Animal Microbiome

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Antimicrobial treatment affects the microbiome and resistome of both treated and untreated rehabilitating harbour seals (*Phoca vitulina*)

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Abstract

Background Antimicrobial use has contributed to the alarming rise of antimicrobial resistance (AMR), posing a significant global health threat. Effective AMR control requires a One Health approach. The presence of AMR in the environment can challenge wildlife conservation, as resistance may reduce treatment success. This study investigates the impact of antimicrobial treatment on the gut microbiome and resistome of harbour seals (*Phoca vitulina*) undergoing rehabilitation at the Sealcentre Pieterburen, the Netherlands. A longitudinal cohort study was conducted with 200 seals, from which 127 were treated with antimicrobials and 73 were not treated. Samples were collected before and during rehabilitation, including before and after treatment and analysed using 16 S rRNA gene sequencing, shotgun metagenomics, and targeted qPCR.

Results We observed a significant but transient decrease in gut microbiome alpha diversity following antimicrobial treatment, with a recovery observed by the time of release. Beta diversity analysis indicated persistent changes in microbial composition post-treatment. An increase in antimicrobial resistance gene load was observed in treated seals, with some resistance genes remaining high at release. Untreated seals cohabiting with treated seals also exhibited increased resistance gene loads, suggesting exposure through environmental transmission.

Conclusions Antimicrobial treatments in rehabilitation settings alter the gut microbiome and enhance AMR gene persistence in seals. The potential risk of antimicrobial resistance transmission among rehabilitating seals suggests the need for antimicrobial stewardship as the risk of antimicrobial resistance contamination by seals returning to the wild is currently unknown.

Keywords Harbour seal, Common seal, Microbiome, Resistome, Antimicrobial, Rehabilitation, qPCR, Alpha diversity, Beta diversity, Composition

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Background

Antimicrobial drugs are essential for fighting bacterial diseases and are used worldwide to treat humans, animals and plants [1–4]. The use of antimicrobial drugs [5, 6] has led to one of the most concerning health problems of our times: antimicrobial resistance (AMR). AMR threatens human and animal health around the globe and its control requires a One Health approach [5, 7, 8]. Antimicrobial stewardship programs in human and veterinary medicine have been shown to be successful in reducing antimicrobial use and consequently also antimicrobial resistance [7]. However, the widespread presence of AMR in the environment might pose a threat for wild animals and might challenge conservation efforts. Coastal ecosystems are becoming increasingly important as reservoirs but may also be sentinels for infectious organisms and AMR as human populations in coastal areas continue to increase [9–12].

Antimicrobial drugs, when orally administrated, do not affect only the targeted bacteria, but also other bacteria present in the digestive tract and may lead to dysbiosis [13–16]. In humans, it has been described that gut bacterial microbiota diversity and its composition decrease with administration of antimicrobials [17, 18]. Similarly, in other animal species, such as dogs, mice, and equids, some of the same changes have been reported [19–23]. In addition, antimicrobial drugs might influence the resistome, leading to an increase of antimicrobial resistance genes (ARG) that can persist over time [13–15, 24–26].

Animals undergoing rehabilitation are often sick or injured when admitted to a rehabilitation centre and regularly need antimicrobial treatment [27]. In the case of stranded wild seals admitted at the Sealcentre Pieterburen, the Netherlands, more than half of them receive antimicrobial therapy to treat bacterial infections like infected wounds, umbilical infections and pneumonia (Sealcentre unpublished data). This antimicrobial treatment could not only have an effect on their gut microbiome like reduced richness [28, 29], but also could potentially affect the gut microbiome of the seals that do not need antimicrobial treatment, as they are often sharing the same rehabilitation environment.

To our knowledge, little is known about the long term effects of antimicrobial treatment on the gut microbiome and resistome of harbour seals (*Phoca vitulina*). In-depth longitudinal studies have not yet been performed. Here we investigate two seal cohorts (total $n = 200$) under rehabilitation at the Sealcentre Pieterburen to reveal the influence of antimicrobial treatment administration on the gut microbiome diversity and composition; the differences in gut microbiome diversity and composition between treated and untreated seals; and the effect of rehabilitation and treatment on antimicrobial resistance gene load. For this we used 16S sequencing, shotgun metagenomics and resistome determination, and targeted qPCR of relevant resistance genes.

Materials and methods

Study design

In this longitudinal cohort study, harbour seals admitted for rehabilitation at the Sealcentre Pieterburen, the Netherlands, were repeatedly sampled during their rehabilitation period. All sampled seals stranded alive along the Dutch coast and islands and were transported to the Sealcentre Pieterburen for rehabilitation. During the summer of 2015, 88 harbour seal pups (seals estimated younger than two months at admission are referred to as ¨pups¨) and between October 2015 and April 2016, 112 harbour seal weaners (age estimated between two and ten months old at admission are referred to as “weaners”) were admitted to the centre. This seal cohort was partly studied by Rubio-Garcia et al., where the effect of long-term rehabilitation on the nontreated seal gut microbiome was described [30]|.

Seals received antimicrobial treatment if indicated because of bacterial infection suspicion. For pups, these were mainly umbilical infections, pneumonia and infected wounds, as for weaners pneumonia and wounds were the main reasons for antimicrobial treatment prescription by the veterinary team. Dosages for each treatment were based on publicly available dosage recommendations for seals [31]. The seals that received at least one antimicrobial oral treatment while in rehabilitation will be referred to as treated (38 pups and 89 weaners). Seals that did not receive any antimicrobial oral treatment will be referred to as untreated (50 pups and 23 weaners) (Table S1).

Every seal included in the study was sampled at admission (before any antimicrobial treatment was administered), during rehabilitation (days 8 and 15), and before release (referred to as t0, t8, t15 and R). Seals that died during rehabilitation were sampled according to the same protocol before death and additionally during post-mortem examination (final sample referred to as D). Regarding the treated seals, a sampling scheme different from the original timepoints was created (in addition to t0 and R), because these animals received treatment at different timepoints during rehabilitation. The following sampling moments were added: before treatment (BT) (when different than t0), and 1 day after the treatment course was completed (AT) (Table S2).

Faecal sample collection

As a proxy for faecal samples, we used rectal swabs (EswabTM: BD Liquid Amies Elution Swab Collection and Transport System) as previously described [32]. During veterinary exams or before feeding or

during post-mortem examination, the seals were manually restrained by a trained caretaker, and a cotton swab was introduced into the rectum of the seal to collect faecal material. Admitted seals were examined and sampled within 1 and 7 h after being found, depending on the location and distance to the Sealcentre Pieterburen. The swab was placed in a container with Amies liquid, and all swabs were stored at -80ºC within 48 h.

Metadata collection

During rehabilitation, all information related to the seals was recorded in the seal's medical file and a digital database. This concerned information on stranding date and location, estimated age, sex, weight, received medication and feeding type.

Age was estimated in the number of days based on the status of the umbilical stump. None of the pups presented lanugo coat at admission, which is considered a sign of prematurity [33]. Only if the stump is open, an accurate estimation (namely, younger than 10 days) can be done. The presence of an umbilical cord or an open stump was categorized as younger than 10 days, a closed stump 10 days or older. Individuals estimated to be weaned were assigned June as their month of birth, which is consistent with harbour seal births in the Wadden Sea [30, 34, 35].

Feeding

Feeding of the seals consisted of salmon emulsion (*Salmo salar*) and whole herring (*Clupea harengus*) and followed the protocol described by Rubio-Garcia et al. [30].

Environment

During their time at the Sealcentre Pieterburen, the seals were kept in different facilities, depending on the rehabilitation phase. Seals were clustered by arrival date (therefore by age) and moved along the different rehabilitation phases (phase 1 to 3) as they grew and medical treatment was discontinued for the treated animals. Treated and untreated seals were not housed in different enclosures if they were housed together because of arrival time. In all these facilities, the seals had access to water in smaller or bigger pools, alone or with more seals in the same pool. The water of the pools of all 3 phases was supplied by a closed water filtration system composed of three basic parts; mechanical filters that remove solids, biological filters or baffles to remove or detoxify chemicals in the water and disinfecting methods consisting of sodium hypochlorite (15gr/L) shock in the filtered water buffer to control or remove micro-organisms with the aim of < 100 CFU/ml following the Sealcentre standards for water quality [31].

DNA extraction and sequencing for amplicon sequencing

DNA extraction and Illumina 16 S amplicon sequencing were performed as described previously [30]. Briefly, DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands). The variable V3 and V4 regions of the 16 S rRNA amplicon were amplified and libraries were prepared following the 16 S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). and paired-end sequencing was performed using the 600 cycles MiSeq Reagent Kit V3 (Ilumina, San Diego, CA, USA), generating 2×300 bp paired-end reads.

Bioinformatics

Sequence data were transformed into an amplicon sequence variant (ASV) table and converted into a Phyloseq object [36] following the DADA2 pipeline [37] tutorial v.1.6 with settings as described by Theelen et al. [23]. Briefly, raw reads (250 bp) obtained from Illumina 16 S rRNA gene were analyzed using DADA2 [37]. Reads were truncated at positions 15–290 for forward, and 15–210 for reverse reads. Post filter and trimming the reads were merged. Merged data was used to create a sequence table. Reads were grouped into amplicon sequence variants (ASVs). After removing chimaeras, taxonomy was assigned using v. 132 of the Silva database [38] and analyzed using Phyloseq [36]. The data visualization and analysis were performed using a variety of R packages: ggpubr [39], microbiome [40], pairwiseAdonis [41], tidyverse [42], and vegan [43].

Microbiome data analysis

Analyses were performed separately for pups and weaners because of biological differences [44, 45].

Alpha diversity (Shannon) and richness (observed species) were calculated on rarefied data. Wilcoxon signed-rank (paired) test were performed to determine alpha diversity differences between treatment stages (at admission, 1 day after treatment and at release). At admission to the Sealcentre, mean alpha diversity was compared between seals that went on to have treatment during rehabilitation and seals that did not receive any antimicrobial treatment using Wilcoxon rank-sum (unpaired) test. Since one of the main aims was to investigate the microbiome before release back into the wild, several analyses were done in relation to the samples taken at the end of the rehabilitation. To compare the potential effect of antimicrobial treatment at the end of rehabilitation, the same comparison between groups as described for admission was done at release. Furthermore, univariable linear regression was done at release to test the effect of several possible influencing factors (age at sampling, days at rehab, sex, treatment yes/no). A p-value cut off below 0.2 was used to select variables for a multivariable

linear regression and after backward elimination the final model was presented. Within the treated seals group, univariable and multivariable linear regression was also done at release to test the effect of several possible influencing factors (age at sampling, days at rehab, sex, days after treatment, number of treatments).

The analysis of the microbiome composition (beta diversity – diversity between samples) and relative abundance was performed on unrarefied data, which was transformed in compositional data prior to analysis. Relative abundance was visualized at phylum and genus levels, throughout the different timepoints in rehabilitation, for both untreated and treated pups and weaners. Non-metric Multidimensional Scaling (NMDS) was performed to visualise (dis)similarities in the bacterial microbiome between treatment stages, and Bray-Curtis dissimilarity index was calculated based on the compositional data. If stress levels were above 0.2, NMDS ordinations were made in three dimensions.

Permutational multivariate analysis of variance (PERMANOVA) was used to test potential changes in the microbiome composition due to the influence of different determinants. First, each possible determinant was tested univariably and if the p-value was below 0.2, then a multivariable PERMANOVA was applied. To identify potential differences among treatment stages pairwise PERMANOVA was used. For each analysis, the homogeneity of dispersion was checked. All beta diversity statistical comparisons were done at the same timepoints and between treatment stages and groups as described for alpha diversity. For comparisons between treatment stages, the variable seal was included to account for host individuality.

Because of the high correlation between variables age at sampling and days at rehab, for multivariable analysis only days in rehab were used (Pearson's r of 0.99 for pups).

Data analysis and visualization were performed in R (version 4.0.5), using RStudio (version 1.4.1103), using the following packages: Phyloseq [36], Microviz (v0.9.1) [46] Microbiome [40], Vegan [47] and PairwiseAdonis (https://github.com/pmartinezarbizu/pairwiseAdonis).

Shotgun metagenomic sequencing & resistome data analysis

To determine the resistome in the microbiome, faecal samples from eight weaners that had undergone tetracycline treatment (one treatment course TID (three times a day) 20 mg/kg during 7 to17 days) were sequenced (24 samples in total: day 0, after treatment and before release). DNA was extracted according to the EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission) protocol [48, 49], DNA concentrations were measured with a Qubit. Illumina sequencing was performed using Illumina NovaSeq 6000 (Useq, Utrecht sequencing facility) with a maximum read length of 2×150 bp and a target depth of 5 gbases. Libraries were prepared with Illumina Nextera XT DNA Library Preparation Kit according to the manufacturers protocol. Afterwards, the reads were trimmed with trim galore [(v0.6.4_dev https://github.com/FelixKrueger/TrimGalore] and the quality was assessed with FastQC [v0.11.4, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/]. The resistome was investigated by using KMA V 1.4.2 utilizing the Resfinder database with a minimum of 80% gene coverage [50, 51]. The resistome reads were first normalised for gene length and resistome read counts were normalized by 16 S rRNA gene count and log transformed. A descriptive analysis was completed, however due to the small number of samples included statistical tests were not applied in this part of the study. Mean relative resistome abundance was described at the antimicrobial class and gene level in three treatment stages: admission, 1 day after treatment and release. This data was also used for the selection of resistance genes for qPCR.

qPCR based quantification of resistance genes

From the shotgun metagenomics results we selected five genes based on relevance for animal and human health [52] and their prevalence in the resistome from faeces from seals one day after treatment. These selected five genes were: *tetO*, *aph3*, *sul1*, *blaTEM* and *ermB*, coding for tetracycline, aminoglycoside, sulfonamide, beta lactam and macrolide resistance respectively, along with the bacterial 16S rRNA gene which was used to normalize the qPCR results. qPCR was performed to quantify the abundance of these five antimicrobial resistance genes, using the methods described [53]. Primer sequences are given in Supplemental Table S16. The Ct values were transformed in CFU equivalent counts by subtracting the 16S Ct value from the resistance gene value, followed by exponential transformation with base 2 and multiplying by 10^8, based on earlier performed standard curves.

Resistance genes data analysis

A random selection of 60 seals (29 treated and 31 untreated (detailed treatments in Table S3) that were included in the microbiome study were analysed using qPCR. Sample points t0, t15/AT and R were included from each group. From the rehabilitated pups a group of eight pups (three from the treated pups group and five from untreated pups group) were readmitted into rehabilitation as weaners and an extra analysis was done comparing qPCR data from release as pups with the new t0 admission qPCR data as weaners. In addition, these eight pups that were readmitted as weaners (referred to as "readmitted weaners") were compared at t0 with

the other 29 (30 minus 1 t0 sample missing) weaners from the study group that had not been previously in rehabilitation.

The abundances of the five selected resistance genes *tetO*, *aph3*, *sul1*, *blaTEM* and *ermB* were compared using GraphPad Prism between treated and untreated groups of seal pups and weaners for each sampling point (t0, t15/AT, R) with an unpaired Mann-Whitney U test, and the Bonferroni method was used to adjust for multiple testing (alpha 0.05/15 = 0.003333). All five resistance genes abundances were compared within each group between each timepoint with paired Wilcoxon signed-rank tests (t0vs t15/AT, t0vs R and t15/AT vs. R) and Bonferroni was used to adjust for multiple testing (alpha 0.05/15 = 0.003333). All five resistance genes´ abundances were compared between pups that were released and were readmitted as weaners using Wilcoxon signed rank test (paired) with Bonferroni adjustment (alpha 0.05/5 = 0.01). In addition, the five genes' abundances were compared between the weaners that had been readmitted and weaners that were admitted for the first time using unpaired Mann-Whitney U test with Bonferroni adjustment (alpha 0.05/5 = 0.01).

Results

Description of the study population

Of the 200 seals included in the study, 88 were pups and 112 were weaners. Fifty seal pups (56.8% of the pups) and 23 seal weaners (20.5% of the weaners) did not receive any oral antimicrobial treatment while 38 pups and 89 weaners received oral antimicrobial treatment. From the untreated group, all the pups were released and from the weaners only one died (95.6% released) (Table S4). Of the treated pups and weaners, 86.6% and 70.7% were released respectively. On average, rehabilitation of released seals was around two to three months (Table 1).

Antimicrobial treatment strongly affects alpha and beta diversity of treated seals

Antimicrobial treatment resulted in a significant decrease of richness and Shannon diversity index at 1 day after treatment, followed by a significant increase at release in both pups and weaners (Fig. 1a and b). Additionally, for pups, richness was significantly different between before treatment and release (Fig S1).

For both pups and weaners, the gut microbiome composition was significantly different between treatment stages (Fig. 1c and d and Tables S5 and S6). At release, microbial composition of pups and weaners was again closer to before treatment composition, but significant differences were still observed between before treatment and release. Overall, beta dispersion results in pups and weaners were non-significant (Tables S5 and S6). Additionally, the pairwise PERMANOVA, that tested individual differences between each treatment phase, showed similar results. Most differences in beta diversity were statistically significant (p = <0.001).

At release, the treated pups microbiome´s alpha diversity was significantly influenced by sex, with males showing higher Shannon diversity (p-value = 0.005) (Tables S7 and S8). For the weaners, the longer after treatment the higher the Shannon diversity index (p-value = 0.005). In the multivariable analysis if a seal had been treated twice, a significantly lower Shannon diversity was observed and the longer it stayed in rehab, the higher Shannon diversity observed (Tables S9 and S10).

Table 1 Descriptive characteristics of the study population

	Untreated		Treated	
	Pups	Weaners	Pups	Weaners
N (male, female)	50 (18 m, 32f)	23 (9 m, 14f)	38 (17 m, 21f)	89 (43 m, 46f) [a]
Age at arrival (t0) (days)	29 < 10d [b] 21 > 10 d	179 (61.66) [c]	23 < 10d 15 > 10 d	159 (50.47)
n at release (R) (male, female) (%)	50 (18 m, 32f) (100%)	22 (9 m, 13f) [d] (95.6%)	33 (13 m, 20f) (86.8%)	63 (33 m, 30f) (70.7%)
Age at release (R) (days) Mean (standard deviation)	102 (11.82)	236 (61.46)	96 (11.07)	231 (57.9)
Rehab duration at release (days) Mean (standard deviation)	95 (12.16)	63 (11.31)	90 (12.6)	74 (26.98)
Days after treatment at release Mean (standard deviation)	n/a	n/a	54 (15.95)	56 (22.5)
n dead (D) (male, female) (%)	0	1(f) (4.4%)	5 (4 m, 1f) (13.2%)	26 (10 m, 16f) [e] (29.3%)

[a] Calculations done with 88 seals because one sample had < than 10,000 reads

[b] Age was assessed as a binary variable (either younger than 10 days or 10 days and older)

[c] Age was assessed as a continuous variable and indicated as mean (standard deviation)

[d] Calculations done with 21 seals because one sample had < than 10,000 reads

[e] Calculations done with 25 seals because one sample was missing

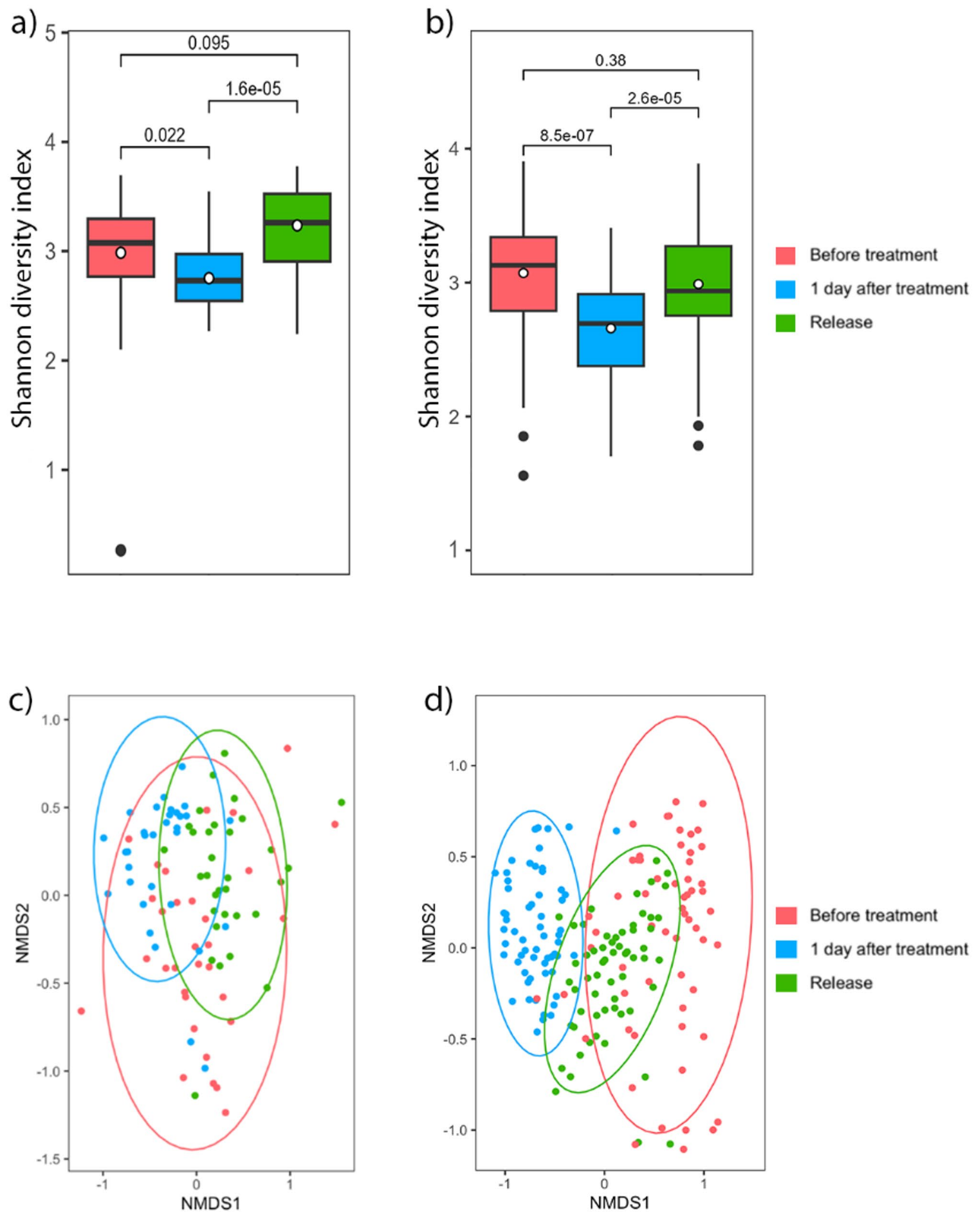

Fig. 1 (See legend on next page.)

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Fig. 1 **a**) Shannon diversity over time index for treated seal pups (*n* per group = 32). **b**) Shannon diversity index for treated seal weaners (*n* per group = 47). For both **a**) and **b**) the open white circles represent the mean and horizontal line the median. Wilcoxon signed-rank p-values indicated above the arches. **c**) Compositional differences in the faecal microbiota of seal pups. Bray Curtis NMDS 3D (stress = 0.16) of beta diversity comparing treatment stages (*n* per group = 32). 1st and 2nd dimensions (1st and 3rd dimensions in Fig S1). **d**) Compositional differences in the faecal microbiota of seal weaners. Bray Curtis NMDS 3D (stress = 0.17) of beta diversity showing treatment stages (*n* per group = 60). 1st and 2nd dimensions are displayed in this figure (1st and 3rd dimensions in Fig S1). The ellipses denote the 95% confidence intervals

The composition (beta diversity) of the treated pups microbiome at release was influenced by sex and the number of treatments (also significant beta dispersion (0.0006)) (Univariable PERMANOVA p-value = 0.001 and 0.012 respectively) (Table S11). For the treated weaners microbiome´s composition, a significant association with sex, number of treatments and age at release was observed at release (multivariable p-values = 0.008, 0.04 and 0.025) (Table S11).

No difference in alpha diversity between treated and untreated seals at admission and release

For both pups and weaners, at admission there were no significant differences seen in alpha diversity between seals that received antimicrobial treatment during rehabilitation and seals that did not receive any antimicrobial treatment (richness p-values = 0.57 and 0.65 for pups and weaners respectively, and Shannon diversity index p-values = 0.97 and 0.47 respectively). We also observed no significant difference in alpha diversity between treated and untreated pups and weaners at release (richness p-values = 0.6 and 0.31 respectively, and Shannon diversity index p-values = 0.46 and 0.32 respectively). Other variables tested with univariable regression models and only showed that sex (male) led to an increased Shannon diversity index (p-value = 1.03e-05) in all pups at release (Tables S12 and S13).

Difference in beta diversity between treated and untreated weaner seals, also after adjustment for confounders

With regards to microbiome composition, PERMANOVA at admission showed no significant differences between seals that went on to have treatment during rehabilitation and seals that did not receive any antimicrobial treatment, in both pups and weaners (p-values = 0.102 and 0.183 respectively). On the contrary, at release PERMANOVA results in all pups showed a significant difference between the two groups in microbial composition. However, if considered in a multivariable analysis with other determinants, then treatment became non-significant although this variable had a p-value of 0.050 (Table 2).

Similar results were observed for the weaners group at release, a significant difference in microbiome composition was identified between those that received antimicrobial treatment and those that did not receive it. However, this difference in microbiome composition was also significant when considered in a multivariable PERMANOVA (Table 2).

Metagenomic sequencing suggests lasting effects of treatment on resistome of treated seals

For a selection of treated seals, we determined the resistome with shotgun metagenomics of the admission (t0), 1 day after treatment (AT) and release samples (R) (Fig. 2). An average of 5.3 gbases per sample was reached. This analysis was used to get an overview of the gut resistome of these treated seals in rehabilitation and as guide for selection of the qPCR targets. No statistical analysis was performed. After treatment (all received tetracycline ranging from 7 to 17 days) there seemed to be an increase of total relative resistome abundance with a small decrease before release. Overall, 70 antimicrobial resistance gene variants belonging to 52 antimicrobial resistant genes (ARGs) have been detected in the analysed samples.

At admission, the *mdf(A)* gene, which belongs to multidrug resistant (MDR) class, had the highest mean value. Other resistance genes identified were *blaTEM-104* (beta-lactams), tetracycline resistance genes *tet(Q)*, *tet(40)* and *tet(A)*, *sul2* and *dfrA1* coding for folate pathway antagonist resistance, *aph(3")-Ib* and *aph(6)-Id* (aminoglycosides), quinolone resistance gene *qnrB4* and lastly, *mph(A)* from MLS (macrolide, lincosamide and streptogramin) class. After tetracycline administration, within samples at one day after treatment, gene *aph(6)-Id* had the highest mean value, followed by two tetracycline resistant genes *tet(L)* and *tet(B)*. At release, the highest mean read counts were assigned to genes of the tetracycline class (Fig. 2).

qPCR based resistance gene quantification suggests treatment but also rehabilitation affects total resistance gene load

To exactly quantify the change in resistance gene counts between groups and timepoints, qPCR quantification was performed on the five selected targets. We selected five genes: *tetO*, *aph3*, *sul1*, *blaTEM*, *ermB* and qPCR was performed to quantify the abundance of these genes, and the results were compared between groups and timepoints. A descriptive table of the study population is given in Tables 3 and 4.

The abundance of genes was significantly higher after treatment (AT) compared with t15 of untreated seals for

Table 2 PERMANOVA of all seal pups and weaners at release

	Permanova	Variables	R^2	*p*-value	Beta dispersion
Pups (*n* = 83)	Univariable	treatment	0.021	0.041	0.315
		age at sampling	0.022	0.029	-
		days at rehab	0.023	0.018	-
		sex	0.057	< 0.001	0.047
	Multivariable	treatment	0.019	0.050	-
		days at rehab	0.020	0.020	-
		sex	0.057	< 0.001	-
Weaners (*n* = 84)	Univariable	treatment	0.024	0.011	0.529
		age at sampling	0.019	0.041	-
		days at rehab	0.014	0.271	-
		sex	0.043	< 0.001	0.0002
	Multivariable	treatment	0.022	0.005	-
		age at sampling	0.019	0.030	-
		sex	0.039	< 0.001	-

three genes out of five for the pups and two genes out of five for the weaners (p-values in Table S14), (Fig. 3).

When comparing the resistance genes abundance within the age groups along time (Fig. 3) (p-values in Table S14) there was a significant increase of gene abundance after treatment compared with t0 for *tetO* for treated pups and for all five genes for treated weaners. For both groups of treated pups and weaners the abundance of the resistance genes decreased when comparing AT with before release R, but this difference was significant only in the case of *tetO* for the pups and *aph3* and *ermB* for the weaners. For all the treated seals groups, pups and weaners, the abundance of the tested resistance genes was significantly higher at release compared with t0 for four genes in the weaners group.

For untreated pups and weaners there was also an increase of the tested resistance genes amount observed. When comparing t15 and t0, there was a significant increase of three genes out of five for both untreated pups and weaners. In most cases the resistance genes abundance decreased when comparing t15 with R (except for *sul1* in untreated pups), but this decrease was significant only in the case of *ermB* in untreated pups. For all the untreated seal groups, pups and weaners, the abundance of the tested resistance genes was higher at release compared with t0, but this difference was significant for *sul1* for the pups, and three out of five genes for the weaners.

For the group of eight pups that were admitted back in rehabilitation as weaners ("readmitted weaners") the comparison between release and t0 when readmitted of resistance gene abundance for the same five genes did not show any significant difference, even though the general trend seems to be a lower gene abundance for all five genes when the seals were admitted back in rehabilitation as weaners (t0 readmitted weaners).

These group of eight readmitted weaners did not show any significant difference for any of the five resistance genes tested when compared with the other 29 weaners that were admitted in rehabilitation for the first time (Fig. 4 (p-values in Table S15)).

Discussion

Antimicrobial treatment of seals undergoing rehabilitation is a commonplace procedure in many seal rehabilitation centres [10]. As treatment may predispose the seal gut microbiome to carry antimicrobial resistant pathogens with the potential to transfer to other seals, this may hamper further rehabilitation efforts, as antimicrobial treatment options for seals and wild animals in general are limited, especially when they are infected with resistant bacterial pathogens. The spread of resistant pathogens may not be limited to seals, as transmission of pathogens between seals and harbour porpoises has been observed in the past [54].

In this study, we see a drastic but partly transient effect of antimicrobial treatment on the microbiome alpha diversity when compared to untreated seals, and a limited effect on the beta diversity that persist longer than just after treatment stops. Interestingly, this change is not entirely mirrored in the quantitative resistome analysis using qPCR, where effects are less transient, and increase of resistant genes last up to release and perhaps longer. Additionally, a bystander effect is observed, where untreated seals also have an increased resistance gene load in their resistome potentially originating from the treated animals. It is important to note that from a limited set of "returning" seals, which entered the centre as pup and later returned as weaner, no increased resistance gene load was observed when compared with seals that were admitted for the first time. Below we discuss specifics of our observations.

Antimicrobial treatment resulted in a significant decrease of the gut microbiome´s alpha diversity of both pups and weaners when comparing before and

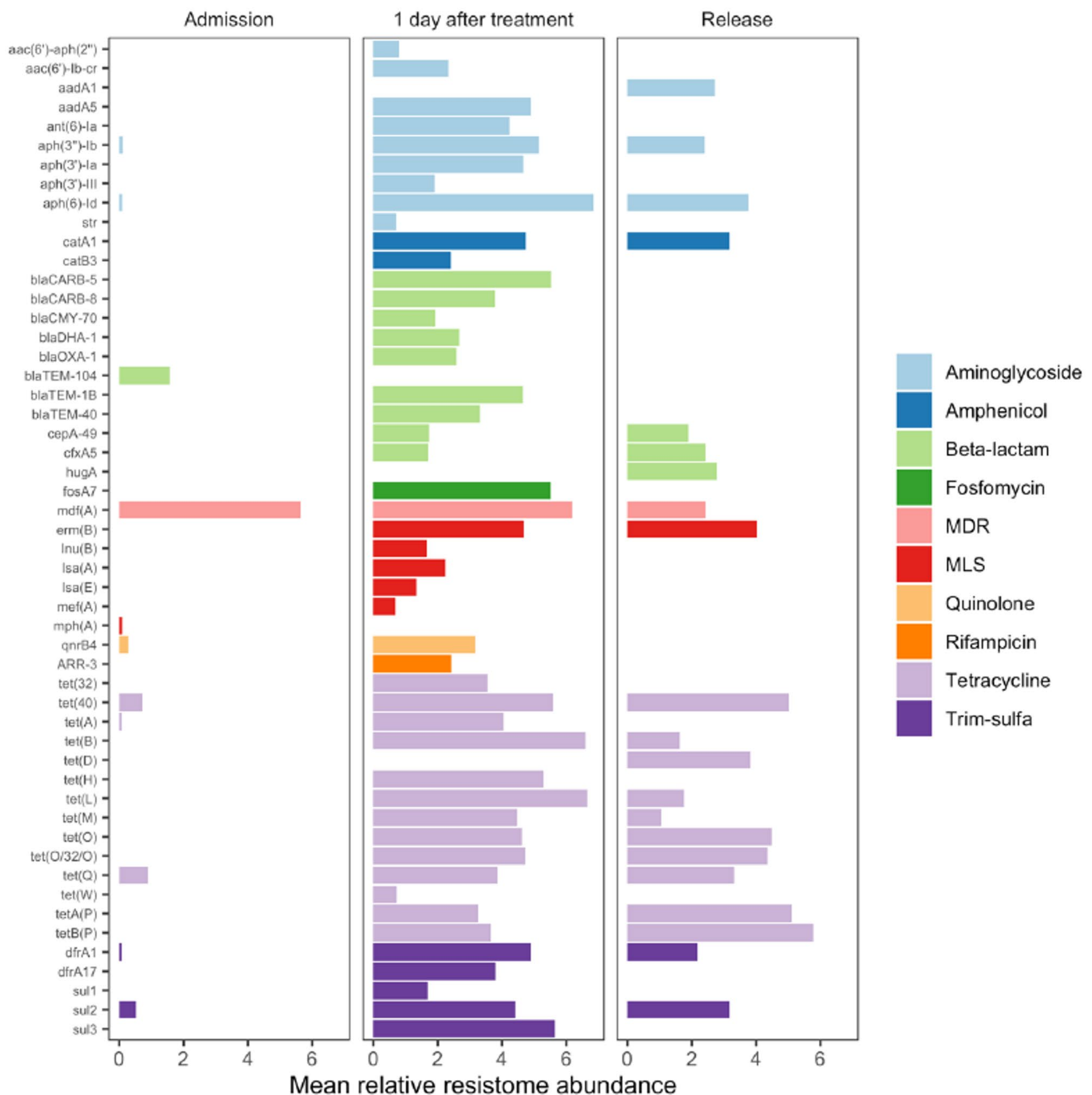

Fig. 2 Relative antimicrobial resistant gene abundances in the selection of seals ($n=8$). In the graph we observe the genes abundance over time at three different points of rehabilitation: admission (t0), one day after treatment (AT) and before release (R). Different colours indicate the different antimicrobial classes the ARGs belong to

after treatment samples. However, alpha diversity before treatment and at release (release was on average 54 and 56 days after treatment respectively for pups and weaners) was similar. Therefore, we conclude that recovery of alpha diversity is possible within 8 weeks. Decrease of alpha diversity following antimicrobial treatment in the microbiome has been described in other seal and animal species and humans [13–15, 19, 21–23, 28]. Similar to our study, a recovery on the alpha diversity was observed for treated ponies sometime after discontinuation of the antimicrobial treatment, six months in this case [23]. However, the loss of bacteria directly after treatment might still have health implications for the seals as it was shown for other mammals [55, 56]. There are very few studies on the effect of antimicrobials on the seals microbiome [28, 29], therefore further research would be needed to elucidate to what extent this effect could have health implications. Surprisingly, Switzer and colleagues

Table 3 Descriptive characteristics of the qPCR study population

	Untreated		**Treated**	
	Pups	Weaners	Pups	Weaners
N (male, female)	18 (6 m,12f)[1]	13 (4 m, 9f)	12 (6 m, 6f)	17 (11 m, 6f)
Age at arrival (t0) (days)	-11 pups younger than 10 days[2] – 7 pups 10 days or older	171 (25.64)[3]	-7 pups younger than 10 days -5 pups 10 days or older	136 (48.74)
Age at release (R) (days) Mean (standard deviation)	98 (9.32)	236 (36.56)	93 (5.93)	210 (54.88)
Rehab duration (days) Mean (standard deviation)	91 (8.91)	65 (13.08)	87 (6.38)	74 (27.48)
Days after treatment at release Mean (standard deviation)	not applicable	not applicable	52 (9.43)	58 (27.44)

[1] f, female; m, male

[2] age was assessed as a binary variable (either younger than 10 days or 10 days and older)

[3] age was treated as a continuous variable and indicated as mean (standard deviation)

Table 4 Descriptive characteristics of the qPCR study population subgroup of seals that were readmitted for rehabilitation

	Pups	**Readmitted weaners**	**Weaners**
N (male, female)	8 (1m,7f)[1]	8 (1 m,7f)	29 (15 m, 14f)
Age at arrival (t0) (days)	not applicable	251 (50.55)[2]	151 (44.26)
Age at release (R) Mean (standard deviation)	95 (8.24)	not applicable	not applicable

[1] f, female; m, male

[2] age was treated as a continuous variable and indicated as mean (standard deviation)

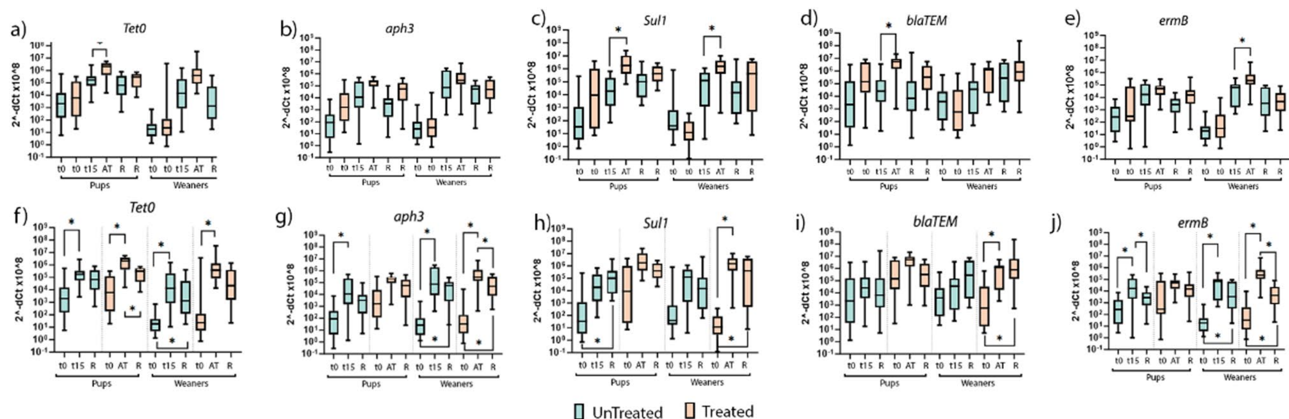

Fig. 3 Comparison of the qPCR results for five resistance gene abundance in CFUeq of **a**) *tetO*, **b**) *aph3*, **c**) *sul1*, **d**) *blaTEM* and **e**) *ermB* between non treated and treated groups of pups and weaners at all sample points (t0, t15, AT, R). Resistance genes abundances were compared with unpaired Mann-Whitney U test with Bonferroni correction for multiple testing and are visualized with box plots. The horizontal black line is the median and the significance * is shown on the top of the figure (alpha 0.05/15 = 0.00333). Comparison of the qPCR results for five resistance gene abundance in CFUeq of **f**) *tetO*, **g**) *aph3*, **h**) sul*1*, **i**) *blaTEM* and **j**) *ermB* within each group along time in rehabilitation. Resistance genes abundances were compared with paired Wilcoxon signed-rank test with Bonferroni correction for multitesting and are visualized with box plots. The horizontal black line is the median and the significance * is shown on the top of the figure (alpha 0.05/15 = 0.00333). All p-values can be found in Table S14

observed a higher alpha diversity in the rectal microbiome of harbour seal pups that received antimicrobial treatment and they hypothesised that antimicrobial treatment could have favoured early acquisition of commensals [29].

We did not observe significant differences in the gut microbiome alpha diversity between the treated and untreated seals in our study at the time of release. This confirms that although a significant effect of treatment on the alpha diversity was observed directly after treatment, there doesn't seem to be an effect of antimicrobial treatment on alpha diversity when the gut microbiome is recolonized after several weeks. Alpha diversity of the seals microbiome at release was significantly associated with sex. This sex effect was also observed in the study of the untreated pups group described in Rubio-García et al. [30] and in the study by Switzer and colleagues in harbour seal pups [29].

Beta diversity was also affected by antimicrobial treatment, and even though we observed a return towards the

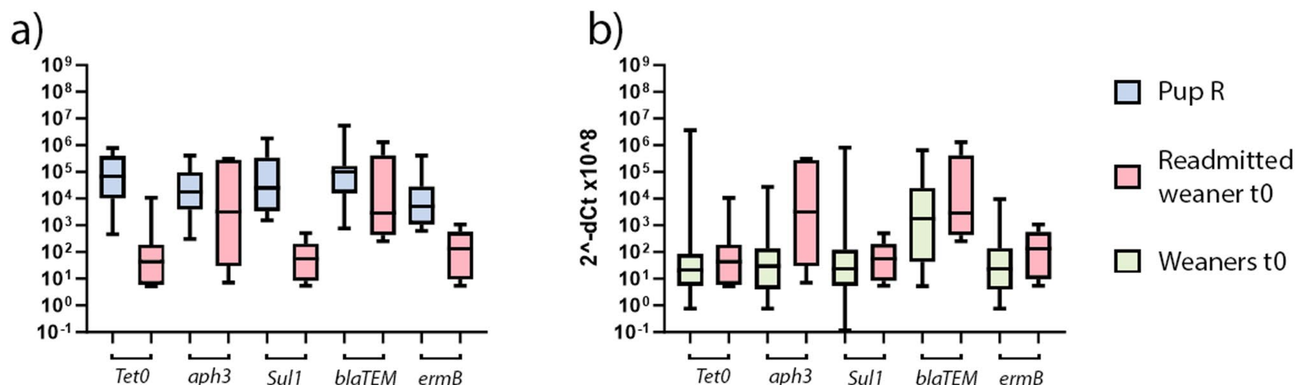

Fig. 4 Comparison of the qPCR results for 5 resistance genes *tetO*, *aph3*, *sul1*, *blaTEM* and *ermB* between released pups that were readmitted as weaners (**a**) (paired Wilcoxon signed-rank test with Bonferroni correction), and the same weaners compared with weaners that were admitted for the first time (**b**) (unpaired Mann-Whitney U test with Bonferroni correction). Resistance genes abundances are visualized by box plots, the horizontal black line is the median and the significance * is shown on the top of the figure (alpha 0.05/5 = 0.01), all p-values can be found in Table S15

before-treatment composition, at release there was still a significant difference compared to samples collected before treatment. The same effect has been observed in foals, ponies and dogs where the antimicrobial treatment had a long-lasting effect on the gut microbiome composition. In the three studies a recovery trend was observed, but at the end of the studies, even after 7 months, the composition was still not completely back to before treatment status [19, 22, 23]. In addition, in our study, at release, univariable results in all pups showed a significant difference in microbial composition (beta diversity) in pups that received antimicrobial treatment compared to the ones that did not. If considered in a multivariable analysis with other determinants, treatment became non-significant but with a p-value of 0.050. However, for the weaner group, differences in composition were also observed at release between the treated and untreated groups, and this difference was still significant when considered in a multivariable analysis. For both pups and weaners, age at sampling and sex were determinant for the composition, similar as for alpha diversity and similar to earlier study results for untreated seals [30], in which the follow up of seals after treatment was limited to several weeks, because for welfare reasons the seals were being released as soon as possible when their health had recovered. However, we now know that they were released with different gut microbiome composition compared to before treatment. Further studies on how long this effect might persist would be valuable to investigate especially if it is linked to survival. However, this is challenging to carry out in wild seals due to the impossibility of satellite tracking longer than a year, as the transponders are lost after moulting season. In addition, live tracking does not assure recapture of individuals [31]. A possibility would be to study tagged seals that are admitted again into rehabilitation or are found dead. However, in that case the cause of readmission/death might have also an impact in the gut microbiome [30], showing the limitations of this analysis.

The antimicrobial resistance gene load in the gut microbiome of these seals was increased after treatment compared to before treatment in both treated pups and weaners. This increase, shown by qPCR measurements for a selection of genes, was still observed before release (average 52 and 58 days after treatment for pups and weaners respectively); however, only significant for four genes in the treated weaners group (Fig. 3). A similar effect has been observed by Theelen et al. in horses, where the resistome even stayed significantly increased 200 days after treatment, as compared to the before treatment resistance gene levels [23]. Stoddard and colleagues also found a higher resistance in *E. coli* isolated from elephant seal pups after rehabilitation compared with *E. coli* isolated at admission of the seals [10].

Unexpectedly, for untreated pups and weaners, a significant increase in load was also observed for three genes out of five. There was a decrease at release but this was significant only in the case of *ermB* in untreated pups. For all the untreated seal groups, pups and weaners, the abundance of the tested resistance genes was higher at release compared to t0 (significant for *sul1* for the pups, and three out of five genes for the weaners). We hypothesize that this could be caused by the untreated seals sharing their environment (enclosures but mainly the pool water) with seals that were receiving or had received treatment. Pool water is not only shared between seals within one enclosure, also between several enclosures since the rehabilitation centre used a shared closed water system. The water of the pools was supplied by a closed water filtration system that removed micro-organisms to < 100 CFU/ml following the Sealcentre standards for water quality [31] but did not aim to eliminate all micro-organisms. Therefore, bacteria harbouring resistant genes could be transferred from treated seals to untreated

seals as has been observed in pigs [57] or resistant genes could be transferred among bacteria, e.g. through DNA uptake or conjugation [58]. Stoddard and colleagues [10] also observed increased resistance in *E. coli* of untreated elephant seals in rehabilitation, next to those who were treated with antimicrobials. Additionally, antimicrobial residues, excreted from treated seals, could also end up in the water. During the sampling period of the 60 seals included in the qPCR study from 1st June 2015 until 30th June 2016, a total of 452 seals were admitted to the Sealcentre. Of those seals, 392 seals that were in rehabilitation and were not included in the qPCR study, 281 received antimicrobial treatment (71.6%) (Sealcentre Pieterburen unpublished data) adding to the presence of resistant bacteria, genes and residues in the water system. Especially antimicrobials that are persistent in the environment, such as tetracycline [59] that select for resistance at low concentrations [60] may be contributing to the increased resistance gene load in the seals. Unfortunately, pool water could not be tested as part of this study, future studies should include water samples in the analysis to investigate this hypothesis.

Although most of the treated seals included in the qPCR resistome quantification were treated with tetracycline, we observed an increase on most of the studied resistance genes *tetO*, *aph3*, *sul1*, *blaTEM* and *ermB*. An explanation could be that the selected genes or a number of them were located in the same bacterial clones, or on the same plasmid or close to each other in the bacterial DNA on an integrative conjugate element [61, 62]. Unfortunately, it was not possible to investigate this in this study, therefore future research should investigate this possibility, and this would require long read sequencing of metagenomes or an extensive selective culturing approach.

From a group of eight pups that were admitted again into rehabilitation as weaners, we observed that the resistance genes load had decreased again compared with their earlier release moment. Even though their resistance genes load seemed higher than that from other weaners admitted for the first time into rehabilitation, there were no significant differences. Although the sample group is not large, it seems that the observed increase on resistance genes during rehabilitation doesn´t persist in the wild. As the returned seals only form a small percentage of the Dutch Wadden and North seas seal population, returning seals are unlikely to form an extensive AMR contamination risk. In addition, the amount of residues excreted from the seals are almost negligible compared to releases from livestock industry or human wastewater [1, 11, 63–66].

Seals admitted into rehabilitation and suffering from bacterial infections caused by resistant pathogenic bacteria pose a real problem as was shown in in Rubio-Garcia et al. [30]. The potential risk of spread of resistant bacteria among seals in rehabilitation or even to care takers should be taken seriously [67–69]. To minimize the effect of antimicrobial treatment in the gut microbiome and resistome, antimicrobial stewardship is of utmost importance. The Sealcentre Pieterburen has reduced the antimicrobial use in the last 15 years by at least 30% (Sealcentre unpublished data), however it is a continuous effort to find the most efficient and safe way of treating the admitted seals. A possible mitigation strategy could be having separated water filtration systems for seals that are receiving antimicrobial treatment and seals that are already recovered and do no longer receive antimicrobials. This might reduce the transmission of resistance genes among pools.

Conclusions

Our study reveals significant implications of antimicrobial treatment on seal gut microbiomes during rehabilitation. Antimicrobial treatment causes a decrease in gut microbiome alpha diversity, though this diversity can recover within approximately 8 weeks. The immediate impact on microbiome composition is substantial and the resistome shows persistent changes, with increased resistance genes observed even after treatment cessation. Importantly, untreated seals also exhibited increased resistance gene loads, likely due to shared environmental factors such as pool water in the rehabilitation centre. This research highlights the potential risk of antimicrobial resistance transmission among rehabilitating seals and suggests the need for antimicrobial stewardship. The findings indicate that while seals returning to the wild may not pose a significant antimicrobial resistance contamination risk, the spread of resistant bacteria within rehabilitation settings remains a concern. Future research should focus on understanding the long-term health implications of these microbiome and resistome changes, and develop strategies to minimize resistance gene transmission, such as implementing separate water filtration systems for treated and recovered seals.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

A.R.-G., A.L.Z., J.W.A.R., J.H.v.Z., and J.A.W. conceptualized the study. A.R.-G., A.L.Z., I.M., and R.E.C.L. curated the data, performed formal analysis, and conducted investigations. A.R.-G., A.L.Z., I.M., and R.E.C.L. developed the methodology. A.R.-G., J.W.A.R., and J.A.W. provided resources. J.W.A.R., J.H.v.Z., and J.A.W. acquired funding. A.R.-G., A.L.Z., and R.E.C.L. wrote the original draft of the manuscript and all authors reviewed and edited the manuscript.

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Data availability

The sequence data is available at European Nucleotide Archive under accession PRJEB76659. All other relevant data are within the manuscript and its supporting information files. The data underlying the results presented in the study are available from https://www.ncbi.nlm.nih.gov/bioproject/PRJEB60284/,https://www.ncbi.nlm.nih.gov/bioproject/PRJEB76659 and https://doi.org/10.5281/zenodo.14501721.

Declarations

Ethics approval and consent to participate

Admission and rehabilitation procedures of different seal species at the Sealcentre Pieterburen were authorized by the government of the Netherlands (permission ID at time of sample-taking: FF/75/2012/015). No invasive sampling was performed; therefore, no special permit was needed (as stated in the directive 2010/63/EU of the European Parliament).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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