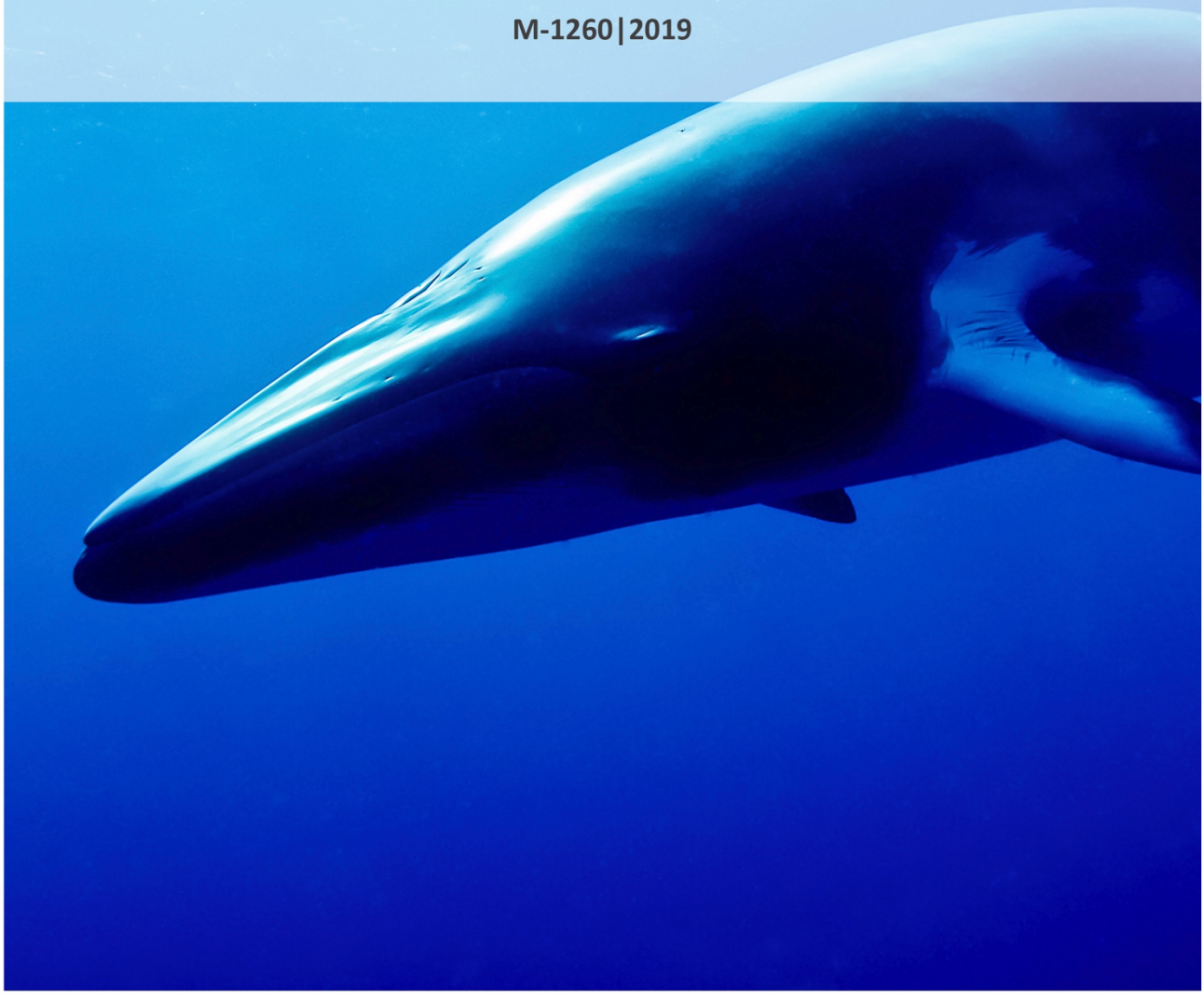
Antimicrobial resistance in the marine environment: MIC profiles of bacteria isolated from whale and seal faeces

M-1260|2019



February 2019

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GenØk-Centre for Biosafety, Tromsø, Norway
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Antimicrobial resistance in the marine environment: MIC profiles of bacteria isolated from whale and seal faeces M-1260|2019

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Suggested citation:
Venter, H. (2018) Antimicrobial resistance in the marine environment: MIC profiles of bacteria isolated from whale and seal faeces. Project report, M-1260|2019, GenØk, Tromsø, Norway.

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This report was commissioned by the Norwegian Environment Agency

Background

Antimicrobial resistance (AMR), particularly antibiotic resistance among bacteria, is globally recognised as a threat to modern medicine, and thus a threat to human and animal health (World Health Organization, 2015). There is increasing appreciation for the fact that the environment plays a dynamic role in the issue of antibiotic resistance. It has become clear that the environment is both a source and a recipient of antimicrobial resistance genes (AMRGs) and resistant bacteria. Environmental compartments are not merely sinks for antimicrobial resistance, but partners with anthropogenic activities in a multidirectional relationship which is facilitated by horizontal gene transfer (HGT), co-selection, and dispersal.

The national strategy of the Norwegian government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a holistic perspective. It is aligned with the One Health principle, and acknowledges that human and animal health and the environment interact and must be seen in context to each other. The national strategy is based on the report "Antibiotikaresistens-kunnskapshull og aktuelle tiltak (2014)" prepared by an expert group. In the report, the identification and monitoring of the presence of antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARG) in different environments is highlighted as one of the areas where more information is needed.

The presence of resistant bacteria in different natural environments, such as soil, fresh water, sea sediments and wild animals, has however only been sporadically studied, although they may contribute to the development of resistance of clinical importance. This implies that there is therefore a need for more knowledge about antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARGs) in different natural environments in
Norway.

GenØk – Centre for Bioseafety

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Acknowledgement

We thank Elisabeth Olsen for assistance in the lab and Katrine Jaklin for editorial work. We also would like thank Kaare Nielsen, OsloMet, for advice, Kathrine Ryeng and Lotta Lindblom, Institute of Marine Research, for providing whale samples, and Morten Tryland, UiT, for the seal samples.

Table of Contents

Summary 1
Sammendrag på norsk 2
Introduction 3
Materials and Methods 4
Sampling 4
Methods 4
Resuscitation of bacteria from frozen samples 4
Phenotypic characterization: Broth Microdilution 5
Molecular Characterization: DNA Isolation and PCR 5
Results and Discussion 7
Phenotypic characterization: Broth Microdilution 7
Molecular Characterization: DNA Isolation and PCR 13
References 18
Appendix 20

Summary

Faecal samples were collected from obtained from whales and seals for analysis of antimicrobial resistance. There were 55 whale samples and 5 seal samples, from which bacteria were cultivated on a variety of culture media. Ten isolates per sample (total 600) were selected for further analysis.

For testing of phenotypic resistance to a selection of antibiotics (ampicillin, cefotaxime, vancomycin, kanamycin, tetracycline, ciprofloxacin, erythromycin and trimethoprim), the standard method for determining minimum inhibitory concentration (MIC) was used. A concentration range of 0.0625 – 32 mg/L was tested. MICs were determined for 285 of the isolates, 239 from whale samples and 46 isolates from seal samples. For each antibiotic, there were isolates which were capable of growing at the maximum concentration tested (32 mg/L). The MIC values for ampicillin and tetracycline were low compared to studies of other environmental samples. The highest number of isolates with an MIC of ≥32 mg/L was seen for trimethoprim, which also had an overall MIC range which was high compared to similar studies. There were observable differences in the distribution of MIC values between the seal and whale samples, though this appears to be due to an overrepresentation of one species in the seal samples.

Molecular testing for a selection of antimicrobial resistance genes (AMRGs) was done using standard PCR methods. The selected AMRGs were *mecA, tetA, ermB, nptII, qnrs, dfrA1, vanA*, as well as two genes which code for multidrug resistance (MDR) efflux pumps, *acrB* and *mexD*. Only *nptII* failed to produce any amplicons. There was not a strong association between high MIC values and the detection of resistance genes (except for vancomycin and *VanA*), though this is likely due to the small selection of genes screened for.

The results of this study reveal a snapshot of the antimicrobial susceptibility and resistance genes present in isolates collected from whale and seal samples, and contribute to a growing body of evidence connecting the environment to dissemination of antibiotic resistance. The present study is limited in terms of scale, and continued research is required to fully understand the role of marine mammals in the context of maintenance and spread of antimicrobial resistance.

Sammendrag på norsk

Avføringsprøver fra vågehval og sel ble samlet inn og analysert for antimikrobiell resistens. Det var 55 hvalprøver og 5 selprøver. Bakteriene ble dyrket i ulike kulturer, og ti isolater pr. prøve (totalt 600) ble valgt for videre analyse.

Ved testing av fenotypisk resistens mot et utvalg av antibiotika (ampicillin, cefotaxim, vancomycin, kanamycin, tetracyklin, ciprofloxacin, erytromycin og trimethoprim) ble standardmetoden for å bestemme minste hemmende konsentrasjon (MIC) benyttet. Et konsentrasjonsområde på 0,0625 - 32 mg/l ble testet. MIC ble fastslått for 285 av isolatene, 239 fra hvalprøver og 46 isolater fra selprøver. For hvert antibiotikum fantes det isolater som var i stand til å vokse i den maksimale konsentrasjonen som ble testet (32 mg/l). MIC-verdiene for ampicillin og tetracyklin var lave sammenlignet med studier av andre miljøprøver. Det høyeste antallet isolater med en MIC på ≥32 mg/l ble observert for trimetoprim, som også hadde et samlet MIC-område som var høyt sammenlignet med lignende studier. Det var observerbare forskjeller i fordelingen av MIC-verdier mellom sel- og hvalprøver, men dette kan skyldes at prøvene kom kun fra en selart.

Molekylær testing av et utvalg antimikrobielle resistensgener (AMRG) ble gjort ved bruk av standard PCR-metoder. De utvalgte AMRG var *mecA, tetA, ermB, nptII, qnrs, dfrA1, vanA*, samt to gener som koder for multidrug resistens (MDR) effluks pumper, *acrB* og *mexD*. Kun i testene for *nptII* ble det ikke produsert noen amplikoner (PCR-produkt). Det var ikke en sterk sammenheng mellom høye MIC-verdier og deteksjon av resistensgener (unntatt for vancomycin og *VanA*), men dette skyldes sannsynligvis det begrensede utvalget av gener som ble analysert.

Resultatene i denne studien viser et øyeblikksbilde av den antimikrobielle resistensen og av de antibiotikaresistensgenene som er tilstede i isolater fra hval- og selprøver. Studien er begrenset i omfang, men bidrar til økt kunnskap om forekomst og spredning av antibiotikaresistens i naturmiljøet. Det er nødvendig med videre forskning for å fullt ut forstå rollen marine pattedyr har i sammenheng med opprettholdelse og spredning av antimikrobiell resistens.

Introduction

In the decades since humans began the mass production of antimicrobial products (especially antibiotics), there has been an increase in cases of antimicrobial resistance (AMR) at the clinical level, which interferes with our ability to treat disease. A large and diverse range of antimicrobial resistance genes and organisms is found in the environment, and it is likely that clinical AMR has its origins in environmental reservoirs (D'Costa *et al.*, 2011; Larsson *et al.*, 2018; Perry *et al.*, 2016). The capacity of microorganisms to share genetic material via horizontal gene transfer (HGT) means that genes present in the environmental resistome have the potential to move between bacterial communities, potentially transferring antimicrobial resistance genes to human and animal pathogens (Finley *et al.*, 2013; Larsson, 2014). There is a need for better understanding of how antimicrobial resistance drivers present in the environment impact the prevalence and spread of AMR, how this intersects with spread facilitated by wildlife and water, and connects with human activities (VKM, 2018).

Minke whales are highly mobile animals, migrating enormous distances from areas in the North Atlantic (around the coast of Norway and Greenland), to as far south as the waters around the Southeastern United States and the Caribbean (Risch *et al.*, 2014). Migratory animals are of particular interest when considering environmental reservoirs of AMR, because in addition to being exposed to multiple sources of antimicrobials and drivers of resistance, they may also play a role in the spread of AMR bacteria and genes between different environments (Viana *et al.*, 2016; VKM, 2018). Minke whales, with their extensive migration patterns, thus make good candidates for investigation of this concept.

This project aims to improve understanding of this situation by mapping antimicrobial resistant bacteria and genes sampled from minke whales. Building on our previous experience, GenØk proposes to map antibiotic resistance by following a culture-based approach, which allows resistance phenotypes to be investigated, and a molecular approach, for the detection of specific genes associated with antimicrobial resistance phenotypes.

Materials and Methods

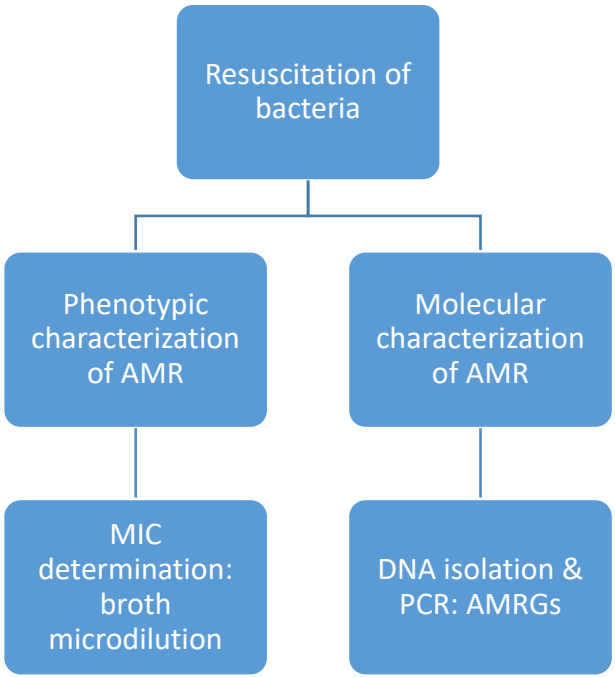
Sampling

Minke whale samples were collected by Lotta Lindblom (Institute of Marine Research (Havforskningsinstituttet)). The samples were collected in Lofoten and transferred to GenØk, where they were kept frozen until use. More information regarding the whales from which the samples were taken can be found in the appendix. The seals samples were collected in Tromsø by GenØk personnel from seal cubs shortly after arrival from the coast of Greenland.

Seal samples were collected with rectal swabs, which were placed in a BHI-glycerol mixture and frozen to preserve them until resuscitation.

Methods

A brief summary of the methods is presented in the diagram below. There were three main phases of work: resuscitation of bacteria from samples, determination of minimum inhibitory concentration of various antibiotics, and screening for selected antimicrobial resistance genes (AMRGs).



Resuscitation of bacteria from frozen samples

Non-selective culturing was used to resuscitate bacteria from the frozen samples. Sample material was plated out on MacConkey agar, Nutrient agar and Brain Heart Infusion agar. After incubation, colonies

were selected for sub-culturing to ensure single cultures (referred to as isolates). Ten isolates per sample were selected for further testing.

Phenotypic characterization: Broth Microdilution

The antimicrobial resistance capabilities of the isolates against selected antimicrobial agents were determined by broth microdilution, based on the ISO 20776-1 international standard method. This method allows for the determination of minimum inhibitory concentration (MIC) for each of the selected antibiotics for each isolate. Four antibiotics were tested per 96-well plate, in duplicate, at a concentration range of 0.0625 mg/L to 32 mg/L (see example of plate setup in figure below). The 8 antibiotics tested were ampicillin, cefotaxime, vancomycin, kanamycin, tetracycline, ciprofloxacin, erythromycin, and trimethoprim.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	Antibiotic 1
B	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	
C	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	Antibiotic 2
D	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	
E	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	Antibiotic 3
F	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	
G	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	Antibiotic 4
H	0.0625 mg/L	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	32 mg/L	GC	NC	

Figure 1 Microdilution plate setup with increasing concentration gradient from left to right. GC = Growth control (no antibiotic), NC = Negative control (no antibiotic and no inoculant).

Molecular Characterization: DNA Isolation and PCR

DNA was extracted from each isolate using the Lucigen QuickExtract method, and diluted before use as a template for PCR. 16S rRNA primers were used as a quality control step to demonstrate the isolated DNA was amplifiable and not subject to inhibition. For molecular characterization of AMR, a selection of genes has been compiled which confer resistance phenotypes to the antibiotics given in table 1. Primers have been selected to detect these genes, which are given in table 1 below. After PCR, electrophoresis to determine the results was done using the E-Gel electrophoresis system (Invitrogen).

Table 1) Selected antibiotic resistance genes and the primers used for their detection in this study

Antibiotic	Gene	Primer Sequences (5' - 3')	Annealing temp (°C)	Amplicon size (bp)	Reference
Tetracycline	*TetA*	CCTGATTATGCCGGTGCT TGGCGTAGTCGACAGCAG	61	200	(Szczepanowski *et al.*, 2009)
Ampicillin	*mecA*	AAAAAGATGGCAAAGATATTCAA TTCTTCGTTACTCATGCCATACA	56	185	(Szczepanowski *et al.*, 2009)
Kanamycin	*nptII*	ATGATTGAACAAGATGGATTGC TCAGAAGAACTCGTCAAGAAGG	55	364	(Börjesson *et al.*, 2009)
Erythromycin	*Erm(B)*	GATACCGTTTACGAAATGG GAATCGAGACTTGAGTGTGC	58	364	(Chen *et al.*, 2007)
Ciprofloxacin	*qnrS*	ATCAAGTGAGTAATCGTATGTACT CACCTCGACTTAAGTCTGAC	61	171	(Berglund *et al.*, 2014)
Trimetoprim	*dfrA1*	ATGGAGTGCCAAAGGTGAAC TATCTCCCCACCACCTGAAA	62	241	(Grape *et al.*, 2007)
Vancomycin	*VanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	54	732	(Biavasco *et al.*, 2007)
Multidrug efflux pump, RND family	*acrB*	ATATCCTACGATTGCACCGC GGTACCCGTGGAGTCACTGT	62	160	(Szczepanowski *et al.*, 2009)
Multidrug efflux pump, RND family	*mexD*	TCAACGGTCTGGGTAACTCC GTCGATCAACAGGCGCAGT	62	182	(Szczepanowski *et al.*, 2009)

Results and Discussion

Phenotypic characterization: Broth Microdilution

After resuscitation and sub-sampling, 600 isolates were selected from 55 whale samples and 5 seal samples. Of the selected isolates, 285 met the criteria for MIC determination using the broth determination method in terms of the amount of growth within the allotted time period of this method. Of these, 239 were isolated from whale samples, and 46 from seal samples. The MIC results are summarized in the graphs below.

The MIC values of the isolates collected from whale and seal faeces samples were lower than initially expected. The original concentration range chosen to test the antibiotics was 0.5 – 128 mg/L. Early experiments indicated that this range was too high, and it was lowered to 0.0625 – 32 mg/L which worked well for the majority of the isolates. In some isolates, even the lowest value of 0.0625 mg/L proved to be enough to inhibit the growth of the bacteria.

Differences can be seen in the MIC profiles between the isolates which were cultured from whale samples compared to those from seals. Caution should be exercised when interpreting these results, since the number of isolates per sample is small, and it is possible that in some cases that several isolates were part of the same clonal lineage (particularly among the seal isolates). The sampling between the whales and seals also differed, which affected the number and type of colonies available for selection at the sub-culturing step. Nonetheless, differences can be observed in the responses of these 2 groups of samples to the selected antibiotics.

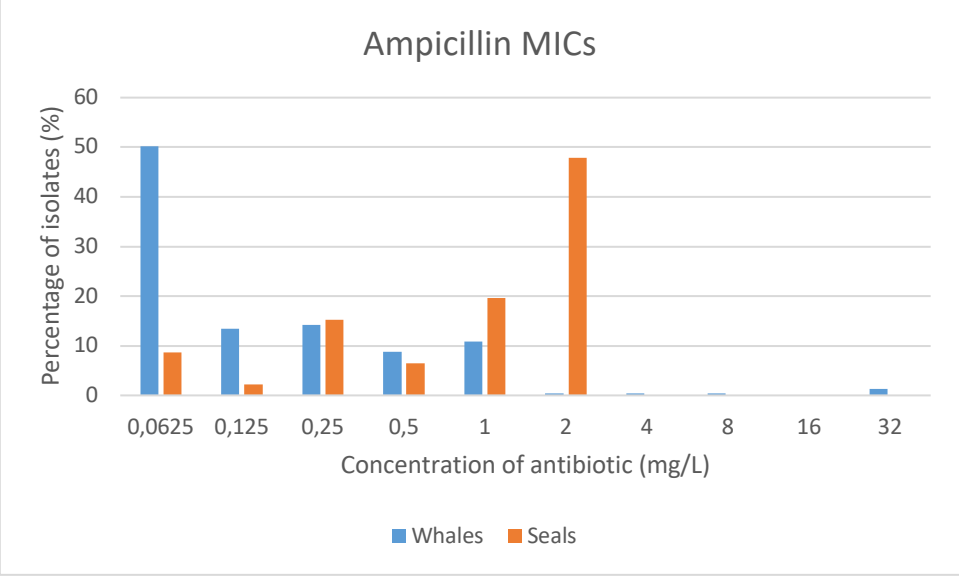


Figure 2 Distribution of MIC values for Ampicillin for isolates cultivated from whale and seal faeces

The whale isolates tended to be more sensitive to ampicillin, with 86.6 % of the isolates having an MIC of 0.5 mg/L or less, with 50.2 % having an MIC of the lowest concentration tested (0.0625 mg/L). One the other hand, most of the seal isolates (67.4 %) had an MIC value of 1 mg/L and above, with almost half (47.8 %) reaching an MIC of 2 mg/L. Both groups of isolates have low MICs for ampicillin compared to the isolates collected from roe deer and reindeer samples by the Norwegian Veterinary Institute, however, of which 70 % had MICs of 4 – 8 mg/L (Sunde *et al.*, 2018). Our observations are consistent with Glad *et al.* (2010), however, who also found phenotypic resistance to ampicillin to be low in isolates cultivated from Arctic and Sub-Arctic seal colonic samples.

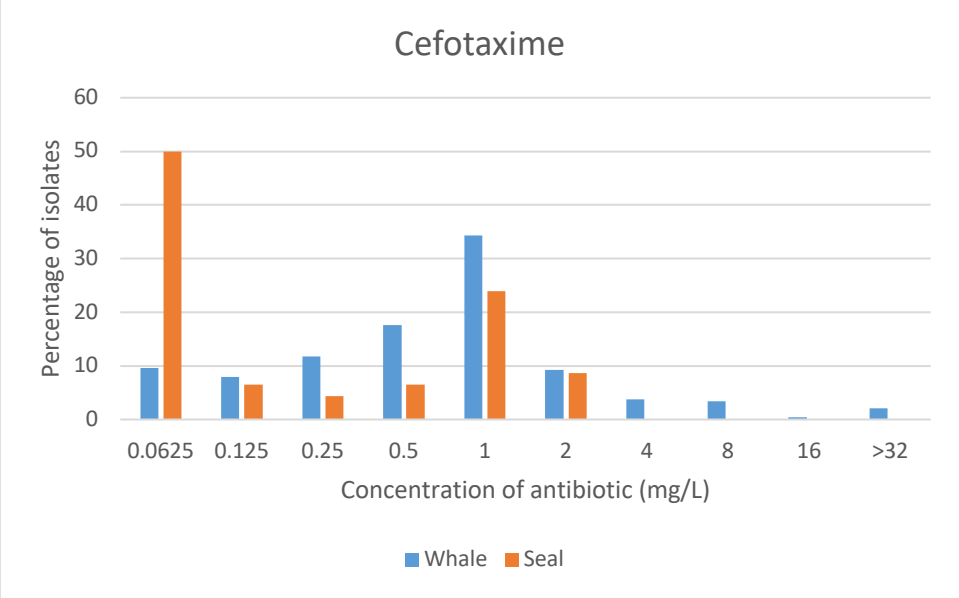


Figure 3 Distribution of MIC values for Cefotaxime for isolates cultivated from whale and seal faeces

A different trend is observed for cefotaxime, where 50% of the seal isolates had MIC values of 0.0625 mg/L, while the whale isolates' MICs were more spread out across the concentration range (see figure 2). Three of the 5 isolates with MIC values of >32 mg/L for cefotaxime also had MIC values of >32 mg/L for kanamycin, and 2 of these (both from the same whale sample, W50) also had an MIC of >32 mg/L for trimethoprim.

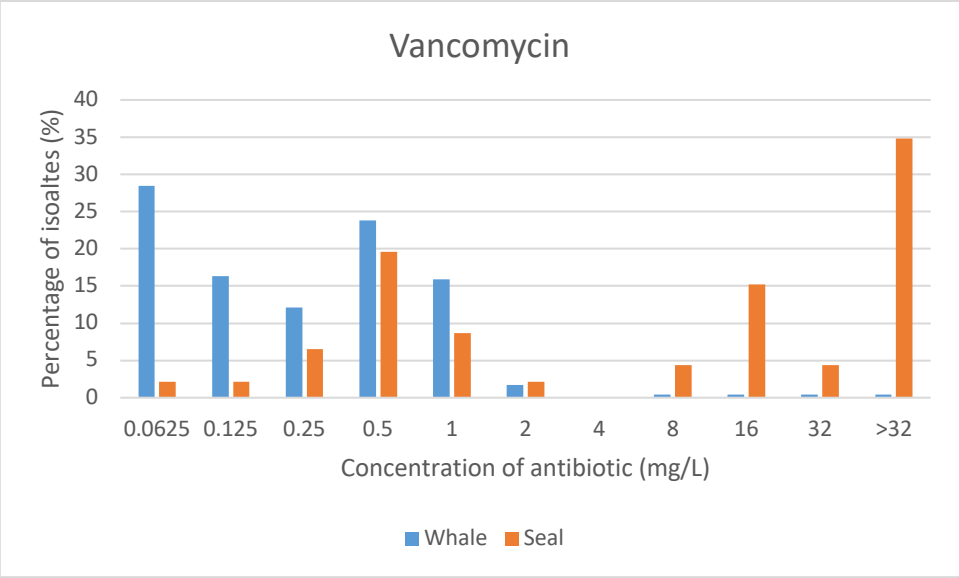


Figure 4 Distribution of MIC values for Vancomycin for isolates cultivated from whale and seal faeces

One of the most stark examples of the differences in MIC values between the whale and seal isolated is for vancomycin (see figure 4). While the majority of the whale isolates had MIC values which fell between 0.0625 mg/L and 0.5 mg/L (80.8% of the isolates), the seal isolates showed a very different pattern. The majority of the seal isolate MIC values (58.7%) were above 8 mg/L, with a large portion (34.8%) exceeding the maximum concentration of 32 mg/L. In fact, the collection of seal isolates responsible for this portion of high MIC values has very similar MIC values across the selected antibiotics, and also bears a very similar profile of PCR results. This group of around 28 isolates, with representatives from all 5 seal samples, accounts for the bulges of MIC values seen in the seal data for ampicillin MIC 1-2 mg/L, cefotaxime and ciprofloxacin MIC 0.0625 mg/L, and erythromycin MIC ≥32 mg/L. The trend is less obvious for tetracycline, kanamycin, and trimethoprim MIC values, which are more evenly distributed across the antibiotic concentrations.

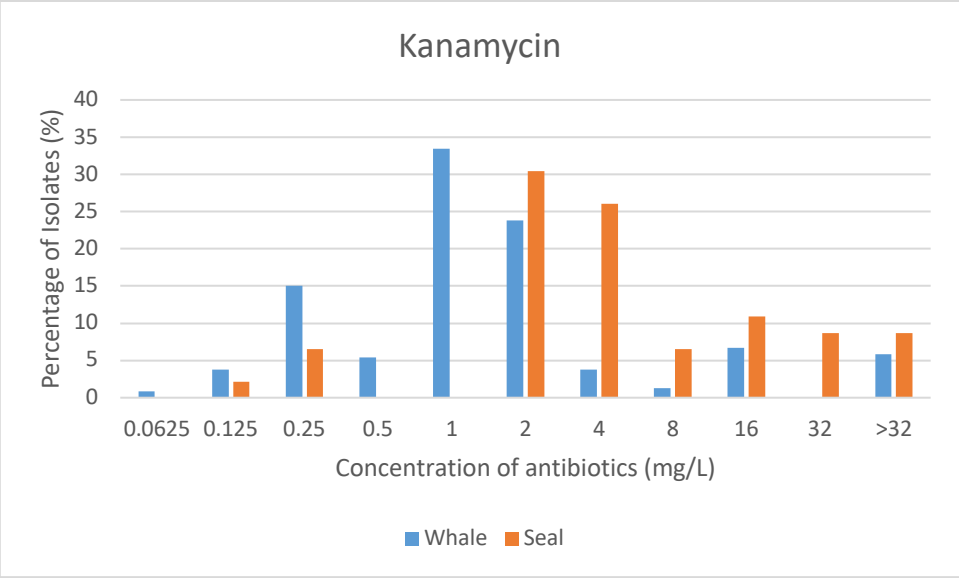


Figure 5 Distribution of MIC values for Kanamycin for isolates cultivated from whale and seal faeces

Kanamycin is the antibiotic with the most similar distribution of MIC values between the whale and seal isolates. Most of the MIC values were in the 1-4 mg/L range, though MICs of 16-≥32 mg/L were measured for both whale and seal isolates (43 isolates in total).

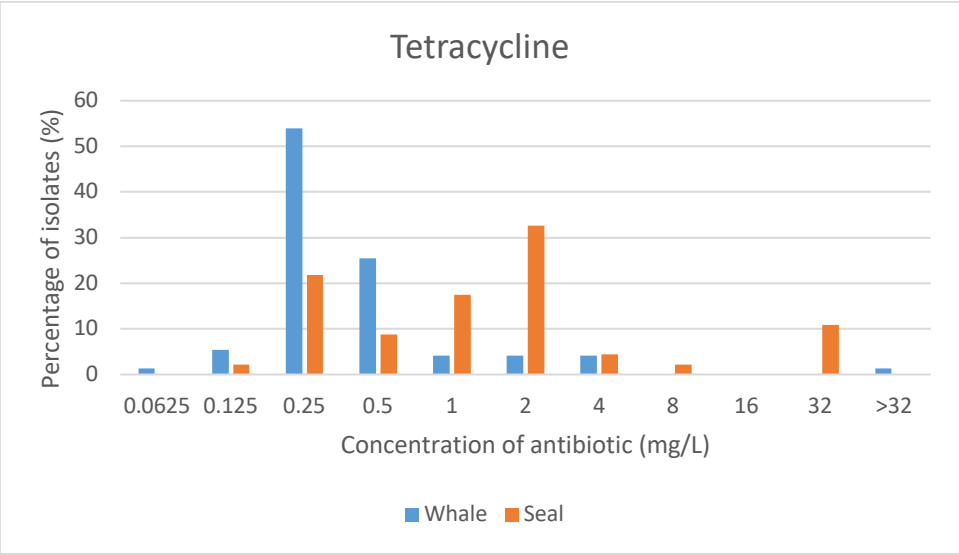


Figure 6 Distribution of MIC values for Tetracycline for isolates cultivated from whale and seal faeces

For tetracycline, 53.9% of the whale isolates had an MIC value of 0.25 mg/L and 25.5% of 0.5 mg/L, reflecting the overall low tolerance to this antibiotic. Sunde *et al.* (2018) reported that 95% of their faecal isolates had an MIC of 2 mg/L for tetracycline. These values are low compared to the screening concentration used by Glad *et al.* (2010), who also reported low resistance to tetracycline in their seal

samples. While the MIC values were generally low, 5 seal isolates and 3 whale isolates had MIC values of ≥32 mg/L for tetracycline. All but one of the isolates with MIC of ≥32 mg/L for tetracycline, also had MIC values of ≥32 mg/L for trimethoprim.

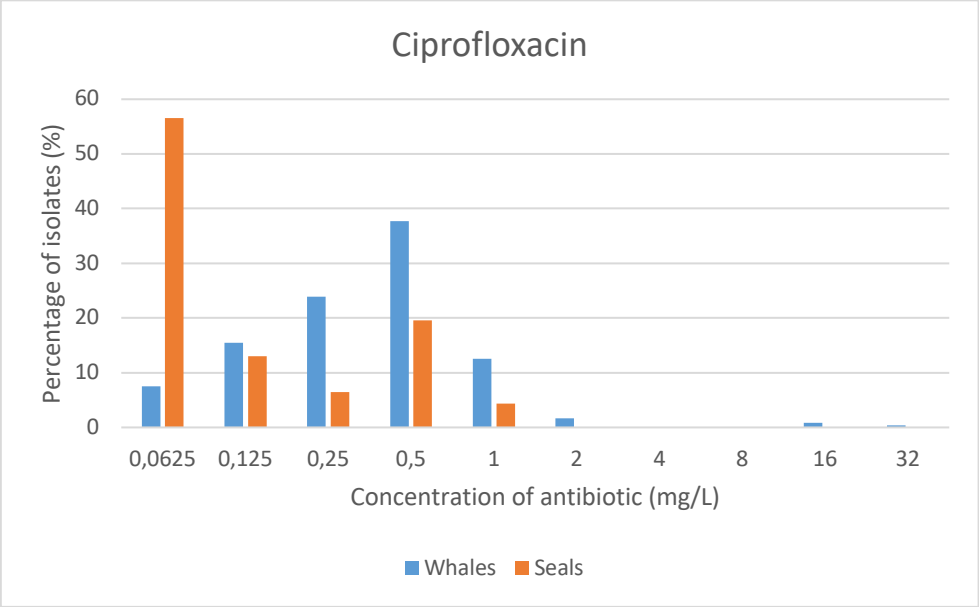


Figure 7 Distribution of MIC values for Ciprofloxacin for isolates cultivated from whale and seal faeces

Ciprofloxacin had the lowest range of MIC values of all the antibiotics testes, with 98.95% of the isolates having an MIC value of 2 mg/L or less. Only 3 isolates (all from different whale samples) had MIC vales of 16-32 mg/L, and all 3 of these also had MIC values of ≥32 mg/L for ampicillin. Two of these isolates also had an MIC of >32 mg/L for trimethoprim, and MICs of 16->32 mg/L for erythromycin.

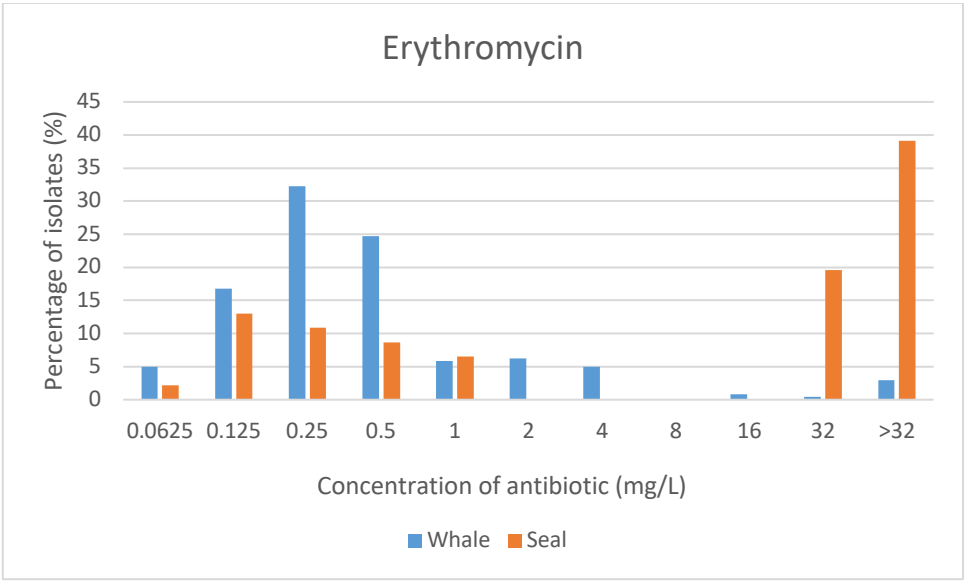


Figure 8 Distribution of MIC values for Erythromycin for isolates cultivated from whale and seal faeces

Of the 35 isolates with MIC values of ≥32 mg/L for erythromycin, 27 are included in the collection of seal isolates with similar MIC patterns mentioned above. Of the 8 whale isolates with erythromycin MIC values of ≥32 mg/L, 5 come from the same whale sample (W33) and have very similar MIC values for the other antibiotics too – possibly these isolates are of the same lineage. Of the three other whale isolates with a high MIC for erythromycin, only one had high MIC values for other antibiotics as well.

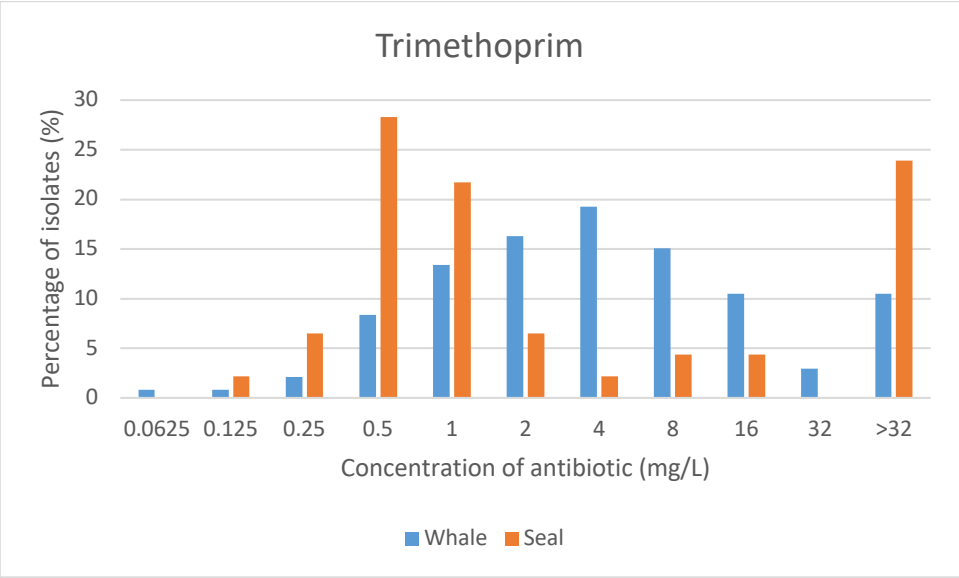


Figure 9 Distribution of MIC values for Trimethoprim for isolates cultivated from whale and seal faeces

The range of trimethoprim MIC values was higher than the other antibiotics, with only 4.6% of the isolates having MIC values of 0.25 mg/L or lower. Trimethoprim also has the highest number of isolates with an MIC value of ≥32 mg/L of all the antibiotics tested. These results differ from those of Sunde *et al.* (2018), since 90% of the isolates in that study (isolated from deer and reindeer faeces) had an MIC of 0.25 mg/L.

Molecular Characterization: DNA Isolation and PCR

Table 2 Summary of PCR results after amplification with primers targeting selected AMRGs.

Gene	Number of amplicons (Whale isolates)	Number of amplicons (Seal isolates)	Total
TetA	5	0	5
mecA	10	1	11
nptII	0	0	0
Erm(B)	2	0	2
qnrS	1	0	1
dfrA1	5	5	10
VanA	54	27	81
acrB	7	27	34
mexD	3	12	15

After DNA isolation and PCR with 16S primers, PCR was done with a selection of primers targeting various antibiotic resistance genes, including two genes targeting multidrug efflux pumps (*acrB* and *mexD*). In the case of the molecular characterization, all 600 selected isolates were screened. Table 2 above is a summary of the PCR results, listing amplicons which correspond to the length of the target sequence. Amplicons of incorrect size or multiple amplicons are not listed.

The highest number of amplicons were obtained for *vanA*, which confers resistance to vancomycin (Biavasco *et al*., 2007), which of all the genes tested also had the best association with high MIC values for the associated antibiotic. The genes coding for efflux pumps, which could potentially confer a degree of resistance to a number of antimicrobial compounds, also produced a high number of amplicons. Many of these amplicons were concentrated among the seal samples, within the group which also accounted for the bulges in MIC values for some antibiotics. This supports the notion that, when taken from the same sample, the isolates may be clonally related to each other rather than representing a number of different species with the same AMR profile.

Amplicons of the correct size were generated for all primer sets except *nptII* (neomycin phosphotransferase II), which codes for kanamycin resistance. This gene is frequently used as an antibiotic resistance marker during the development of genetically modified crops (Nordgård, 2016). Though thought to be virtually ubiquitous in some environments, *nptII* was not detected in the present study, nor in Nordgård *et al.* (2017) who screened for the presence of the gene in reindeer faeces. To the best of our knowledge, it is only the present study and that of our colleagues Nordgård *et al.* (2017)

which have investigated the presence of antibiotic reistance marker genes in wild animals. Detection of high MIC values suggest that other genes associated with kanamycin resistance are present in the isolates, which could be tested for at a later date.

Association of the presence of amplicons of the expected size with MICs which indicate a level of resistance to a given antibiotic was not a given. For example, none of the isolates which displayed MIC values of 8->32 mg/L for tetracycline produced an amplicon of the correct size when amplified with *tetA* primers. Of the 5 *tetA* amplicons of the correct size, three belonged to isolates which came from the same whale sample (W61), and were associated with MIC values of 2-4 mg/L. However, we only screened for *tetA*, and there is a plethora of other genes associated with tetracycline resistance (Roberts & Schwarz, 2016), including several multidrug efflux pumps such as *acrB* and *mexD* (McArthur *et al.*, 2013). Neither *acrB* nor *mexD* amplicons were associated with high tetracycline MIC values either, however, suggesting that other genes were responsible. Ampicillin and cefotaxime MIC values were also not closely associated with positive results with any of the primer sets.

Several isolates produced amplicons with primer sets targeting multiple genes conferring resistance to antibiotics. The cluster of around 28 seal isolates which is responsible for the grouping of MIC values for seals for ampicillin, cefotaxime, vancomycin, ciprofloxacin and trimethoprim, also has a pattern of PCR results which is very similar. These isolates account for 24 of the 34 *acrB* amplicons, 20 of the *vanA* amplicons and 12 of the *mexD* amplicons listed in table 2. The high MICs seen in this group towards vancomycin, erythromycin and trimethoprim may be explained by these results.

The limited association of the selected target genes with the MIC values of the antibiotics suggests that a wider net should be cast in the detection of AMRGs in such samples. A metagenomics analysis following next generation sequencing of total DNA isolated from these samples is recommended. A similar approach, followed by Nordgård (2017a), demonstrated that such an untargeted examination of AMRGs was very useful in analysing environmental samples. A non-culture based approach would also compliment the current culture-based approach, since the limitations of cultivation in terms of community representation (particularly in cases such as this study when samples had endured periods of freezing at non-ideal temperatures) can be overcome. Follow-up studies which place greater emphasis on genes associated with mobile genetic elements, such as intergrases and transposon genes, would also compliment the current list of genes screened for.

The recent VKM report noted a tendency towards *directionality* in the patterns of antimicrobial resistance in wildlife populations. The reviewed studies showed a trend of higher AMR in wildlife

populations in close proximity and association with humans, compared to those with minimal contact (VKM, 2018), though more study was needed. The present study, which considered a limited number of isolates and antimicrobial resistance genes, can currently contribute little towards better understanding this scenario. However, we have now analysed 2 years' worth of whale samples. If similar studies were to be done in the future, with a greater number of AMRGs, a clearer picture may emerge.

Limitations

There are many limitations/uncertainties related to environmental studies and antimicrobial resistance (AMR). Many of the uncertainties arise due to technical limitations, data limitations and lack of standardized protocols. In this study, some of the listed limitations are also due to the limited project period and can easily be followed up, while others are connected to sampling procedures and lead rather to recommendations for future studies. This study represents a snapshot of the samples tested, and cannot provide all details.

- Sampling and sample preservation: there are practical aspects connected to sampling in the environment which can mean that sample preservation methods are not ideal. In this case, the whale samples were collected as part of a larger field study, and could not be returned to the lab for analysis immediately, not could they be stored at ≤-60°C which would have preserved the sample better. The practicalities of the sampling trip meant that the samples were frozen at -20°C in the field, which made resuscitation of the bacteria in those samples tricky. It may also have contributed to a sampling bias, favouring genera with survival strategies which can overcome cold and adverse conditions. Less hardy bacteria may have been eliminated, preventing their representation in the study (even if they were prevalent in the original sample). The wide range of colony morphologies seen at the resuscitation step does suggest that many species are represented however, despite the sampling limitations. However, it should also be noted that the slow-growing nature of many of these isolates made them unsuitable candidates for the MIC method selected for this study, which favours fast-growing organisms. This resulted in usable MIC results for 239 isolates, approximately half of the isolates cultivated.
- The sampling procedure and storage of the seal samples differed from that of the whales, The seal samples, which were taken by rectal swab, were placed in BHI with glycerol as a protectant before being frozen. While this made resuscitation of the bacteria easier, it did seem to favour a faster-growing, though less diverse, set of isolates than the whale samples.

- Cultivation based methods themselves introduce biases and limitations to a study, and are only capable of recovering a small percentage of the total community in such diverse samples. We used a variety of culture media to try to recover a good representation of the community present. This approach would be complimented by a metagenomics approach, which would provide more details about the bacteria present and the genes they carry, which were not represented using the current methodology. This would also allow detection of a greater number of resistance genes than the list chosen for this study.
- The isolates in this study were not identified. This limits interpretation of the MIC results in particular. Sequencing or MALDI-TOFF identification of the isolates, particularly those which had high MIC values and/or positive PCR results for resistance genes, would be a valuable addition to the study, and one which could easily be accomplished in follow-up studies.
- Likewise, amplicons obtained through PCR screening for the selected AMRGs have not been sequenced to confirm their identities, though this could also easily be achieved in follow-up.

Recommendations

This study has provided a foundation of results relating to the antimicrobial capabilities and presence of resistance genes in these samples. The whale faeces samples in particular are rare and not easily obtained, so it is strongly recommended to get the most out of these samples, since they provide a view into the AMR profiles of highly mobile marine mammals.

Follow-up work which could be done with the current samples:

- Total DNA isolation from the samples, followed by either next generation sequencing and metagenomic analysis, or screening with a wider selection of antimicrobial resistance genes (with more emphasis on mobile genetic elements) using standard PCR or Q-PCR methods.
- Sequencing of PCR amplicons to confirm the presence of the AMRGs detected, as well as to identify the isolates carrying them (using the 16S amplicons).

Recommendations for future studies

- Inclusion of more modern methods to maximise the information which can be obtained from the samples. Metagenomic analysis and comparison with databases such as CARD, similar to the methods used by Nordgård (2017a), are well established approaches which provide a great deal of information. Other recent advances include epicPCR and plasmidome analysis, which could provide more targeted data from the total DNA sample.

- A standardized sampling procedure for collection of faecal samples during field studies, which is tailored to the needs of the study but also takes into account the practicalities of field expeditions, should be developed. These differ depending on whether the analysis will be culture-based or non-culture based. If the samples are to be collected by field scientists who are not involved with the microbiological analysis, better support and training could be provided so that the samples are collected in a way which results in good characterization of the microbial community. On the other hand, knowing the limitations of the field situation may mean that the microbiological analysis needs to be planned to take these into account.
- Currently, we have analysed 2 years' worth of whale samples. If possible, faecal samples for microbiological analysis should become part of the compliment of samples taken during the research hunt, with screening for AMRGs becoming part of the standard regimen of tests done on such samples. More data, provided over a longer time period, would help to fill knowledge gaps about environmental antimicrobial resistance reservoirs and the directionality of the spread of AMRGs.

Conclusion

The results of this study reveal a snapshot of the antimicrobial susceptibility and resistance genes present in isolates collected from whale and seal samples. For each antibiotic, there were some isolates which were capable of growing at concentrations of ≥ 32 mg/L, the maximum concentration tested in this study. Of the selected antimicrobial resistance genes, only *nptII* had no positive results. While detection of AMRGs in environmental settings is not unusual, it does contribute to a growing body of evidence connecting the environment to dissemination of antibiotic resistance. It is also important to highlight that the present study is limited in terms of scale, and continued research is required to fully understand the role of marine mammals in the context of maintenance and spread of antimicrobial resistance.

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Appendix

Supplementary table 1: information regarding whales from which samples were taken

Whale number	Sampling year	Length	Diameter	Sex	F-length	Comments
20	2017	760	360			
22	2017	790	380	1	37	
23	2017	820	420	2	51	
24	2017	880	460	2	52	
25	2017	830	400	2	42	
26	2017	790	400	1	63	
28	2017	740	400	1	32	
29	2017	900		1	90	
30	2017	840	410	2	53	
32	2017	860	400	2	60	
33	2017	820	400	2	65	
35	2017	930	420	1	25	
36	2017	800	410	1	57	
37	2017	910	450	2	65	
38	2017	830	430	2	44	
39	2017	850	450	2	78	
40	2017	810	430	1	89	
41	2017	840	410	2	86	
44	2017	920	490	2	86	
45	2017	840	450	2	43	
46	2017	840	450	1	65	
47	2017	820	400			
48	2017	710	350			
49	2017	840	410	1	19	
50	2017	690	340			
51	2017	740	400	2	76	
52	2017	840	400	1	67	
54	2017	910	420	1	71	
55	2017	790	400	2	59	
56	2017	820	430	1	65	
57	2017	810	410	2	76	
58	2017	650	340			
59	2017	770	410	1	62	
60	2017	820	420			Pregnant, but no foetus yet
61	2017	650	350			
62	2017	800	360	1	34	
63	2017	730	320			
64	2017	740	370			
65	2017	750	360			
86	2018	670	270			

88	2018	800	420	1	78	Liver parasite
90	2018	690	300			
92	2018	650	350			
93	2018	810	450	2	48	
94	2018	850	500	2	139	External abdominal parasite
96	2018	780	440	2	124	
97	2018	800	440	1	117	
98	2018	820	440	1	120	Liver parasite
99	2018	870	440	2	60	
100	2018	810	460	1	131	
102	2018	860	460	1	120	
103	2018	860	480	2	151	
104	2018	820	460	2	149	
105	2018	810	470	2	173	
107	2018	810	470	2	160	