important to investigate both intrinsic and acquired resistance in marine bacteria. There are currently no established or validated marine indicator organism for antimicrobial resistance in the same way as it is for antimicrobial resistance surveillance in production animals and meat (e.g., *E. coli* and *Enterococcus* spp.).

Vibrio is a true marine genus containing about 140 species, where 12 species are known to be pathogenic to humans and several more pathogenic to marine animals, such as fish and oysters. The ability of vibrios to tolerate elevated levels of copper depends on their habitation and environmental conditions. Shi et al. found that the vibrio-host oysters, accumulate copper for antibacterial effect [16], and Vanhove et al. found that copper resistance genes *cusCBAF* and *copA* gave beneficial virulent traits in vibrios and were necessary for surviving intracellularly when infecting the host [17]. Different *Vibrio* species have intrinsic resistant features developed by evolution, and not by recent acquired genes, such as the tetracycline resistance gene *tet(34)*, the chloramphenicol resistance genes *catB* and *bcr/cflA*, and the multidrug efflux pump gene *emrD* [18, 19], however, these resistance traits are not necessarily expressed phenotypically if not needed [20]. Furthermore, resistance to ampicillin and colistin in *V. anguillarum* have been found to be related to whether it is serovar O1 or O2 [21]. Both the human pathogenic and non-pathogenic *Vibrio* species are also competent to acquire antimicrobial resistance genes. For instance, other *tet* genes coding for efflux pumps, conferring resistance to tetracyclines, have been found in *V. splendidus* and *V. tasmaniensis* [22], and on an R plasmid in *V. anguillarum* [23]. Other genes, such as bla_{NDM-1}[24], bla_{CTX-M} and *qnrA* [25, 26] encoding beta-lactam and quinolone resistance, respectively, have also been found in human pathogenic vibrios. Some vibrios also harbour the metallo-beta-lactamase *varG* gene, which has its strongest activity against meropenem [27].

Antimicrobial resistance associated with the aquaculture industry is a recurring topic. Since it has been shown that heavy metals can induce and sustain antimicrobial resistance, there is a need to identify whether bacteria have acquired resistance to antimicrobials used in the 1980s and 1990s, as well as if the bacteria present at aquaculture locations are impacted by the continuous discharge of heavy metals into their environment. Hence, the aim of this project was to screen for antimicrobial and heavy metal resistance among marine *Vibrio* species and the faecal indicator organism *E. coli*.
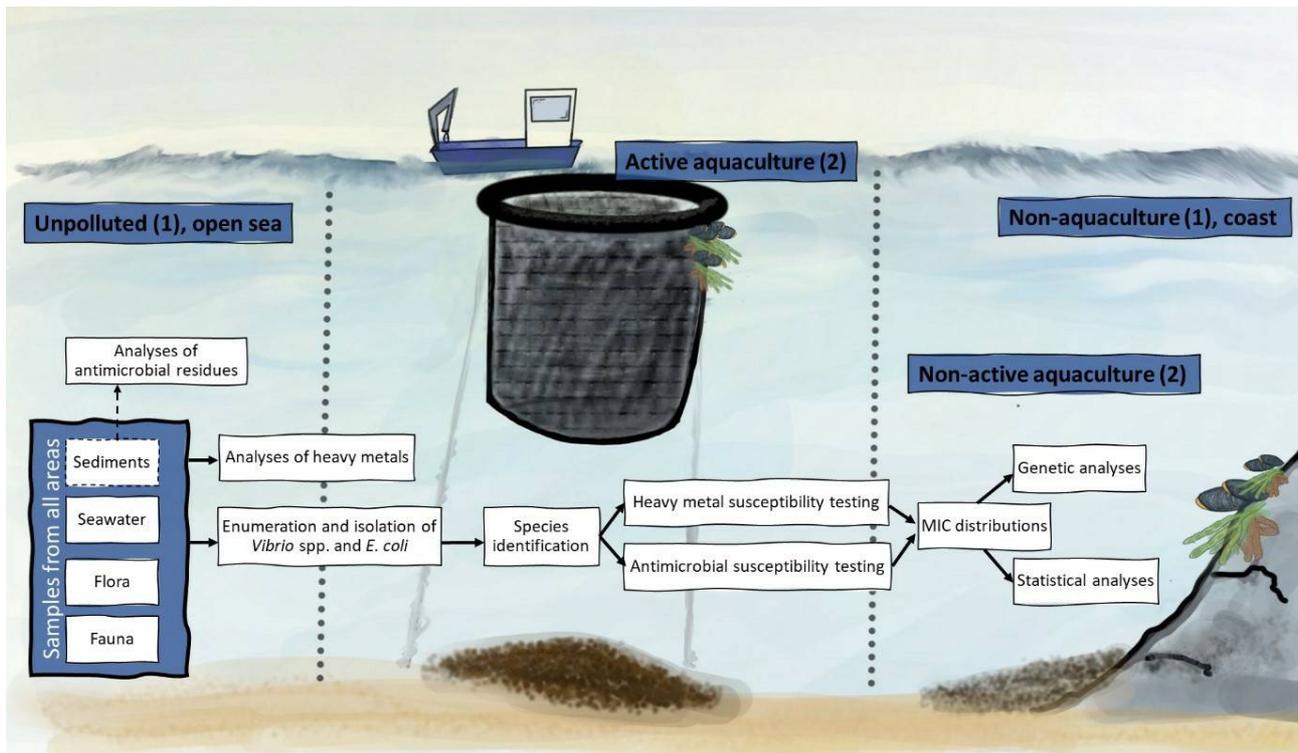
Figure 1. Flow chart of the presented project. The blue boxes show the four examined areas; Active aquaculture, Non-active aquaculture, Non-aquaculture and Unpolluted with number of locations in brackets. Key analyses are presented in white boxes.

3 - Materials and methods

3.1 - Locations

To investigate the presence of antimicrobial and heavy metal resistant bacteria in the marine environment and the possible relationship to copper discharges, samples were collected from two active aquaculture sites, referred to as Active aquaculture areas. These sites had known use of fish nets with anti-fouling agents and historical high concentrations of copper in sediments (information obtained from Fylkesmannen in Hordaland). Two closed aquaculture locations that were investigated for antimicrobial resistance in the early 90's were included to see if the resistance pattern changes after the production stops, referred to as Non-active aquaculture areas. Further two areas were included, both far away from aquaculture, one located close to shore (Non-aquaculture) and one in the open sea (Unpolluted).

3.2 - Sampling

Each location was sampled twice and from each sampling we aimed for six samples consisting of two samples of sediments, two water samples, one fauna sample (molluscs), and one flora sample (macroalgae). The sediments at the aquaculture locations were sampled with a large Van Veen Grab used for recipient analyses. At other locations, a small Van Veen Grab was used (Figure 2). Molluscs and macroalgae samples were collected in sterile plastic bags using sterile glows and sometimes tweezers and knives, into sterile plastic bags. The water samples were collected by a clean 3 litre Van Dorn water sampler and transferred to acid washed Teflon bottles. The fauna and flora samples were collected in sterile plastic bags using sterile gloves and tweezers. All samples were kept cold during transport, and microbial analyses started within 24 hours.


Figure 2. Van Veen Grab sediment sampler.

3.3 - Chemical analyses

3.3.1 - Analyses of heavy metals

The concentration of heavy metals in samples of sediments, fauna and flora were analysed in inductive-coupled plasma mass spectrometer (ICPMS) by a standardised accredited protocol for sample preparation and heavy

metal detection [28]. All samples were freeze-dried prior to analyses, and the sediment samples were additionally sieved through a 500µm mesh, and the smaller fraction was analysed. The seawater samples were examined by inductive-coupled plasma sector field mass spectrometer (ICP-SFMS) according to SS EN ISO 17924-1,2 and inductive-coupled plasma atomic emission (ICP-AES) according to ISO 11885, and the analyses were performed by ALS Laboratory Group Norway. The data sets were analysed for outliers, and one-way Anova (GraphPad Prism v8) was used for statistical comparison between areas.

3.3.2 - Screening for antimicrobial residues

The samples were examined by Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) screening for multiple antimicrobials. It is expected that antimicrobials will have longer half-life in sediments compared to water, fauna and flora samples. Sediment samples from all locations were therefore examined for residues of the antimicrobial's ciprofloxacin, enrofloxacin, oxolinic acid, flumequine, trimethoprim, and florfenicol. Additionally, four samples from Active aquaculture areas and two samples from Unpolluted areas, were also analysed for tetracycline, oxytetracycline, doxycycline and chlortetracycline.

3.4 - Quantitative and qualitative isolation

3.4.1 - Isolation of *Vibrio* spp.

1) Each sample of sediment, fauna and flora was quantitatively examined for Vibrios using one or both of the following selective methods: a) 10-fold dilutions with peptone water and spread on VibrioChromo-select agar and incubated at 25 °C for 48 hours. The limit of quantification (LOQ) was 10 colony forming units (CFU)/g. b) Most probable number (MPN) for Vibrios with 3x5 tubes dilution series with alkaline peptone water [29] for 48 hours and confirmation on VibrioChromo-select agar grown at 25 °C for 48 hours. The LOQ was 18 bacteria/100g.

2) Seawater samples were filtered through a 0.45µm filter in aliquots of 2x 100 ml and 2x 50 ml per filter and grown on VibrioChromo-select agar at 25 °C for 48 hours. LOQ was 1 CFU/50 ml.

3) From each sample, up to 20 different colonies were grown to pure culture and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, MALDI Biotyper®, Bruker) at the Norwegian Veterinary Institute (NVI), Bergen, using a combination of the reference library from Bruker and an «in-house» library created by the NVI. The bacteria were frozen in 25% glycerol and stored at -80 °C.

3.4.2 - Isolation of *Escherichia coli*

1) Each sediment sample, fauna sample and flora sample were analysed a) Quantitatively for *E. coli* using standardized MPN methodology [30] confirmed on TBX agar. The LOQ was 18 CFU/100g. b) Qualitatively for Enterobacteriaceae on MacConkey agar grown at 37 °C for 24 hours. Typical pink colonies were verified on Chromagar before making pure cultures. LOQ was 10 CFU/g.

2) Water samples were filtered in aliquots of 1x 200 ml and 1x 500 ml per filter and grown on MacConkey agar at 37 °C for 24 hours. The LOQ was 1 CFU/200 ml.

3) Up to 20 *E. coli* isolates from each sample were grown to pure culture and identified by MALDI-TOF MS at the NVI, Oslo, before frozen in 25% glycerol and stored at -80 °C.

3.5 - Phenotypic susceptibility testing

The bacterial isolates were tested for susceptibility to 1) at least eight antimicrobials and 2) the heavy metals copper, zinc and cadmium.

3.5.1 - Heavy metal susceptibility testing

Investigation of heavy metal resistance was carried out in 96-well plates with increasing concentration of copper, zinc or cadmium. For each heavy metal, a stock solution was prepared and used to make two-fold dilutions to obtain the range from 2 048 mg/L to 16 mg/L for copper (Figure 3) and zinc, and 1 024 mg/L to 8 mg/L for cadmium. The metals were dissolved in 50 ml dH2O and subsequently added 50 ml double strength Mueller Hinton broth. For *Vibrio splendidus*, the Mueller Hinton broth contained 4% NaCl. Overnight cultures were dissolved in sterile water with or without 2% NaCl to obtain 0.5 MacFarland and 30 µl were transferred to tubes with 5 ml Mueller Hinton broth with or without 2% NaCl. The plates were filled by using AIMs dispenser by first dispensing the different concentrations of the heavy metals followed by dispensing the sample. The plates were covered with plastic film for 24-48 hours. The first well without growth were recorded as minimum inhibition concentration (MIC) [31, 32].
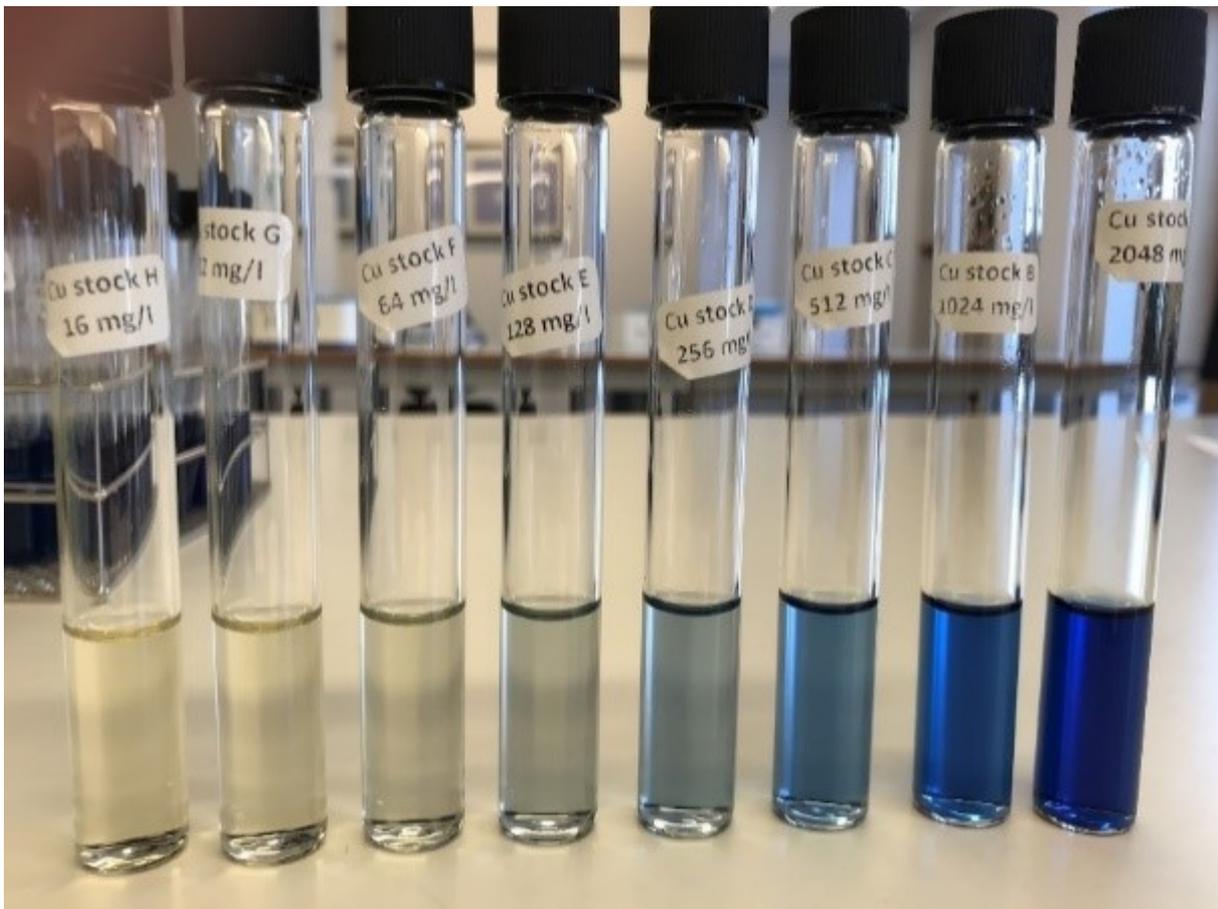
Figure 3. Two-fold dilution series of copper dissolved in Mueller Hinton broth with concentrations from 16 mg/L to 2 048 mg/L.

3.5.2 - Antimicrobial susceptibility testing

Susceptibility to antimicrobials were tested using standard broth microdilution methods. For susceptibility testing of *E. coli* isolates, the Sensititre® EUVSEC panel (TREK Diagnostics, LTD.) was used. This panel contains the following substances, ampicillin, cefotaxime, ceftazidime, meropenem, tetracycline, tigecycline, sulfamethoxazole, trimethoprim, nalidixic acid, ciprofloxacin, gentamicin, chloramphenicol, colistin and azithromycin. Epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, accessed 23.03.2020) were used, except for azithromycin for which cut-off values are not defined. See Table A2. for definitions of cut-off values.

There are no similar standardized panel for *Vibrio* spp., and custom-made panels from Sensititre® (TREK

Diagnostics, LTD.) were made. These plates contain ampicillin, cefotaxime, ceftazidime, meropenem, oxytetracycline, tigecycline, sulfamethoxazole, trimethoprim, oxolinic acid, ciprofloxacin, gentamicin, florfenicol, colistin, azithromycin and nitrofurantoin. Prior to analysis, different *Vibrio* spp. were tested for cultivation on Mueller Hinton media with or without 2% NaCl. For *V. splendidus*, the addition of salt was needed to obtain growth. Plates were incubated at 25 °C for 24-48 hours. Since there are still no breakpoint values for *Vibrio* spp., an ECV was calculated for the combination of each of the *Vibrio* spp./antibiotics by ECOFFinder [33] based on the obtained MIC distribution data. ECV at 97.5% were applied, see Table A2. for definitions of cut-off values. Isolates with MIC above ECV are referred to as resistant, even though there is an uncertainty in the defined break-point as it is based on the data obtained in this project only.

3.6 - Data processing

Data from antimicrobial sensitivity testing (AST) were presented with 95% confidence intervals calculated by the exact binomial test using R version 3.5.2 for Windows (R Development Core Team, 2019). For *Vibrio* spp., the MIC distributions were analysed for differences in respect of sampling area for the tested antimicrobials and heavy metals by nonparametric OneWay Anova with KruskWallis test in GraphPad Prism 8.4.3 (©1992-2020 GraphPad Software, LLC). Susceptibility data for *E. coli* were recorded and stored as discrete values (MIC). Data management and analysis were performed in SAS-PC System® v 9.4 for Windows (SAS Institute Inc., Cary, NC, USA) and in R v4.03 [34].
Graphs were made in GraphPad Prism 8.4.3 (©1992-2020 GraphPad Software, LLC) and Microsoft® Excel® for Microsoft Office 365® ProPlus. Pearson correlation test was performed to tests for possible pairwise correlations between the occurrence of specific resistances towards antimicrobials and the heavy metals copper, zinc and cadmium in samples where both *Vibrio* spp. and *E. coli* had been isolated (N=147).

3.7 - Genotypic resistances testing

Bacterial isolates with special resistance patterns were subjected to whole-genome sequencing. DNA from the *Vibrio* isolates were extracted with Qiagen Blood and Tissue kit (Qiagen). Library preparations were performed applying Swift Turbo 2S flex prior to sequencing with Illumina MiSeq with 2 x 300 bp, both steps performed at the Norwegian Sequencing Centre (Oslo). Paired end reads were trimmed in bbMAP 31 [35] and assembled applying SPAdes v.3.13.0 [36]. Genomes were blasted against BacMet predicted database v2.0 [37] to assess heavy metal resistance genes. The genomes were also uploaded to National Center for Biotechnology Information, where the genomes were annotated prior to manual assessment of resistance genes.
DNA from the *E. coli* isolates were extracted using Qiagen QIAamp® Mini kit (Qiagen). The DNA extracts were sequenced at the NVI using Illumina® MiSeq with Nextera DNA Flex library prep. Paired end reads were trimmed and assembled using the Bifrost pipeline [38]. Assemblies were subjected for analysis for both acquired genes and chromosomal point mutations using the ResFinder v4.1 pipeline at the Centre for Genomic Epidemiology web site (https://cge.cbs.dtu. dk/services/ResFinder/) [39, 40].

3.8 - Microbiome

From each sediment sample, microbiome analyses were prepared for 16S rRNA gene amplicon sequencing, to compare the overall microbial (bacterial and archaeal) richness and diversity between locations. Sequence data was processed using the DADA2 [41] software implemented in the Qiime2 [42] pipeline, to construct ASVs (Absolute Sequence Variants), where each ASV represents one bacterial or archaeal species. Statistical analyses to compare sample diversity between locations was done using R (R core team, 2020, https://www.R-project.org) calculating Shannon index for each sample. This index is a function of number of species observed,

and the evenness of their distribution within a community. A high Shannon diversity is usually considered to be a sign of a healthy environment, whereas low Shannon diversity can be a sign of the opposite.

4 - Results

4.1 - Sampling

Successful sampling was carried out twice at six different locations representing four different areas. Table 1 shows sampling position, description of the site, obtained samples and date of sampling. We failed to get a complete sample set at two locations and this was compromised by increasing the number of samples that were accessible. In total, 70 samples were collected.Table 1. Information about locations, sampling dates and samples taken twice at four different areas.

Table 1. Information about locations, sampling dates and samples taken twice at four different areas.

Area	Location	Positions	Description	Depth	Water temp.	Samples	Date Sampled
Active aquaculture	**1**	Anonymous	Active fish farming site licenced since 1973, with annual fish production of 2600 tonnes using on average 3300 tonnes fish feed.	500-600 m, steep rocky bottom with sludge sediment pockets.	12 °C, 8 °C	Water(2+2) Sediment(1+2) Fauna(2+1) Flora(2+1)	30.10.2018 26.03.2019
	2	Anonymous	Active fish farming site licenced sin 1973, located 20 km from Location 1. Average fish production is 1800 tonnes using approx. 2250 tonnes fish feed.	100-250 m, gravel and sludge sediment pockets.	12 °C, 11 °C	Water(2+2) Sediments(2+2) Fauna(0+0) Flora(2+1)	14.11.2018 07.10.2019
Non-active aquaculture	**5**	N60° 07' E5°13'	Kobbavika, Austevoll. Aquaculture site closed in 2008.	7-15 m, shell sand	5 °C, 17 °C	Water(2+2) Sediments(2+2) Fauna(1+1) Flora(1+1)	05.03.2019 13.08.2019
	6	N60° 16' E5°05'	Skogsvågen, Sotra. Closed aquaculture site examined for antimicrobial resistant *Vibrio salmonicida* in 1988 [1].	4-13 m, gravel	5 °C, 15 °C	Water(2+2) Sediments(2+2) Fauna(1+1) Flora(1+1)	05.03.2019 09.09.2019
Non-aquaculture	**4**	N59° 55' E5°15'	Pilapollen, Fitjar, a semi-closed, shallow bay protected from aquaculture impact, with small boat travellers during the summer months.	6-10 m, muddy bottom	5 °C,18 °C	Water(2+2) Sediments(2+2) Fauna(1+1) Flora(1+1)	17.02.2019 13.08.2019
Unpolluted	**3**	N58°58' E02°11' and N58°21' E1°49'	North Sea (3A) and Norwegian Sea (3B) (Control location)	70 m and ca.100 m, gravel	10 °C, 10 °C	Water(2+2) Sediments(2+2) Fauna(0+2 fish) Flora(0+0)	16.11.2018 30.05.2019 01.10.2019

4.2 - Sample description

4.2.1 - Water samples

Water samples were obtained from all locations and the aim was to have sampling points with different water temperatures. However, for three of the locations, the temperatures were quite similar. The sampling at these locations was carried out between 5 – 11 months apart. For Location 3 this is as expected as the temperature in the North Sea does not vary that much.

4.2.2 - Sediment samples

Sediment samples were also collected from all locations, with one sample missing at Location 1. This was caused by the uneven bottom topography at the location, where all grab hauls except one, came back empty. The bottom at this location is characterised as "rocky bottom". Furthermore, the sediments from the different locations varied in composition where the Active aquaculture areas had sludge sediments, the Non-active aquaculture areas had shell sand and gravel, the Non-aquaculture area had mud sediments, and the Unpolluted areas contained gravel sediments. This means that the sediments also differed in grain size distribution (not measured) which impact the sediments ability to retain heavy metals and antimicrobials. Furthermore, the sludge sediments have high production of hydrogen sulphide (H_2S) having anaerobic conditions (Figure 4) that could impact on the retrieved species.

Figure 4. Grab haul with sediments from an Active aquaculture area (left) and shell sand from Non-active aquaculture area (right).

The amount of organic material in the sediments were examined by subtracting the ash content (g/100g) from the dry matter content (g/100g), showing that the sludge samples from Active aquaculture had higher content of organic material (Figure 5).
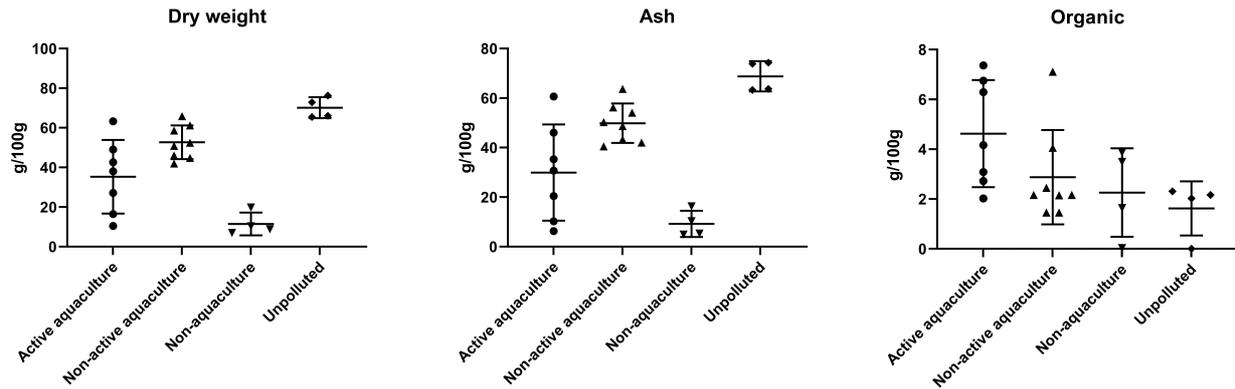
Figure 5. Mean ± SD and individual values of measured dry weight, ash content and organic material in sediments, all in g/100g for the four tested areas, Active aquaculture (n=7), Non-active aquaculture (n=8), Non-aquaculture (n=4) and Unpolluted (n=4).

4.2.3 - Marine fauna samples

The marine fauna samples comprised mostly of blue mussels (*Mytilus edulis*). At one Non-active aquaculture location, only limpets (*Patella vulgata*) could be found. They are grassing and not filtrating animals such as blue mussels but still represent quite stationary fauna from this location. Since there are no blue mussels growing in the North Sea, only fish were included as fauna. The fish samples were divided into fish skin/muscle and fish intestine.

4.2.4 - Marine flora samples

The flora samples comprised algae species that were accessible at the locations. For most areas this included brown algae such as bladder wrack (*Fucus vesiculosus*), sugar kelp (*Saccharina latissima*), channelled wrack (*Pelvetia canaliculata*) and rock weed (*Ascophyllum nodosum*). At one of the aquaculture locations, no brown algae were found, whereas scrapings of green algae (family Chlorophyta) were collected.

Further on, the samples are discussed according to area of origin.

4.3 - Microbiome in sediments

The effects of the aquaculture activity on the diversity and composition of the entire microbial community in the sediment samples was assessed using the 16S rRNA gene of bacteria and archaea.

A clear difference in microbial community structure was observed between sediment samples from Active aquaculture, Unpolluted and Non-active aquaculture areas (Figure 14). We also found higher overall Shannon diversity in samples obtained from Unpolluted and Non-active aquaculture areas than we did in the Active aquaculture areas (Figure 6), indicating a less healthy environment.
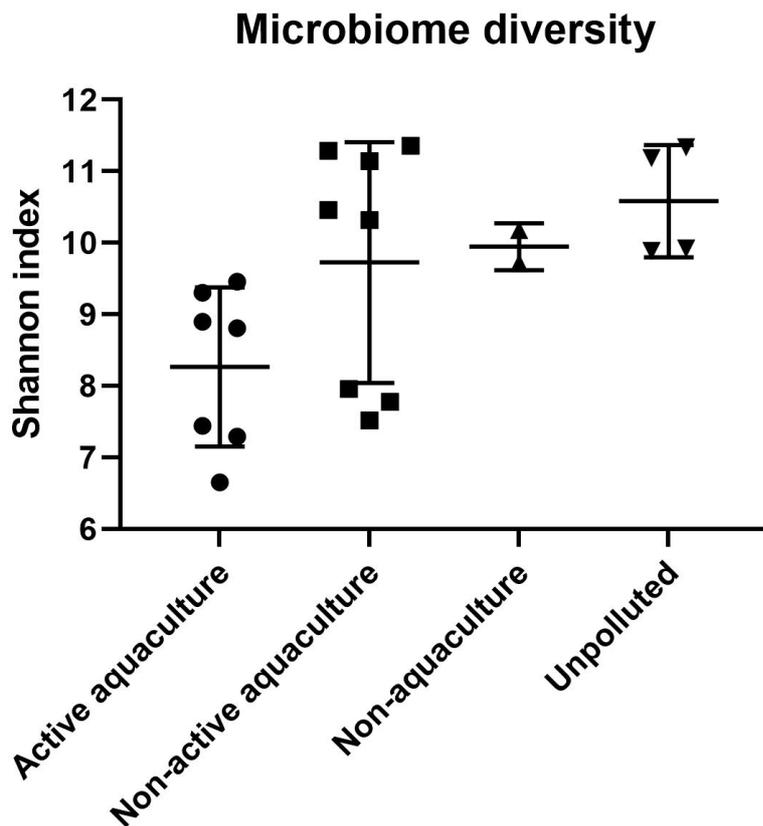
Figure 6: Mean ± SD and individual values of calculated Shannon index, as a measure of bacterial diversity at the different examined areas, Active aquaculture (n=7), Non-active aquaculture (n=8), Non-aquaculture (n=4) and Unpolluted (n=4).

However, the sediment types also differed between the sampling areas (Figure 7). Sediment type and composition can be an important determinant of microbial community structure because of differences in nutrient and carbon availability. In addition, the sediments obtained from Active aquaculture areas had high H_2S content, indicating they were most likely anaerobic. There are usually large differences in bacterial community compositions found in anaerobic sediments compared to aerobic sediments. It is therefore difficult to determine

if the observed differences in this dataset is due to aquaculture activity or differences linked to sediment type (Figure A1).
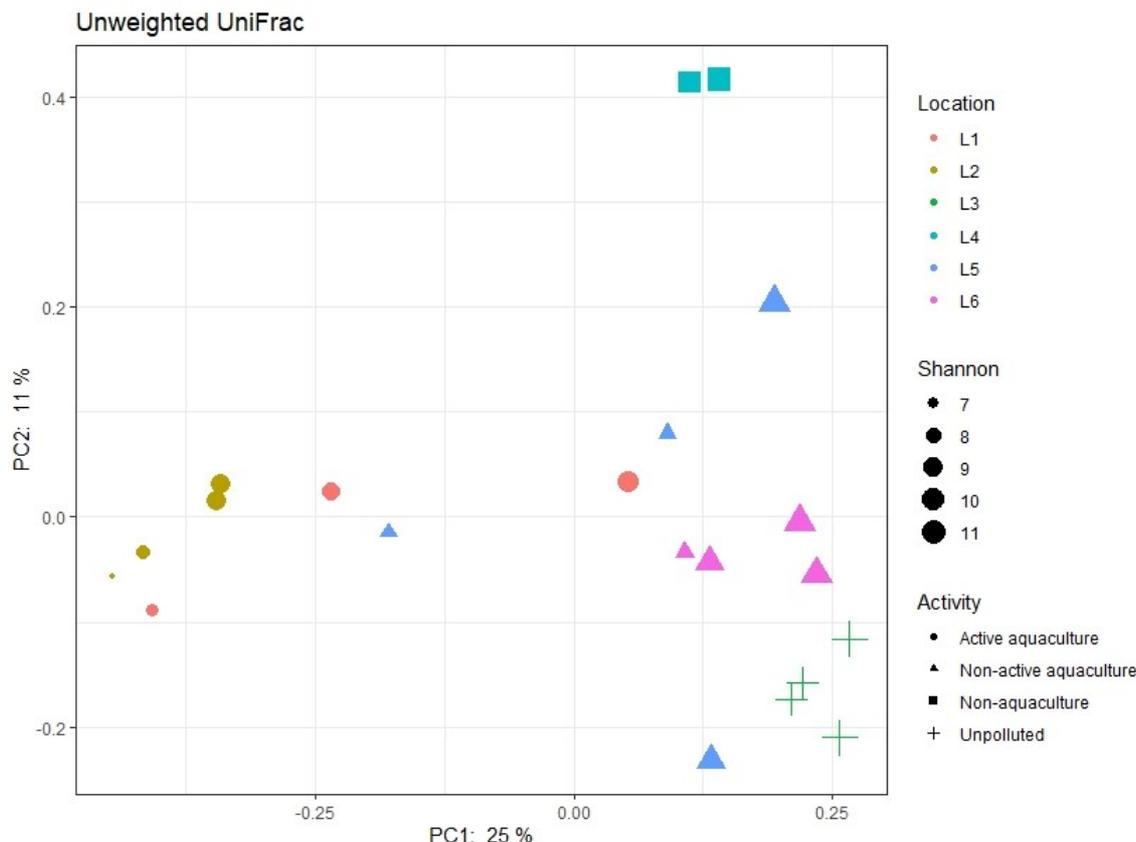
Figure 7. Principal component analyses indicating similarity in bacterial composition of samples regarding the activity of the sampling site (shape of symbols) and Shannon values (size of symbols) indicating the diversity of 16S sequences observed in the samples. The closer two points are on the plot, the more similar they are in microbial community composition. L1-L6 refers to the locations described in Table 1.

4.4 - Heavy metals and antimicrobial residues in samples

All samples were examined for the heavy metals copper, zinc and cadmium by ICP-MS. One sample from a Active aquaculture area had the highest measured concentration of heavy metals with 2868.0 mg/kg d.w. copper and 774.4 mg/kg of zinc. Despite that this samples was considered an outlier, and were not inclueded in the mean concentrations, were sediment samples from Active aquaculture areas significantly higher than all other areas for both copper and zinc with mean of 272.3 mg/kg d.w. and 424.6 mg/kg d.w., respectively (Figure 8). This corresponds to extensively toxic (Class V) and moderate toxic (Class III) (Table A1), according to the classification of polluted environments [43]. Both the Non-active aquaculture and Unpolluted areas were classified with no toxic effect with mean concentrations of 9.8 mg/kg and 2.3 mg/kg, respectively, for the copper. The Non-aquaculture location had high levels of cadmium, which could be related to the mechanical industry with previous registered discharge of metals within 10 kilometres (Miljøstatus.no). Among the samples of blue mussels, the ones from Active aquaculture contained the highest concentration of copper (19.1 mg/kg d.w.), whereas for zinc and cadmium, the Non-aquaculture area were highest.
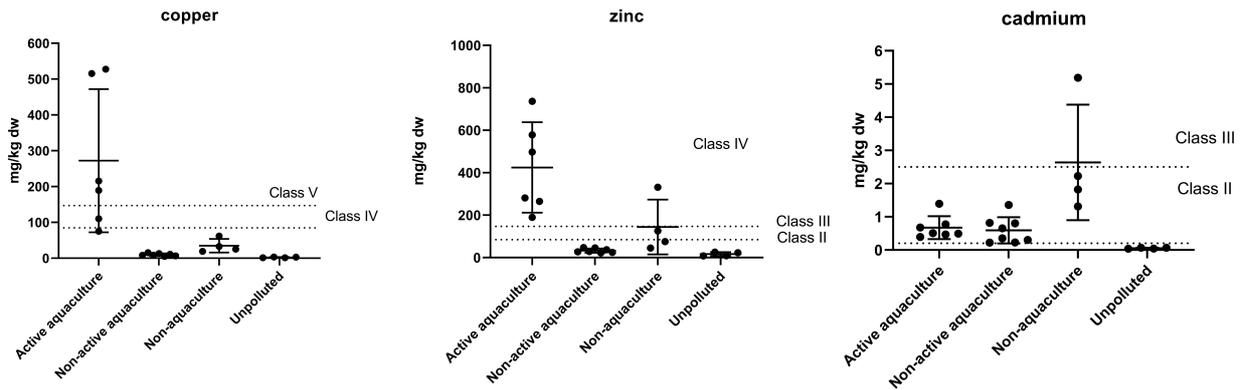
Figure 8: Mean ± SD and individual values of copper, zinc and cadmium (mg/kg d.w.) in sediments from the areas Active aquaculture (n=6), Non-active aquaculture (n=8), Non-aquaculture (n=4) and Unpolluted (n=4). Dotted line indicates limits for classification of polluted environments [43].

Analyses of heavy metals in seawater showed that the highest levels of copper and zinc were found at the Active aquaculture areas. None of the samples contained cadmium above the LOQ at 0.5 µg/l (Figure 9)
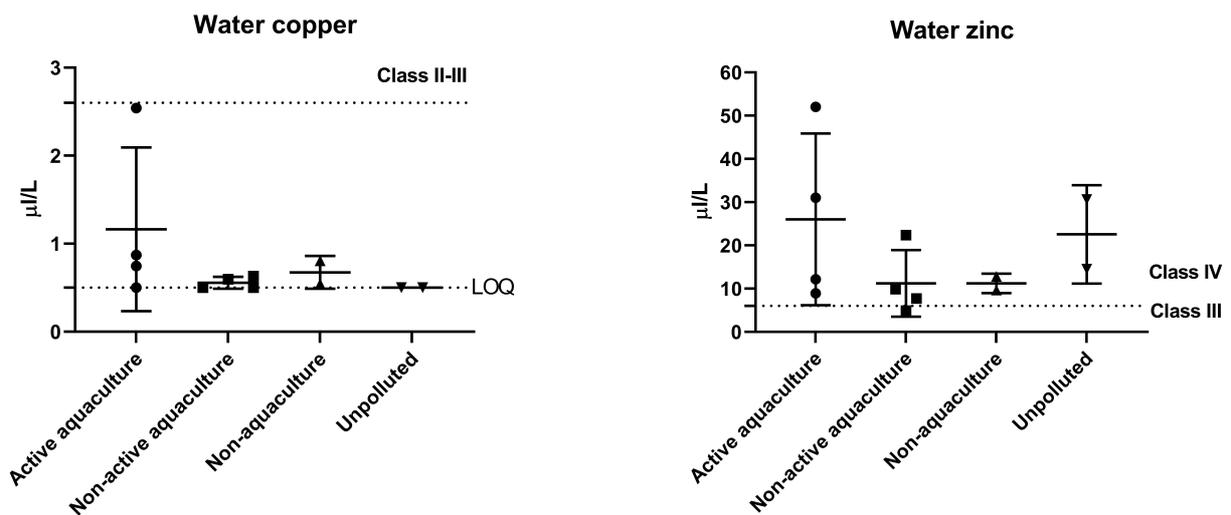
Figure 9. Mean ± SD and individual concentrations (µg/L) of copper and zinc in seawater from the areas Active aquaculture (n=8), Non-active aquaculture (n=8), Non-aquaculture (n=4) and Unpolluted (n=4). Dotted line indicates limits for classification of polluted environments [43].

Among the sediment samples that were analysed for antimicrobial residues, none of the samples contained any antimicrobials above the LOQ of the applied method. List of examined samples, results and LOQ for the different antimicrobials are found in Table A3.

4.5 - Quantitative analyses and Culture collection

The prevalence of *Vibrio* spp. and *E. coli* were assessed quantitatively from all sample types. The results showed highest number of *Vibrio* spp. in flora and fauna sample at Active aquaculture areas (Figure A2), and highest number of *E. coli* in flora and fauna samples at the Non-aquaculture areas (Figure A3). However, in sediment samples and water samples analysed for *E. coli*, the highest concentrations were found in the Active

aquaculture areas, 220 CFU/100ml and 31 CFU/100ml, respectively. The concentrations of *E. coli* indicate faecal pollution. In the water samples, the median value of *E. coli* was 7.1 CFU/100ml for Active aquaculture, 5 CFU/100ml for Non-active aquaculture, 6 CFU/100ml for Non-aquaculture and 0 CFU/100ml for Unpolluted. A clear difference was seen for samples sampled during October-March and April-September, where a 14-fold increase were seen in warmer months for Active aquaculture areas. For the Non-active aquaculture areas and the Non-aquaculture area, the increase were 5 and 6 folds higher during the warmer months.
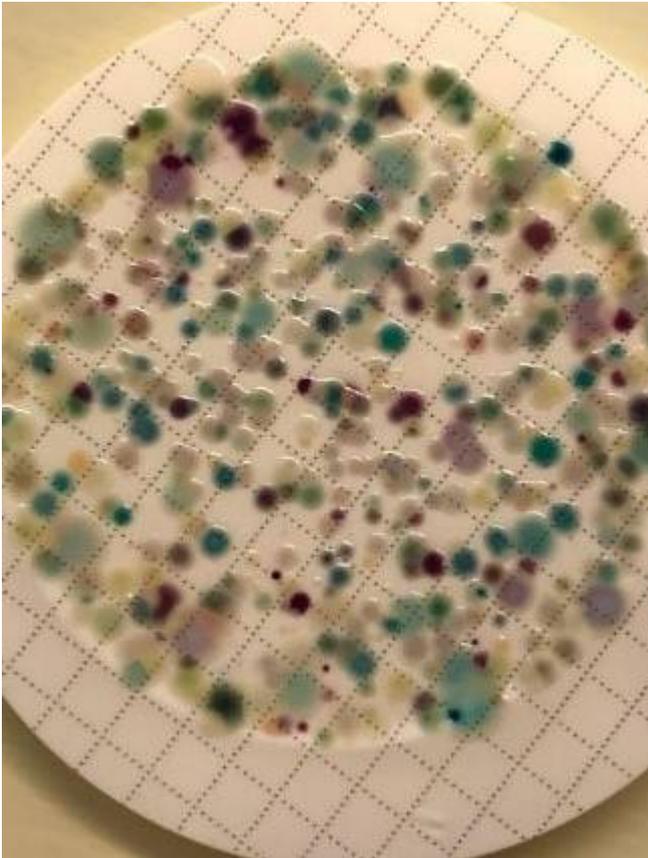
Figure 10. Growth of different Vibrio species on VibrioChromoSelect agar.

From the same samples, 690 *Vibrio* isolates were obtained, and the two dominating species were selected for further analyses, and comprised 350 *V. splendidus*, 73 *V. anguillarum*. It was particularly challenging to sample *Vibrio* spp. from sediments with strong H2S-production.

Forty-three samples were presumptive positive for *E. coli* and 346 isolates were collected from these. Of these, 336 were confirmed to be *E. coli*. No *E. coli* were found from the samples originating from the Unpolluted areas at sea, which was expected.

Figure 11 shows the number of isolates from the different sample types.
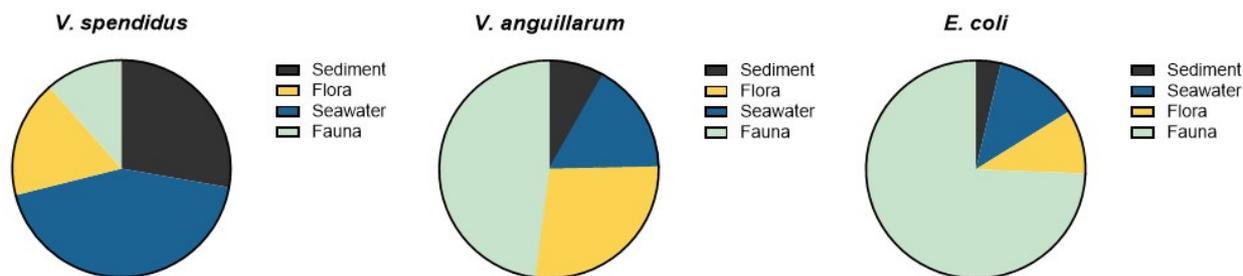
Figure 11. Distribution of V. splendidus, V. anguillarum and E. coli isolates obtained from sediments, flora, seawater and fauna, respectively.

4.6 - Phenotypic antimicrobial susceptibility testing

4.6.1 - Susceptibility according to areas

A total of the 759 isolates were tested for antimicrobial susceptibility. This included 350 *V. splendidus*, 73 *V. anguillarum* and 336 *E. coli*. An overview of the number of *V. splendidus* and *E. coli* isolates above ECV for the three species is shown in Figure 12. The isolates belonging to *V. anguillarum* showed only few isolates above ECV and the date are briefly presented in chapter 4.6.3 and in Table A4.

For *V. splendidus* 56.0% [95% CI: 50.6-61.3] were susceptible to all the antimicrobial classes tested for. The Active aquaculture areas had highest proportion of susceptible isolates with 71.0% [95% CI: 63.0-78.9], followed by Non-aquaculture areas with 57.0% [95% CI: 39.5-72.9], Unpolluted areas 56.3% [95% CI: 44.0-68.1] and Non-active aquaculture areas 37.0% [95% CI: 27.7-46.5].

For *E. coli*, 91.7% [95% CI: 88.2-94.4] of the isolates were susceptible to all of the antimicrobial classes tested for, with a lower proportion in Active aquaculture areas and Non-active aquaculture areas with; 88.2% [95% CI: 82.0-92.9] and 92.0% [95% CI: 85.3-96.3], respectively, as compared to Non aquaculture areas with 98.6% [95% CI: 92.4-100.0].
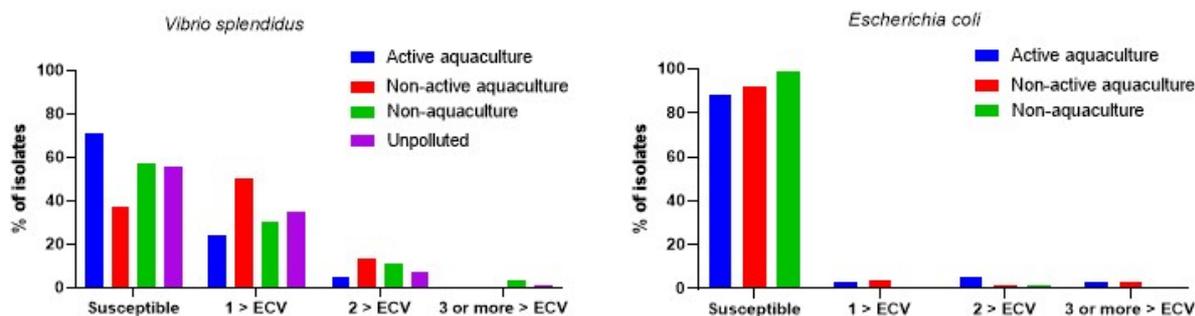
Figure 12. Proportion (%) of V. splendidus (n= 350) and E. coli (n=336) isolates with MIC values above the Epidemiological cut-off values for antimicrobial classes shown according to areas of origin.

4.6.2 - *Vibrio splendidus*

The antimicrobial susceptibility testing of 350 *V. splendidus* isolates (Figure 13) showed that growth above ECV were most common for sulfamethoxazole 44.9% [95% CI: 39.5-50.2], ampicillin [95% CI: 11.1 [8.0-14.9]] and trimethoprim 4.0% [95% CI: 2.2-6.6]. The Non-aquaculture areas had the highest proportion of isolates above ECV for ceftazidime, colistin, meropenem and trimethoprim. The Non-active aquaculture areas had the highest proportion of isolates above ECV for sulfamethoxazole, however, all the areas had high proportions above ECV

for this agent. The Unpolluted areas had highest percentage above ECV for ampicillin. The Active aquaculture areas were the only areas that were above ECV for oxytetracycline and had four isolates showing one and two-fold higher MICs compared to all the other areas. However, a significant difference was seen for the MIC distribution of *V. splendidus* isolated from the Unpolluted areas compared to all the other areas, and not only for the Active aquaculture areas. None of the isolates were above the calculated ECV for azithromycin, cefotaxime, ciprofloxacin, florfenicol, nitrofurantoin, oxolinic acid and tigecycline. MIC distributions are presented in Table A5.
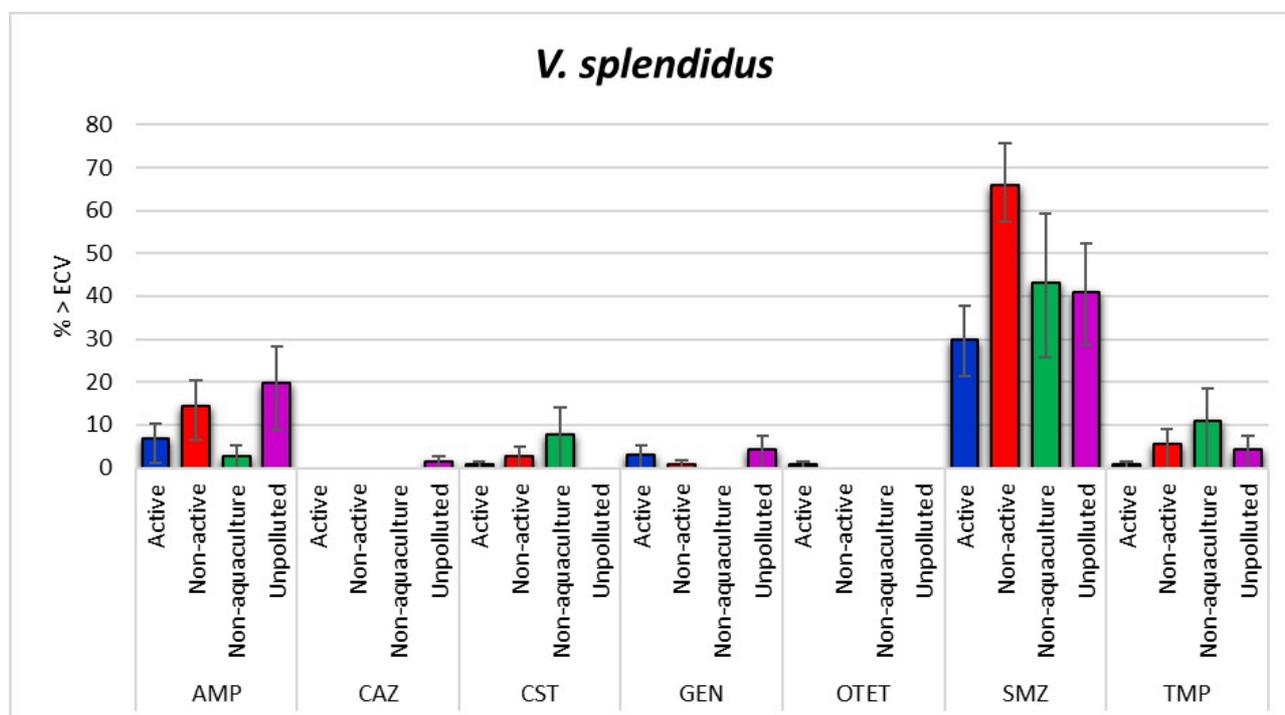
Figure 13. Proportion (%) of V. splendidus isolates with MIC values over the epidemiological cut-offs (ECV) with 95% confidence intervals. Proportions are shown according to isolates belonging to Active aquaculture (n=133), Non-active aquaculture (n=109), Non-aquaculture (n=37) and Unpolluted (n=71) areas. AMP=ampicillin, CAZ=ceftazidime, CST=colistin, GEN=gentamicin, OTET=oxytetracycline, SMZ=sulfamethoxazole, TMP=trimethoprim.

4.6.3 - *Vibrio anguillarum*

Antimicrobial susceptibility testing of 73 isolates belonging to *V. anguillarum* (Table A4) showed that MIC values were below the calculated ECV for 14 of the 15 agents included in the test panel. Seven isolates had MIC values above the ECV for colistin of 4 mg/L, and three of these were found in the Unpolluted areas. However, only four isolates were from these areas, thus the results are inconclusive.

4.6.4 - *Escherichia coli*

The most common resistances observed among the 336 *E. coli* isolates were to tetracyclines (5.4%; [95% CI: 3.2-8.3]), sulfonamides (4.2%; [95% CI: 2.3-6.9]) and ampicillin (3.6%; [95% CI: 1.9-6.2]) (Table A6). The proportion of resistance in the Active aquaculture and Non-active aquaculture areas was quite similar whereas only 1.4% of the isolates were resistant to ampicillin and tetracycline in the Non-aquaculture area. Only one isolate, which originated from the Active aquaculture areas, was resistant to third generation cephalosporins. However, as shown in Figure 14, any significant differences between the locations could not be detected. This is probably mainly explained by the low number of isolates obtained in the Non-aquaculture area. Four isolates were resistant to quinolones, ciprofloxacin and/or nalidixic acid. There were no isolates with resistance to gentamicin, meropenem or tigecycline observed.
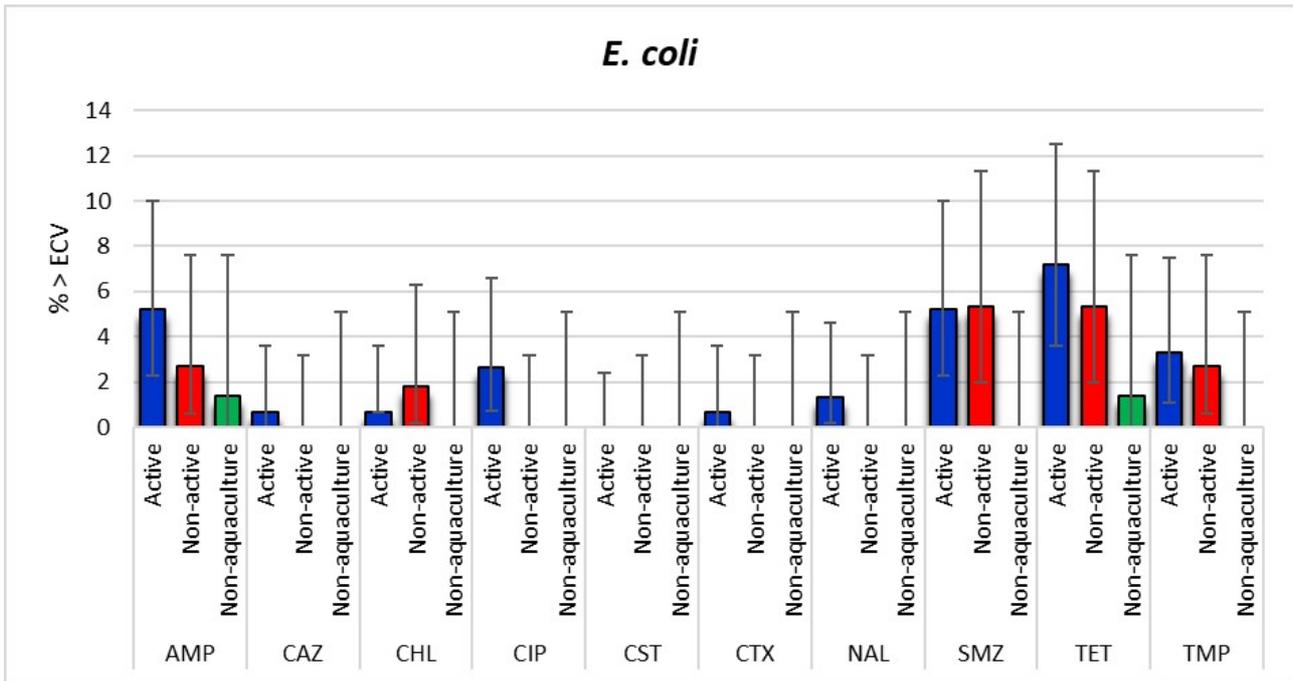
Figure 14. Proportion (%) of E. coli isolates with MIC values above the epidemiological cut-offs (ECV) with 95% confidence intervals. Proportions are shown according to isolates belonging to Active aquaculture (n=153), Non-active aquaculture (n=112), and Non-aquaculture (n= 71) areas. No isolates were found in the Unpolluted areas. AMP=ampicillin, CAZ=ceftazidime, CHL=chloramphenicol, CIP=ciprofloxacin, CST=colistin, CTX=cefotaxime, NAL=nalidixic acid, SMZ=sulfamethoxazole, TET=tetracycline, TMP=trimethoprim.

4.7 - Heavy metal susceptibility testing

4.7.1 - *V. splendidus* and *V. anguillarum*

Both the *V. anguillarum* (Table A7) and *V. splendidus* (Table A8) were tested for susceptibility to the heavy metals copper, zinc and cadmium. Two *V. splendidus* isolates from Active aquaculture areas showed MIC above ECV for copper at >1024 mg/kg, and were two-fold higher than the isolates from the Unpolluted areas (Figure 15). However, only the Non-active aquaculture area showed significant higher MIC distribution for copper, compared to the Unpolluted areas, and only a trend was seen for the Active aquaculture areas. Resistance to zinc were seen in all areas with highest percentage of isolates from Unpolluted areas, followed by the Non-aquaculture area.
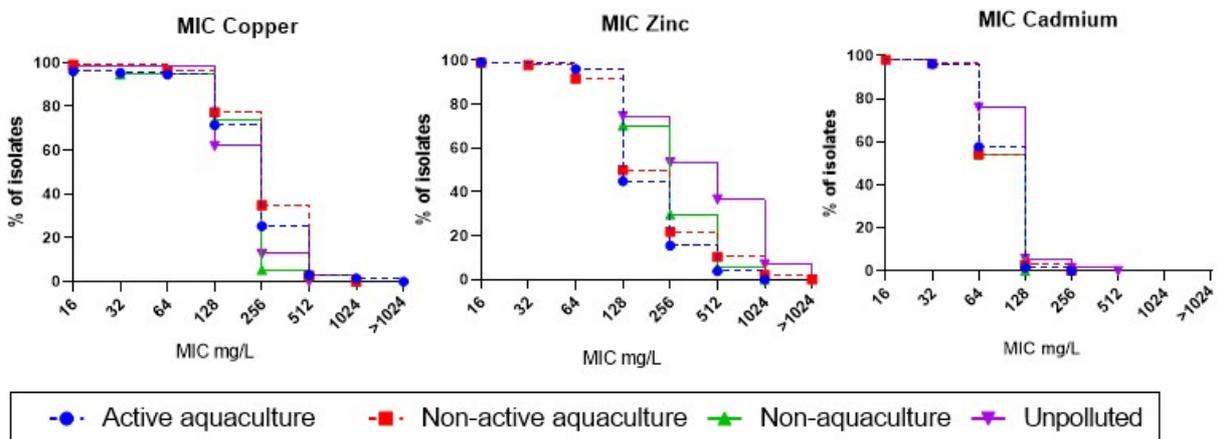
Figure 15. MIC distribution for V. splendidus tested against the heavy metals copper, cadmium and zinc.

4.7.2 - *E. coli*

All *E. coli* isolates were tested for heavy metal susceptibility with the same concentrations as for the vibrios. Because of low variability of the results, no ECVs were calculated. Among the 336 isolates that were tested, 98% and 99% showed MIC above 1024 mg/L for copper and zinc, respectively. For cadmium, 95% had MIC of 128 mg/L, whereas 1.5% had MIC of 256 mg/L.

4.8 - Whole genome analyses

Whole genome sequencing was performed on a total of eleven *V. splendidus*, two *V. anguillarum* and four *E. coli*. All the *Vibrio* genomes harboured the tetracycline resistance gene *tet(34)*, the chloramphenicol resistance gene *catB* and the multidrug efflux pumps encoded by the *emrD* gene and the *bcr/cflA* gene, the cadmium resistance gene *cadA* and the arsenite resistance gene *acr3*, all which are found in the reference genome of *V. splendidus* and *V. anguillarum*. Furthermore, some of the isolates harboured the trimethoprim resistance gene *dfrA*, beta-lactamase genes *bla* and *ampC*, and the quinolone resistance gene *qnrS*, in combination or alone (Table 2).

Searching for HMRGs revealed that all *V. splendidus* harboured several multidrug efflux systems such as RND (*vmeD*, *vmeF*, *vmeI*, *vmeV*, *vmeY*, *vmeZ*) and *norM* (*vcmA*), whereas some of the isolates carried additional multidrug efflux system genes, such as *mtrF* and *vcrM* (Table 5). The two *V. anguillarum* isolates carried the multidrug MFS transporter *vceB*. The arsenate resistance genes *acr3* and *aioB/aoxA* were present in both *V. splendidus* and *V. anguillarum* isolates. One of the isolates from Active aquaculture areas harboured the *nlpE* gene encoding a copper sensing lipoprotein that induces stress response and activates efflux pumps. None of the known plasmid borne HMRGs such as *zneB* (zinc), *czcC* (cadmium, zinc), *pcoA*, *copA*, *copB*, *copJ*, *cusB*, (copper) [44], were detected.

Table 2. Overview of genes detected in the 13 sequenced genomes of V. splendidus and V. anguillarum.

Areas	Isolate No.	Species	>ECV AB	ARGs				HMRGs					
				dfrA	*bla*	*ampC*	*qnrS*	*mtrF*	*vcrM*	*aioB/aoxA*	*vmeY*	*nlpE*	*vceB*
Active aquaculture	*2019-397-21*	*V. splendidus*	*STX, TRIM*	X				X	X	X			
Active aquaculture	*2019-399-11*	*V. splendidus*	*CST*		X			X	X	X			
Active aquaculture	*2018-2091-12*	*V. splendidus*	*OTET*					X	X	X	X	X	
Non-active aquaculture	*2019-184-12*	*V. anguillarum*				X							X
Non-active aquaculture	*2019-184-17*	*V. anguillarum*	*STX*			X							X
Non-active aquaculture	*2019-188-18*	*V. splendidus*	*STX, TRIM*	X									
Non-active aquaculture	*2019-190-8*	*V. splendidus*	*STX, TRIM*	X	X		X						
Non-active aquaculture	*2019-193-10*	*V. splendidus*	*CST*				X	X	X	X			
Non-active aquaculture	*2019-195-7*	*V. splendidus*	*CST*				X	X	X	X			
Non-active aquaculture	*2019-196-14*	*V. splendidus*	*STX, TRIM*	X				X					
Non-active aquaculture	*2019-195-20*	*V. splendidus*	*STX, TRIM*	X			X	X	X	X			
Non-aquaculture	*2019-955-15*	*V. splendidus*	*CST, STX*					X	X	X			
Unpolluted	*2019-1572-18*	*V. splendidus*	*STX, TRIM*	X	X			X					

CST=colistin, STX=sulfamethoxazole, TRIM=trimethoprim, OTET=oxytetracycline

Among the four *E. coli* isolates which were subjected to whole genome sequencing, three were sequenced due

to elevated MIC values for ciprofloxacin and/or nalidixic acid, while the last isolate expressed resistance to extended spectrum cephalosporins as well as quinolones and tetracyclines. In the three quinolone-resistant *E. coli* isolates, the resistance was due to one or two mutations in the chromosomally located *gyrA* gene (p.S83L and p.D87N) in two isolates and due to a plasmid mediated gene qnrS1 in the last isolate (Table 3). The resistance to extended spectrum cephalosporins was due to the $bla_{CTX-M-15}$ gene. This isolate also harboured the *qnrS1* and *tet(A)* genes conferring resistance to ciprofloxacin and tetracyclines, respectively.

Several genes associated with heavy metal resistance were detected (Table A9), and most of the genes are also found in reference *E. coli* genomes, including *cusA*, *zntA*, and several efflux pumps (*acrB*, *acrD*, *mdtA*, *marA*) involved in copper and zinc efflux. Two isolates harboured the plasmid borne *pcoA* gene, which encodes oxidoreductase proteins that detoxifies copper in the periplasm [45].

Table 3. Overview of genes detected in sequenced genomes of E. coli.

Areas	Isolate	>ECV AB	ARGs					HMRGs	
			$bla_{CTX-M-15}$	qnrS1	tet(A)	gyrA (p.S83L)	gyrA (p.D87N)	pcoA	pcoS
Active aquaculture	2019-399-2	CIP, NAL				X			
Active aquaculture	2019-400-1	CIP, NAL				X	X	X	
Active aquaculture	2019-400-9	CIP		X					X
Active aquaculture	2019-604-8	AMP, TET, NAL, CIP, FOT, TAZ	X	X	X			X	X

CIP=ciprofloxacin, NAL=nalidixic acid, AMP=ampicillin, TET=tetracyclines, FOT=cefotaxime, TAZ=ceftazidime

4.9 - Methodological considerations

This project tried to identify different *Vibrio* spp. that could function as indicator for antimicrobial resistance in the marine environment. Such an indicator should comply with some key characteristic being; 1) present in the tested environment, 2) being able to grow under laboratory conditions, and 3) express antimicrobial resistance relevant for surveillance.

Vibrio spp. showed some limitations, as they were not present in the anaerobic sediments containing H_2S. This made it difficult to compare sediments from the different examined areas. Therefore, for further assessment of antimicrobial resistance in sediments, an alternative target bacterium needs to be identified.

Furthermore, methods to perform AST in *Vibrio* spp. are not standardised in the same way as it is for surveillance of bacteria in food, veterinary and human medicine. AST testing of vibrios were therefore performed in custom-made 96-well Sensititre® plates (ThermoFisher) with protocol according to what is used in surveillance set up with adjustments of incubation temperature and time. The same adjustments were applied to isolates belonging to *V. anguillarum* or *V. splendidus*, whereas *V. splendidus* also needed additional salt added to the standardised Mueller Hinton growth media, and good growth was obtained by adding 2% NaCl to the broth. Furthermore, *V. splendidus* had poor growth after only 24 hours, hence incubation was extended to 48 hours to obtain good growth in the positive control well of the Sensititre® plates. The temperature was set at 25 °C which gave best growth for *V. splendidus* (Figure 16).
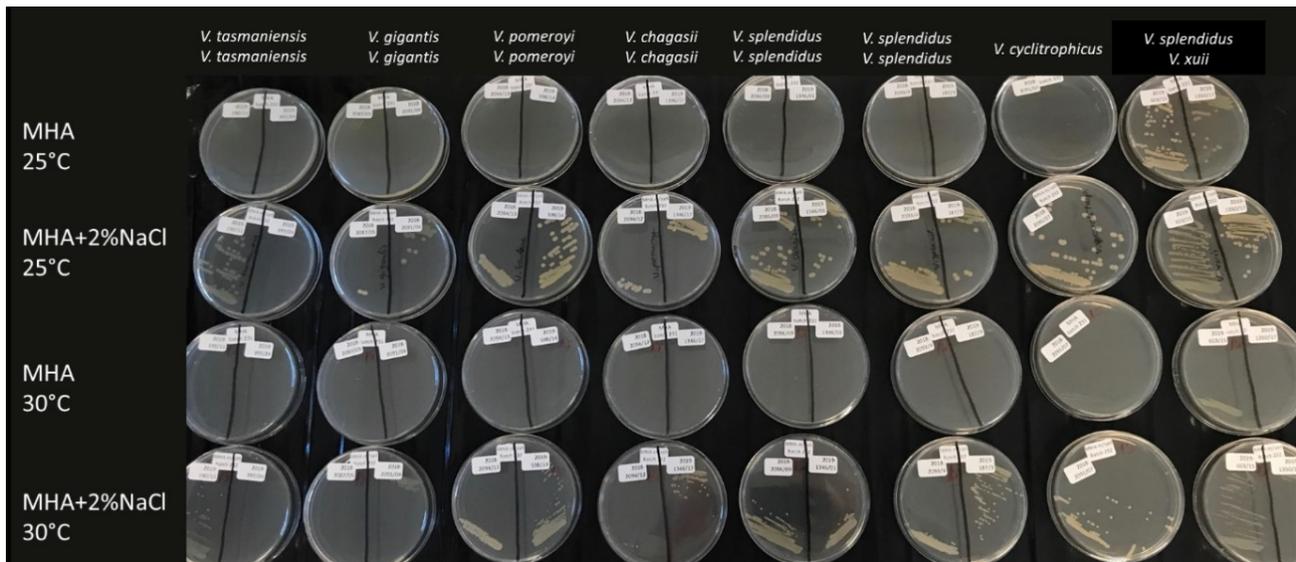
Figure 16. Growth performance test of Vibrio spp. on Mueller Hinton Agar with and without 2% NaCl, tested at 25 °C and 30 °C incubated for 48 hours.

A complete sensitive strain of either *V. splendidus* or *V. anguillarum* was applied as control for both AST and heavy metal sensitivity testing. The controls were stable for all antimicrobial agents, and for zinc and cadmium. More variation was seen for copper. The use of *Vibrio* control strains needs more optimisation. As there is a need for low variability among similar analyses, having a fixed incubation time is crucial. A workaround for this could be to apply spectrophotometry to determine growth density and normalise the measured results according to the variations of the control strain.

The protocol for heavy metal susceptibility testing was performed in broth dilution according to Deus et al. [46], and differed only with the use of broth instead of agar as applied in previous work done on marine bacteria from Norway [32]. The major challenge when preparing growth media with high levels of heavy metals, such as for copper and zinc with 1024 mg/L, is the drop in pH, (pH 4 in copper solutions) when the metals are dissolved in water, and further their complex binding ability when pH increases too rapidly. This was solved by adjusting the solution with Na-Citrate, a weak base that slowly increased the pH to final pH 7. We applied the same concentrations for both *Vibrio* spp. and *E. coli*, but for *E. coli* this was not suitable. Higher metal concentrations should have been applied for this purpose.

5 - Discussion

This study aimed to examine antimicrobial and heavy metal resistance among *Vibrio* spp. and *E. coli* from locations with known heavy metal pollution. Active salmonid fish farms were chosen based on previous reported levels of copper in sediments below the net pens. In addition, two locations with previous aquaculture activity, one location without aquaculture activity, and one unpolluted location, were examined. Chemical analyses of the heavy metals copper, zinc and cadmium in sediments (Figure 8), confirmed significant elevated concentrations at the Active aquaculture areas, with a mean of 272.3 mg/kg d.w. copper and 474.6 mg/kg d.w. zinc, compared to the other tested areas. This corresponds to extensively toxic (Class V) levels of copper and moderate toxic (Class III) of zinc (Table A1). Sediments from Non-active aquaculture, Non-aquaculture and Unpollue areas held 24.4 mg/kg d.w. 34.8 mg/kg d.w., and 2.28 mg/kg d.w. copper, and 70.7 mg/kg d.w., 144.6 mg/kg d.w. and 16.4 mg/kg d.w. zinc, respsectively. Furthermore, the microbiome analyses (Figure 7) showing a decrease in microbial species diversity in the Active aquaculture areas, support that these areas are impacted by the fish farming activity.

Isolates belonging to *V. anguillarum* (n=73) and *V. splendidus* (n=350) were tested for antimicrobial susceptibility. The majority of *V. anguillarum* isolates were fully susceptible to all the antimicrobials tested for. Whereas, among the *V. splendidus* isolates, 56% were susceptible to all agents, 35.1%, 8.3% and 0.6% were above ECV for one, two and three or more antimicrobial classes, respectively. The overall high prevalence of susceptible isolates identified, reflects the low usage of antimicrobials in today's fish farms and in Norway in general. During the 80s and 90s where high amounts of antimicrobials were used, both presence [1] and absence [47] of resistant bacteria was found beneath the net pens. The continued high use of antimicrobials can explain observed resistance in other aquaculture nations [48], such as Chile [49, 50] and China [51].

The Active aquaculture area had highest percentage of overall susceptible *V. splendidus* isolates. Active aquaculture areas were the only location that had one isolate with MICs above ECV for oxytetracycline (Figure 13). However, the isolates had overall MIC distribution similar to the MIC distribution of Non-active aquaculture and Non-aquaculture, and these three areas had all significant MIC distributions higher than the MIC distribution for isolates from the Unpolluted areas. The observed resistance to oxytetracycline could be related to *tet(34)* gene encoding a tetracycline inactivation enzyme that all vibrios harbour. The overexpression of *tet(34)* through regulatory response could have been induced from copper or zinc, as increased resistance to tetracycline have been seen after exposure to high [52] and even sublethal [11] concentrations of copper or zinc, rather than exposure of tetracycline itself. Håkonsholm et al. also found the *tet(34)* gene in all their *Vibrio* spp. that were isolated from open sea and herring, whereas none of the isolates expressed resistance after testing with disk diffusion [20]. One of the studies from aquaculture in the 90s found resistance to oxytetracycline in the majority of examined bacteria, and demonstrated that the tetracycline resistance mechanism from *Vibrio* spp. was transferable to *E. coli* [53]. Whether the increased resistance to oxytetracycline back then was linked to the historical use of oxytetracycline, exposure to heavy metals or both in combination, is not known, but this project did not identify any of such mobile tetracycline resistance genes.

Vibrio spp., including *V. splendidus*, have a unique ability to store multiple resistance genes that could easily be expressed whenever needed through cassettes in super integrons. This might explain the beta-lactamase genes, *bla* and *ampC*, and quinolone resistance gene *qnrS*, that were detected without the isolates showing any phenotypic resistance. Such silent resistance genes stored in cassettes are important for the robustness of the bacterium, being able to rapidly express genes necessary for survival in a changing environment. Hence, discharge of heavy metals to the marine environment can select for bacteria with such resistance genes, and

also decrease bacterial diversity [54]. Further analyses including susceptible isolates will gain knowledge about unexpressed resistance genes.

The Active aquaculture areas were the only areas that had isolates above ECV for copper at >1024 mg/kg, however, the Non-active and Non-aquaculture areas had isolates with MIC at 1024 mg/kg, and no differences were seen between these area, and had were significantly higher MIC than what was found for the isolates from Unpolluted areas. Since we don't know the true MIC for the isolates from Active aquaculture with MIC above the tested rage, these isolates should be examined more in detail.

Genes encoding multidrug efflux pumps and the arsenate efflux gene *aioB/aoxA* were found in isolates across the examined areas. One isolate from the Active aquaculture areas harboured an additional nlpE gene encoding copper sensing lipoprotein, which stimulates stress response and activates efflux pumps excreting both antimicrobials and heavy metals [55]. Unlike the other detected HMRGs, *nlpE* is associated to copper exposure, as it stimulates increased efflux of multiple drugs. This gene was recently detected in several *Vibrio* spp. genomes from open seawater in the North Sea [20], showing that this gene is not exclusive to Active aquaculture areas. Further examinations including whole genome sequencing and analyses of an extended number of isolates is needed to explore whether this or other HMRGs are more related to any of the areas included in the present survey.

The quantification of *E. coli* in all sample types showed that all areas, except for the Unpolluted areas where no *E. coli* was detected, were to some extent exposed to faecal pollution through sewage or run-offs from mainland. When comparing *E. coli* levels in seawater from all areas, a slightly increased, but not significant, inflow of faecal pollutions was implied at the Active aquaculture areas. These areas are located further inshore where less seawater is mixed, and thereby a lower dilution of faecal contamination is facilitated. The majority of the *E. coli* were fully susceptible to all antimicrobials tested for (91.7%). One of the *E. coli* showed resistance towards the extended spectrum cephalosporins, cefotaxime and ceftazidime, and the resistance was conferred by a $bla_{CTX-M-15}$ gene. This isolate also harboured the *qnrS1* gene conferring resistance to ciprofloxacin and tet(A) conferring resistance to tetracyclines. In one of three additional isolates expressing resistance to quinolones, the resistance was due to the plasmid mediated quinolone resistance gene *qnrS1*. *E. coli* with similar resistance pattern and genes as found in this study, have previously been found in marine bivalves in Norway [56]. The $bla_{CTX-M-15}$ is part of the bla_{CTX-M} resistance gene group 1, occasionally detected from humans and animals, both wild, domestic and livestock, in Norway [57-60]. The *qnrS1* gene alone confers low level resistance to ciprofloxacin below the clinical cut offs set by EUCAST, but above the ECV. This gene has also occasionally been detected in samples from livestock and wild animals in Norway [60, 61]. Furthermore, two of the isolates harboured the plasmid borne *pcoA* gene encoding a copper efflux pump [62]. These four isolates were detected in water samples from the Active aquaculture areas. None of the *E. coli* isolated from the other areas showed any resistance to cephalosporins or quinolones. Furthermore, no correlation was seen between the phenotypic resistance results from *V. splendidus* and *E. coli*, indicating no link to faecal contamination. The *E. coli* results might among others be affected by weather conditions [63], such as precipitation that leads to increased run-off from land. Whether exposure to copper have affected the *E. coli* results could not be determined. More extensive studies on a higher number of Active aquaculture areas are needed to investigate these aspects further.

In conclusion, this project applied *Vibrio* spp. as indicators for antimicrobial- and heavy metal resistance in copper contaminated aquaculture areas compared to old aquaculture areas, a near shore non-aquaculture and a unpolluted off shore area. A possible association was seen in Active aquaculture areas where increased MIC values to oxytetracycline and copper were detected. Though slightly lower MIC values were seen for the Non-

active and Non-aquaculture areas, no statistical differences could be found. Furthermore, antimicrobial resistance in *V. splendidus* did not correlate with the resistance found in *E. coli*. Importantly, selection for any resistance mechanism presupposes that the mechanism is present in the current environment. Given the high proportion of susceptible isolates found in this project, the overall prevalence of such selectable resistance mechanisms vibrios from copper contaminated areas assumed to be low.

This study has elucidated some challenges applied to the examination of antimicrobial resistant bacteria in copper contaminated aquaculture areas. We therefore suggest seven key points that should be considered in future surveys; 1) Identify indicator bacteria that are present in sediments containing high levels of H_2S, 2) Increase number of locations to detect possible differences and changes, 3) Include multiple isolates from each location, 4) Establish species specific protocols for heavy metal susceptibility testing, 5) Perform genetic analyses of both susceptible and non-susceptible isolates, 6) Perform metagenomics on samples to investigate for both antimicrobial and heavy metal resistance genes, and 7) Combine future sampling with ongoing surveillance of aquaculture sites, such as Modelling-Ongrowing fish farms-Monitoring (MOM) or sampling associated with the "Risk assessment report of aquaculture".

6 - Acknowledgement

7 - Appendicies

Table A1. Summary of environmental classification scheme for sediments, seawater, brown algae and blue mussels, according to chemical pollution of copper, zinc and cadmium. Class I: Background levels, Class II:No toxic effects, Class III: Moderate toxic effects after long-time exposure, Class IV: Acute toxic effects, Class V: Extensively toxic effects

		Class I	Class II	Class III	Class IV	Class V	Reference
Sediments (mg/kg d.w.)	Copper	<20	84	84	147	>147	[43]
	Zinc	<90	139	750	6690	>6690	
	Cadmium	<0.2	2.5	16	157	>157	
Seawater (µg/L)	Copper	<0.3	2.6	2.6	5.2	>5.2	
	Zinc	<1.5	3.4	6	60	>60	
	Cadmium	<0.03	0.2	*	*	*	
Brown algae (mg/kg w.w.)	Copper	<5	15	50	150	>150	[64]
	Zinc	<150	400	1000	2500	>2500	
	Cadmium	<1.5	5	20	40	>40	
Blue mussels (mg/kg w.w.)	Copper	<10	30	100	200	>200	
	Zinc	<200	400	1000	2500	>2500	
	Cadmium	<2	5	20	40	>40	

Table A2. List of antimicrobials included in antimicrobial susceptibility testing for E. coli and Vibrio spp.
Epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing
(EUCAST, accessed 25.03.2019) for E. coli and calculated by ECOFFinder for V. splendidus and V. anguillarum.
ND = not determined

Antimicrobials	Escherichia coli	Vibrio splendidus	Vibrio anguillarum
Ampicillin	> 8	> 4	Intrinsic
Azithromycin*	ND	> 8	> 8
Cefotaxime	> 0.25	> 0.5	> 0.5
Ceftazidime	> 0.5	> 0.25	> 4
Chloramphenicol	> 16	ND	ND
Ciprofloxacin	> 0.064	> 0.5	ND
Colistin	> 2	> 1	> 4
Florfenicol		> 2	> 1
Gentamicin	> 2	>2	> 4
Meropenem	> 0.125	> 0.25	> 1
Nalidixic acid	> 16	ND	ND
Nitrofurantoin	ND	> 32	> 0.12
Oxolinic acid	ND	> 0.5	> 0.12
Oxytetracycline	ND	> 0.5	> 0.5
Sulfamethoxazole	> 64	> 8	> 8
Tetracycline	> 8	ND	ND
Tigecycline	> 0.5	ND	ND
Trimethoprim	> 2	> 4	> 2

Table A3. Sediment samples analysed for residues of antimicrobials (mg/kg w.w.).

Areas	DO*	CL-TET*	OXYTET*	TET*	ENRO	FLOR	FLUM	OXO	TRIM	CIP
Aquaculture	< 50	< 50	< 50	< 50	< 10	< 4	< 40	< 40	< 2	NA
Aquaculture	< 50	< 50	< 50	< 50	< 10	< 4	< 40	< 40	< 2	< 10
Aquaculture	< 50	< 50	< 50	< 50	< 10	< 4	< 40	NA	< 2	< 10
Unpolluted	< 50	< 50	< 50	< 50	< 10	< 4	< 40	< 40	< 2	< 10
Unpolluted	< 50	< 50	< 50	< 50	< 10	< 4	< 40	< 40	< 2	< 10
Non-aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Non-active aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-active aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-active aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	< 10
Non-active aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	< 10
Aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	NA
Aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Non-aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Non-active aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-active aquaculture	NA	NA	NA	NA	< 10	< 4	< 40	< 40	< 2	< 10
Non-active aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Non-active aquaculture	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Unpolluted	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA
Unpolluted	NA	NA	NA	NA	NA	< 4	< 40	< 40	< 2	NA

Abbreviations: DO=doxycycline, CL-TET=Chlortetracycline, OXYTET=oxytetracycline, TET=Tetracycline, ENRO=Enrofloxacin, FLOR=Florfenicol, FLUM=Flumequine, OXO=Oxolinic acid, TRIM=Trimethoprim, CIP=Ciprofloxacin, NA=Not Analysed.
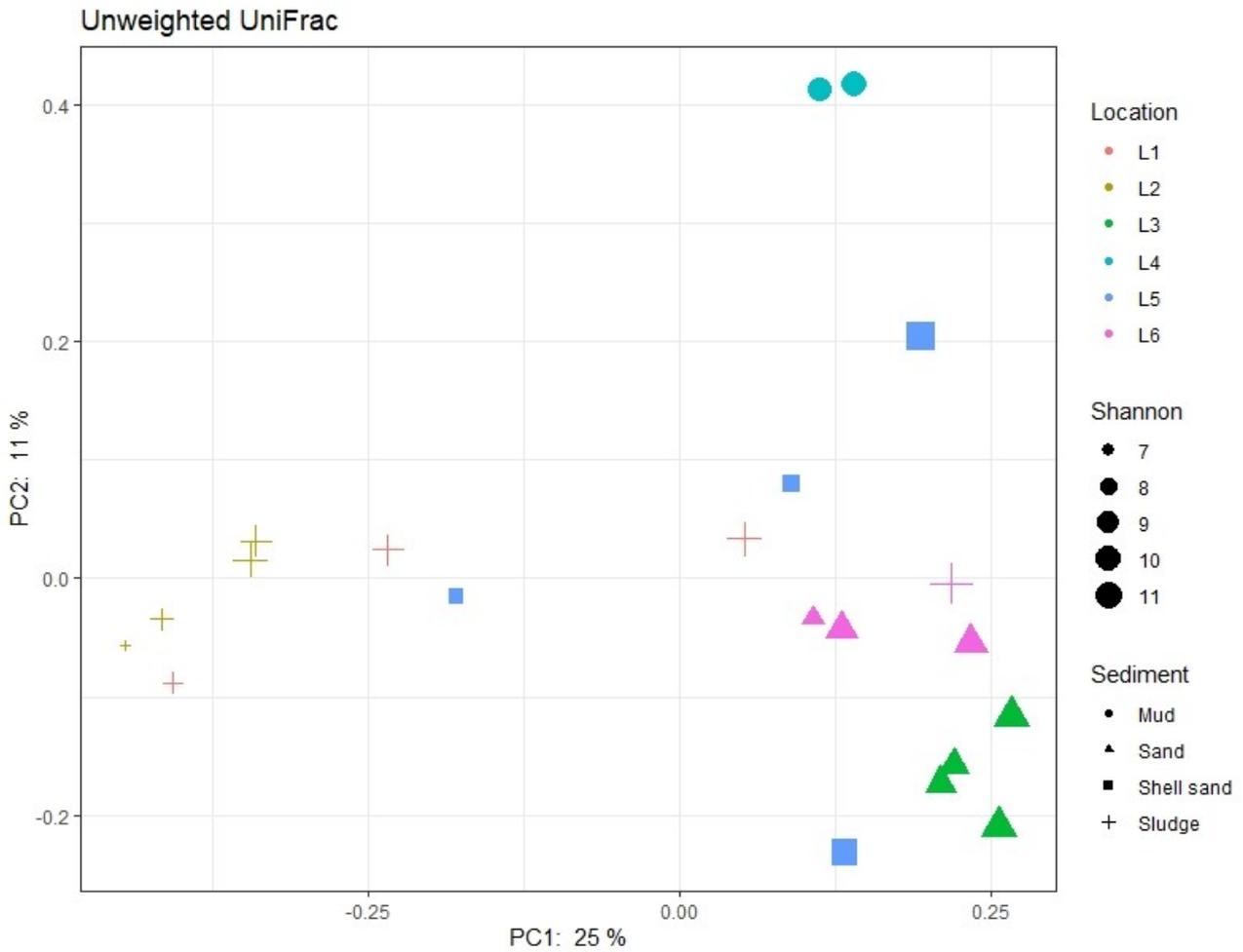
Figure A1. Principal component analysis illustrating similarity of samples regarding the activity of the sampling site and sediment type (shape of symbols) respectively. Shannon values (size of symbols) indicate the diversity of 16S sequences observed in the samples. The closer two points are on the plot, the more similar they are in microbial community composition. L1-L6 indicates locations as described in Table 1.
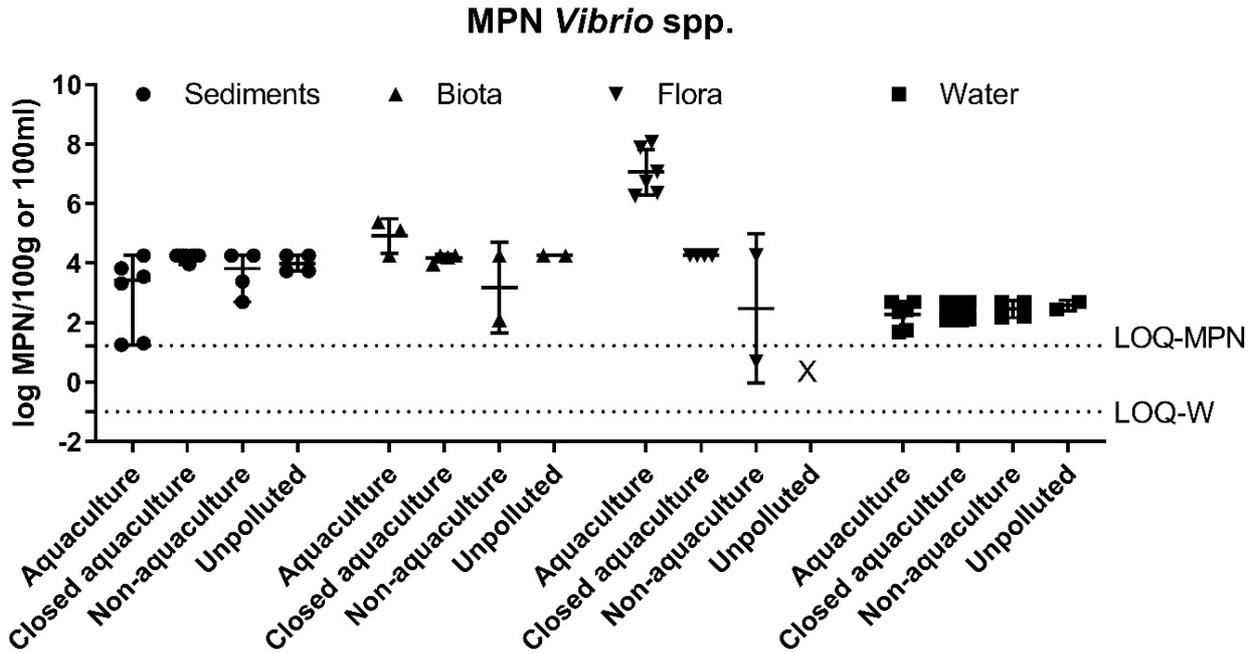
Figure A2. Quantitative analysis of *Vibrio* spp. in sediments, fauna, flora (mean ± range MPN/100g) and water (mean ± range CFU/100ml). The LOQ-MPN and the LOQ-W indicates the LOQ for the MPN method, and filtering method of water, respectively.
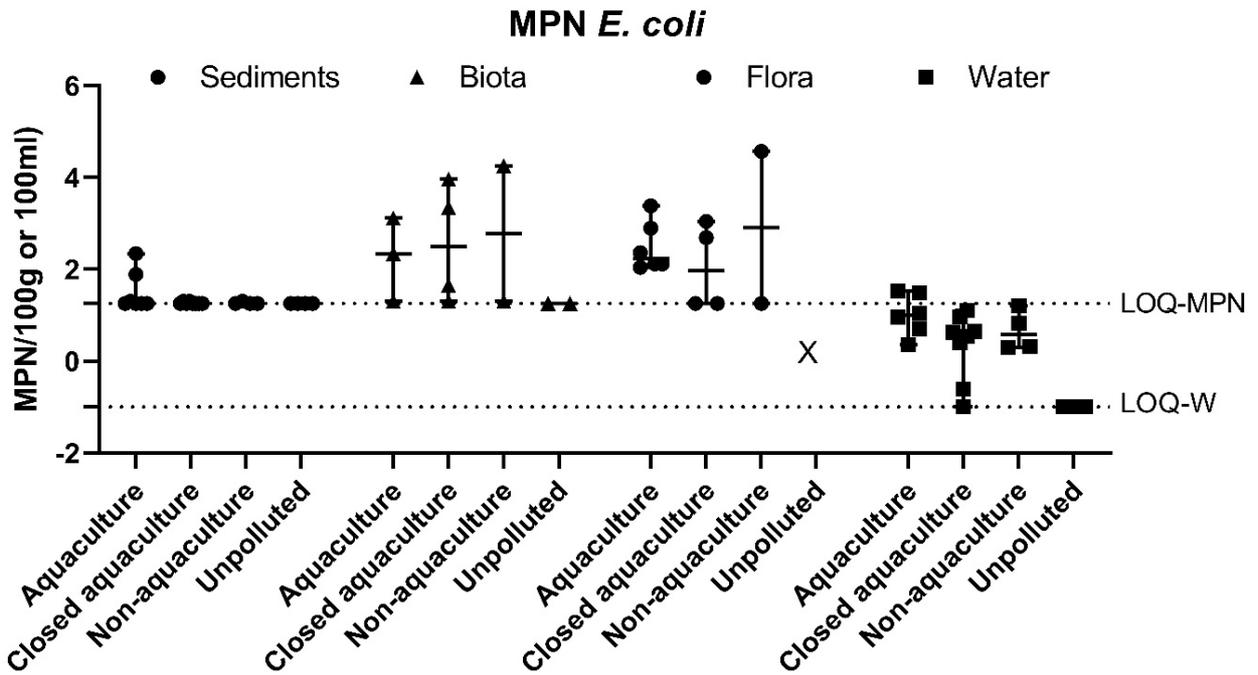
Figure A3. Quantitative analysis of *E. coli* in sediments, fauna, flora (mean ± range MPN/100g) and water (mean ± range CFU/100ml). The LOQ-MPN and the LOQ-W indicates the LOQ for the MPN method, and filtering method of water, respsectively.

Table A4. MIC distribution for V. anguillarum . White fields indicate the tested range of antimicrobials concentrations.

			Number of isolates (n) of MIC values (mg/L)											
		% >ECV [95%CI]	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
AMP	Active aquaculture	Intrinsic											27	3
ECV:ND	Non-active aquaculture	Intrinsic											1	13
	Non-aquaculture	Intrinsic											7	18
	Unpolluted	Intrinsic											4	
	Total	Intrinsic											39	34
AZM	Active aquaculture	0.0 [0.0-11.6]							28	2				
ECV:>8	Non-active aquaculture	0.0 [0.0-23.2]							14					
	Non-aquaculture	0.0 (0.0-13.7)							25					
	Unpolluted	0.0 [0.0-60.2]							4					
	Total	0.0 [0.0-4.9]							71	2				
CTX	Active aquaculture	0.0 [0.0-11.6]			27		3							
ECV:>0.5	Non-active aquaculture	0.0 [0.0-23.2]			2	1	11							
	Non-aquaculture	0.0 (0.0-13.7)			14	6	5							
	Unpolluted	0.0 [0.0-60.2]			4									
	Total	0.0 [0.0-4.9]			47	7	19							
CAZ	Active aquaculture	0.0 [0.0-11.6]					2	26	1	1				
ECV:>4	Non-active aquaculture	0.0 [0.0-23.2]						2	12					
	Non-aquaculture	0.0 (0.0-13.7)						14	11					
	Unpolluted	0.0 [0.0-60.2]						4						
	Total	0.0 [0.0-4.9]					2	42	24	1				
CIP	Active aquaculture	ND	30											
ECV:ND	Non-active aquaculture	ND	14											
	Non-aquaculture	ND	25											
	Unpolluted	ND	4											
	Total	ND	73											
CST	Active aquaculture	3.3 [0.1-17.2]				11	5	10	2	1	1			
ECV:>4	Non-active aquaculture	7.1 [0.2-33.9]						8	5		1			
	Non-aquaculture	8.0 [1.0-26.0]						11	9	3	2			
	Unpolluted	75 [19.4-99.4]							1		3			
	Total	9.6 [3.9-18.8]				11	5	29	17	4	7			
FLO	Active aquaculture	0.0 [0.0-11.6]				28	2							
ECV:>1	Non-active aquaculture	0.0 [0.0-23.2]				1	13							
	Non-aquaculture	0.0 (0.0-13.7)				11	14							
	Unpolluted	0.0 [0.0-60.2]					4							
	Total	0.0 [0.0-4.9]				39	32							

	Category	% [CI]	1	2	3	4	5	6	7	8	9	10
GEN	Active aquaculture	0.0 [0.0-11.6]					2	19	8	1		
ECV:>4	Non-active aquaculture	0.0 [0.0-23.2]					3	10	1			
	Non-aquaculture	0.0 (0.0-13.7)					9	14		2		
	Unpolluted	0.0 [0.0-60.2]					3	1				
	Total	0.0 [0.0-4.9]					17	44	9	3		
MEM	Active aquaculture	0.0 [0.0-11.6]			9	21						
ECV:>1	Non-active aquaculture	0.0 [0.0-23.2]				14						
	Non-aquaculture	0.0 (0.0-13.7)			3	20	2					
	Unpolluted	0.0 [0.0-60.2]				4						
	Total	0.0 [0.0-4.9]			12	59						
NIT	Active aquaculture	ND									30	
ECV:ND	Non-active aquaculture	ND									14	
	Non-aquaculture	ND									23	
	Unpolluted	ND									4	
	Total	ND									71	
OXA	Active aquaculture	0.0 [0.0-11.6]	30									
ECV:>0.12	Non-active aquaculture	0.0 [0.0-23.2]	12	1	1							
	Non-aquaculture	0.0 (0.0-13.7)	24	1								
	Unpolluted	0.0 [0.0-60.2]	4									
	Total	0.0 [0.0-4.9]	70	2	1							
OTE	Active aquaculture	0.0 [0.0-11.6]		29	1							
ECV:>0.5	Non-active aquaculture	0.0 [0.0-23.2]			14							
	Non-aquaculture	0.0 (0.0-13.7)		4	21							
	Unpolluted	0.0 [0.0-60.2]		2	2							
	Total	0.0 [0.0-4.9]		35	37							
SMZ	Active aquaculture	0.0 [0.0-11.6]						14	9	7		
ECV:>8	Non-active aquaculture	0.0 [0.0-23.2]						14				
	Non-aquaculture	4.0 (0.0-20.4)					2	12	10		1	
	Unpolluted	0.0 [0.0-60.2]						4				
	Total	1.4 [0.0-4.9]					16	39	16		1	
TGC	Active aquaculture	ND				30						
ECV:ND	Non-active aquaculture	ND				14						
	Non-aquaculture	ND				25						
	Unpolluted	ND				4						
	Total	ND				73						
TMP	Active aquaculture	0.0 [0.0-11.6]			5	13	12					
ECV:>2	Non-active aquaculture	0.0 [0.0-23.2]				2	10	2				

	Category	% >ECV	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
	Non-aquaculture	0.0 (0.0-13.7)					3	22						
	Unpolluted	0.0 [0.0-60.2]						4						
	Total	0.0 [0.0-4.9]					5	18	48	2				

Aquaculture(n=30), Non-active aquaculture (n=14), Non-aquaculture (n=25) and Unpolluted (n=4).

ECV=Epidemiological cut-off value. White fields indicate the tested range of antimicrobials concentrations. ND= not determined.

Table A5. MIC distribution data for V. splendidus. White fields indicate the tested range of antimicrobials concentrations.

			Distribution (%) of MIC values (mg/L)											
		% >ECV [95%CI]	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
AMP ECV:>4	Active aquaculture	6.8 [3.1-12.4]						71.4	9.0	12.8	3.8	2.3		0
	Non-active aquaculture	13.8[7.9-21.7]						62.4	13.8	10.1	10.1	1.8	0.9	0.9
	Non-aquaculture	2.7 [0.1-14.9]						73.0	16.2	8.1	2.7			
	Unpolluted	19.7 [11.2-30.9]						26.8	11.3	42.3	12.7			7.0
	Total	11.1 [8.0-14.9]						59.2	11.5	17.5	7.3	1.4	0.3	2.8
AZM ECV:>8	Active aquaculture	0 [0.0-2.7]							87.2	11.3	1.5			
	Non-active aquaculture	0 [0.0-3.3]							70.6	28.4	0.9			
	Non-aquaculture	0 [0.0-9.5]							21.6	70.3	8.1			
	Unpolluted	0 [0.0-5.1]							63.4	35.2	1.4			
	Total	0 [0-1.0]							70.3	27.7	2.0			
CTX ECV:ND	Active aquaculture	0 [0.0-2.7]			100									
	Non-active aquaculture	0 [0.0-3.3]			100									
	Non-aquaculture	0 [0.0-9.5]			100									
	Unpolluted	0 [0.0-5.1]			100									
	Total	0 [0-1.0]			99.7	0.3								
CAZ ECV:>0.25	Active aquaculture	0 [0.0-2.7]		85.0	15.0									
	Non-active aquaculture	0 [0.0-3.3]		74.3	23.9	1.8								
	Non-aquaculture	0 [0.0-9.5]		59.5	37.8	2.7								
	Unpolluted	1.4 [0.0-7.6]		74.6	15.5	8.5	1.4							
	Total	0.3 [0.0-1.6]		76.9	20.3	2.6	0.3							
CIP ECV:>0.5	Active aquaculture	0 [0.0-2.7]	10.5	21.8	42.9	23.3	1.5							
	Non-active aquaculture	0 [0.0-3.3]	5.5	14.7	47.7	22.9	9.2							
	Non-aquaculture	0 [0.0-9.5]	5.4	8.1	27.0	54.1	5.4							
	Unpolluted	0 [0.0-5.1]	36.6	9.9	29.6	21.1	2.8							
	Total	0 [0.0-1.0]	13.7	15.7	40.0	26.0	4.6							
CST ECV:>1	Active aquaculture	0.8 [0.0-4.1]				96.2	3.0				0.8			
	Non-active aquaculture	2.8 [0.6-7.9]				92.6	4.6				2.8			
	Non-aquaculture	7.9 [1.7-21.4]				92.1				2.6	5.3			

Antibiotic	Group	% [CI]	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
	Unpolluted	0 [0.0-5.1]				94.4	5.6							
	Total	2.0 [0.8-4.1]				94.3	3.7			0.3	1.7			
FLO ECV:>2	Active aquaculture	0 [0.0-2.7]				40.6	59.4							
	Non-active aquaculture	0 [0.0-3.3]				19.3	77.1	3.7						
	Non-aquaculture	0 [0.0-9.5]				16.2	78.4	5.4						
	Unpolluted	0 [0.0-5.1]				35.2	64.8							
	Total	0 [0-1.0]				30.3	68.0	1.7						
GEN ECV:>8	Active aquaculture	3.1 [0.8-7.6]						13.0	45.8	30.5	7.6	3.1		
	Non-active aquaculture	0.9 [0.0-5.0]				1.8		11.9	36.7	39.4	9.2	0.9		
	Non-aquaculture	0 [0.0-9.5]						5.4	37.8	29.7	27.0			
	Unpolluted	4.2 [0.9-11.9]						9.9	29.6	29.6	26.8	4.2		
	Total	2.3 [1.0-4.5]				0.6		11.2	38.8	33.0	14.1	2.3		
MEM ECV:ND	Active aquaculture	ND		100										
	Non-active aquaculture	ND		100										
	Non-aquaculture	ND		100										
	Unpolluted	ND		100										
	Total	ND		100										
NIT ECV:ND	Active aquaculture	0 [0.0-2.7]										99.2	0.8	
	Non-active aquaculture	0 [0.0-3.3]										99.1	0.9	
	Non-aquaculture	0 [0.0-9.5]										100		
	Unpolluted	0 [0.0-5.1]										97.2	2.8	
	Total	0 [0-1.0]										98.9	1.1	
OXA ECV:>0.5	Active aquaculture	0 [0.0-2.7]	8.2	41.8	44.8	5.2								
	Non-active aquaculture	0 [0.0-3.3]	3.6	33.3	54.1	9.0								
	Non-aquaculture	0 [0.0-9.5]	7.7	15.4	74.4		2.6							
	Unpolluted	0 [0.0-5.1]	35.2	28.2	33.8	2.8								
	Total	0 [0.0-1.0]	12.1	33.5	48.7	5.4	0.3							
OTE ECV:>0.5	Active aquaculture	0.8 [0.0-4.1]		8.3	50.4	38.3	2.3	0.8						
	Non-active aquaculture	0 [0.0-3.3]		2.8	48.6	48.6								
	Non-aquaculture	0 [0.0-9.0]		2.7	32.4	64.9								
	Unpolluted	0 [0.0-5.1]		11.3	69.0	19.7								
	Total	0.3 [0.0-1.6]		6.6	51.7	40.6	0.9	0.3						
SXT ECV:>8	Active aquaculture	30.1 [22.4-38.6]					0.8	6.0	27.1	16.5	19.5	6.0	10.5	13.5
	Non-active aquaculture	66.1 [56.3-74.8]						0.9	8.3	12.8	11.9	11.9	21.1	33.0
	Non-aquaculture	43.2 [27.1-60.5]						18.9	24.3	10.8	2.7	2.7	27.0	13.5
	Unpolluted	40.8 [29.3-53.2]					1.4	4.2	25.4	14.1	14.1	12.7	16.9	11.3
	Total	44.9 [39.5-50.2]					5.4	20.6	14.3	14.3	8.9	16.9	19.1	5.4

		% Res [95% CI]	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
TGC	Active aquaculture	ND					100									
ECV:ND	Non-active aquaculture	ND					100									
	Non-aquaculture	ND					100									
	Unpolluted	ND					100									
	Total	ND					100.0									
TMP	Active aquaculture	0.8 [0.0-4.1]					5.3	12.0	53.4	25.6	3.0	0.8				
ECV:>4	Non-active aquaculture	5.5 [2.1-11.6]					0.9	7.3	40.4	40.4	5.5	0.0				5.5
	Non-aquaculture	10.8 [3.0-25.4]					5.4	8.1	56.8	16.2	2.7	2.7				8.1
	Unpolluted	4.2 [0.9-11.9]					4.2	26.8	45.1	19.7		1.4				2.8
	Total	4.0 [2.2-6.6]					3.7	13.1	48.0	28.0	3.1	0.9				3.1

Aquaculture (n=133), Non-active aquaculture (n=109), Non-aquaculture (n=37) and Unpolluted (n=71). ECV=Epidemiological cut-off value. White fields indicate the tested range of antimicrobials concentrations. ND= not determined.

Table A6. MIC distribution data for E. coli. White fields indicate the tested range of antimicrobials concentrations.

		% Res [95% CI]	MIC distribution (%) of MIC values (mg/L)													
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
AMP	Active aquaculture	5.3 [2.3 -10.0]							2.0	30.1	57.5	5.2	0.7			4.6
ECV:>8	Non-active aquaculture	2.7 [0.6 -7.6]								29.5	62.5	5.4				2.7
	Non-aquaculture	1.4 [0.0 -1.4]								11.3	77.5	9.9				1.4
	Total	3.6						0.9		25.9	63.4	6.3	0.3			3.3
AZM	Active aquaculture	ND								3.9	32.7	59.5	3.9			
ECV:ND	Non-active aquaculture	ND								10.7	41.1	42.9	4.5			0.9
	Non-aquaculture	ND								7.0	45.1	45.1	2.8			
	Total	ND								6.9	38.1	50.9	3.9			0.3
CTX	Active aquaculture	0.7 [0.1 -3.6]					99.4					0.7				
ECV:>0.25	Non-active aquaculture	0.0 [0.0 -3.2]					100									
	Non-aquaculture	0.0 [0.0 -0.05]					100									
	Total	0.3 [0.0 -1.6]					99.7					0.3				
CAZ	Active aquaculture	0.7 [0.1 -3.6]						99.4					0.7			
ECV:>0.5	Non-active aquaculture	0.0 [0.0 -3.2]						100								
	Non-aquaculture	0.0 [0.0 -0.05]						100								
	Total	0.3 [0.0 -1.6]						99.7					0.3			
CHL	Active aquaculture	0.7 [0.1 -3.6]										90.2	9.2			
ECV:>16	Non-active aquaculture	1.8 [0.2 -6.3]										92.0	6.3	1.8		
	Non-aquaculture	0.0 [0.0 -0.05]										94.4	5.6			
	Total	0.9 [0.2 -2.6]										91.7	7.4	0.6		
CIP	Active aquaculture	2.8 [0.7 -6.6]	76.5	20.3	0.7	0.7	0.7	0.7					0.7			
ECV:>0.06	Non-active aquaculture	0.0 [0.0 -3.2]	75.0	24.1	0.9											
	Non-aquaculture	0.0 [0.0 -0.05]	87.3	12.7												

	Group	% >ECV [95%CI]															
	Total	1.2 [0.3 -3.0]	78.3	20.0	0.6	0.3	0.3	0.3						0.3			
CST	Active aquaculture	0.0 [0.0 -2.4]						99.4	0.7								
ECV:>2	Non-active aquaculture	0.0 [0.0 -3.2]						99.1	0.9								
	Non-aquaculture	0.0 [0.0 -0.05]						98.6	1.4								
	Total	0 [0.0 -1.2]						99.1	0.9								
GEN	Active aquaculture	0.0 [0.0 -2.4]						50.3	43.1	6.5							
ECV:>2	Non-active aquaculture	0.0 [0.0 -3.2]						69.6	27.7	2.7							
	Non-aquaculture	0.0 [0.0 -0.05]						76.1	22.5	1.4							
	Total	0 [0.0 -1.2]						62.2	33.6	4.2							
MEM	Active aquaculture	0.0 [0.0 -2.4]		100													
ECV:>0.12	Non-active aquaculture	0.0 [0.0 -3.2]		100													
	Non-aquaculture	0.0 [0.0 -0.05]		100													
	Total	0 [0.0 -1.2]		100													
NAL	Active aquaculture	2.0[0.4 -5.6]								94.8	3.3	0.7					
ECV:8>	Non-active aquaculture	0.0 [0.0 -3.2]								99.1	0.9						
	Non-aquaculture	0.0 [0.0 -0.05]								100							
	Total	0.9 [0.2 -2.6]								97.3	1.8	0.3					
SMZ	Active aquaculture	5.3 [2.3 -10.0]												82.4	11.1	1.3	
ECV:>64	Non-active aquaculture	6.3 [2.5 -12.5]												54.5	33.9	5.4	0.9
	Non-aquaculture	0.0 [0.0 -0.05]												71.8	23.9	4.2	
	Total	4.5 [2.5 -7.3]												70.8	21.4	3.3	0.3
TET	Active aquaculture	7.2 [3.6 -12.5]							85.6	7.2					3.3	3.9	
ECV:>8	Non-active aquaculture	5.4 [2.0 -11.3]							80.4	12.5	1.8				1.8	3.6	
	Non-aquaculture	1.4 [0.0 -1.4]							80.3	18.3						1.4	
	Total	5.4 [3.2 -8.3]							82.7	11.3	0.6				2.4	3.0	
TGC	Active aquaculture	1.3 [0.2 -4.6]					98.7	1.3									
ECV:>0.5	Non-active aquaculture	1.8 [0.2 -6.3]					98.2	1.8									
	Non-aquaculture	0.0 [0.0 -0.05]					100										
	Total	1.2 [0.3 -3.0]					98.8	1.2									
TMP	Active aquaculture	3.3 [1.1 -7.5]					35.3	55.6	4.6	1.3						3.3	
ECV:>2	Non-active aquaculture	2.7 [0.6 -7.6]					28.6	50.9	15.2	2.7						2.7	
	Non-aquaculture	0.0 [0.0 -0.05]					29.6	62.0	7.0	1.4							
	Total	2.4 [1.0 -4.6]					31.9	55.4	8.6	1.8						2.4	

Aqua=Aquaculture(n=153), Non-active=Non-active aquaculture (n=112), Non-aqua=Non-aquaculture (n=71). ECV=Epidemiological cut-off value. White fields indicate the tested range of antimicrobials concentrations. ND= not determined

Table A7. Heavy metal susceptibility test data for V. anguillarum.

			Distribution (n) of MIC values (mg/L)								
		% >ECV [95%CI]	8	16	32	64	128	256	512	1024	>1024
Cadmium	Active aquaculture	0 [0.0-11.5]			4	14	11				

ECV:>512	Non-active aquaculture	0 [0.0-23.1]		2	12				
	Non-aquaculture	0 [0.0-14.8]		12	11	2			
	Unpolluted	0 [0.0-60.2]			4				
	Total	0 [0.0-5.0]	4	28	38	2			
Copper ECV:>1024	Active aquaculture	0 [0.0-11.5]		1	2	24	3		
	Non-active aquaculture	0 [0.0-23.2]				14			
	Non-aquaculture	0 [0.0-14.8]				23	2		
	Unpolluted	0 [0.0-60.2]				4			
	Total	0 [0.0-5.0]		1	2	65	5		
Zinc ECV:>1024	Active aquaculture	0 [0.0-11.5]						11	19
	Non-active aquaculture	0 [0.0-23.2]							14
	Non-aquaculture	0 [0.0-14.8]						1	24
	Unpolluted	0 [0.0-60.2]							4
	Total	0 [0.0-5.0]						12	61

Active aquaculture (n=30), Non-active aquaculture (n=14), Non-aquaculture (n=23) and Unpolluted (n=4).

ECV=Epidemiological cut-off value. White fields indicate the tested range of antimicrobials concentrations.

Table A8. Heavy metal susceptibility test data for V. splendidus.

			Distribution (%) of MIC values (mg/L)								
		% >ECV [95%CI]	8	16	32	64	128	256	512	1024	>1024
Cadmium	Active aquaculture	0 [0.0-2.7]	1.5		2.3	38.5	56.2	1.5			
ECV:>512	Non-active aquaculture	0 [0.0-3.2]	0.9	0.9	1.9	41.7	51.9	2.8			
	Non-aquaculture	0 [0.0-9.0]				45.9	54.1				
	Unpolluted	0 [0.0-5.0]				23.9	70.4	4.2	1.4		
	Total	0 [0.0-1.4]	0.9	0.3	1.4	37.0	57.5	2.6	0.3	0.0	0.0
Copper	Active aquaculture	1.5 [0.0-5.4]	1.5	2.3	0.8	0.8	23.1	46.2	22.3	1.5	1.5
ECV:>1024	Non-active aquaculture	0 [0.0-3.3]	0.9		0.9	2.8	18.5	42.6	31.5	2.8	
	Non-aquaculture	0 [0.0-9.0]			2.7		21.6	70.3	2.7	2.7	
	Unpolluted	0 [0.0-5.0]	1.4				36.6	49.3	12.7		
	Total	0 [0.0-2.0]	1.1	0.9	0.9	1.1	24.2	48.7	20.8	1.7	0.6
Zinc	Active aquaculture	5.3 [0.0-10.7]			3.1	51.5	28.5	11.5	3.8		1.5
ECV:>256	Non-active aquaculture	11.0 [0.0-18.8]	1.0	1.0	6.1	42.9	27.6	10.2	8.2	1.0	2.0
	Non-aquaculture	10.3[0.0-24.2]				29.7	40.5	24.3	5.4		
	Unpolluted	36.6[0.0-48.9]				25.4	21.1	16.9	29.6		7.0
	Total	14.1[0.0 -12.3]	0.0	0.3	2.9	40.6	28.2	13.8	10.6	0.3	3.2

Active aquaculture (n=130), Non-active aquaculture (n=108), Non-aquaculture (n=37) and Unpolluted (n=71). ECV=Epidemiological cut-off value. White fields indicate the tested range of antimicrobials concentrations.

Table A9. List of HMRGs detected in the genomes of V. anguillarum , V. splendidus and E. coli .

Species	LIMS no.	HMRGs
V. anguillarum	2019-184-12	*emrD-3, fabV, ceB, vpoC, vmeF(acrBDF), vexF, vcmA, vcaM*
V. anguillarum	2019-184-17	*emrD-3, fabV, vceB, vpoC, vmeF(acrBDF), vexF, vcmA, vcaM*
V. splendidus	2019-188-18	*emrD-3, fabV, vceB, vpoC, vmeF(acrBDF), vexF, vcmA, vcaM, vcaM*
V. splendidus	2019-190-8	*vpoC, ibpA, vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-193-10	*mtrF, acr3, vcrM, fabV, aioB/aoxA, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-195-7	*mtrF, acr3, vcrM, fabV, aioB/aoxA, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-196-14	*mtrF, acr3, fabV, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-195-20	*mtrF, acr3, fabV, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-397-21	*mtrF, acr3, vcrM, fabV, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM,*
V. splendidus	2019-399-11	*mtrF, acr3, fabV, aioB/aoxA, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-955-15	*mtrF, acr3, aioB/aoxA, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2019-1572-18	*mtrF, fabV, aioB/aoxA , vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM*
V. splendidus	2018-2091-12	*mtrF, mtrF, acr3, vcrM, fabV, pfr, aioB/aoxA, vmeY, vpoC, ibpA, vmeI(acrBDF), vmeV, vmeZ, vmeF(acrBDF), vmeD, vexF, vcmA, vcaM, nlpE*
E. coli	2019-604-8	*acrB, acrF/envD, actP/yjcG, corA, cpxA, cusA/ybdE, cutE/lnt, dsbA, dsbB, dsbC, emrB, emrD, evgA, evgS, fecD, gadA, iclR, kpnO, marA, mdfA/cmr, , mdtC/yegO, mdtF/yhiV, mdtG/yceE, mdtK/ydhE, mdtM/yjiO, mgtA, mntP/yebN, modC, ostA/lptD, pcoA, pcoS, phoB, pstS, robA, silE, tehA, tolC, ychH, ydeI, YdeP, yqjH, zinT/yodA, zntA/yhhO, zntR/YhdM, znuA/yebL, zraR/hydH, zraS/hydG*
E. coli	2019-399-2	*acrB, acrF/envD, corA, corB, cpxA, cusA/ybdE, dsbC, emrB, emrD, evgS, gadA, gadC/xasA, iclR, mdfA/cmr, mdtC/yegO, mdtF/yhiV, mdtM/yjiO, mgtA, modC, ostA/lptD, oxyRkp, pitA, pstA, robA, tehA, tolC, zntR/YhdM, zraS/hydG*
E. coli	2019-400-1	*cutC, acrF/envD, actP/yjcG corA, cpxA, mdtK/ydhE, dsbA, dsbC, emrB, emrD, evgA, evgS, gadA, pcoA, znuA/yebL, gadC/xasA, zraS/hydG, mdtF/yhiV, merE, mgtA, modC, nfsA, ostA/lptD, pitA, pstA, rpoS, zraR/hydH, zntR/YhdM, tolC, ydeP, yqjH*
E. coli	2019-400-9	*acrB, mdtC/yegO, mdtK/ydhE, mdtM/yjiO, silA, acrD/yffA, zinT/yodA, zntA/yhhO, znuA/yebL, zraS/hydG, zntR/YhdM, zraR/hydH, zur/yjbK, bcr, cueO, acrF/envD, cusA/ybdE, actP/yjcG, corA, corB, cpxA, cutA, cutE/lnt, dsbA, dsbB, dsbC, mdfA/cmr, marA, emrB, emrD, evgS, gadA, gadC/xasA, iclR, kexD, kpnO, mdtF/yhiV, merE, mgtA, mntP/yebN, mntR, modC, nfsA, ostA/lptD, phoB, pitA, robA, soxR, tehA, terC, tolC, ychH, ydeP*

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HAVFORSKNINGSINSTITUTTET

Postboks 1870 Nordnes
5817 Bergen
Tlf: 55 23 85 00
E-post: post@hi.no
www.hi.no