EFFECT OF OXYTETRACYCLINE TREATMENT ON THE GASTROINTESTINAL MICROBIOME OF CRITICALLY ENDANGERED WHITE ABALONE (*Haliotis sorenseni*) TREATED FOR WITHERING SYNDROME

Christine A. Parker-Graham, Ameen Eetemadi, Zeinab Yazdi, Blythe C. Marshman, Malina Loeher, Christine A. Richey, Samantha Barnum, James D. Moore, and Esteban Soto

Highlights

- OTC is the drug of choice for treating abalone for *Ca*Xc, which is associated with Withering Syndrome.
- Gut microbiome α-diversity is reduced following OTC treatment but recovers by day 203.
- *Fusobacteria* remains absent in OTC-treated animals, even after α-diversity recovers.
- OTC appears safe for immersion treatment of Withering Syndrome for white abalone.

EFFECT OF OXYTETRACYCLINE TREATMENT ON THE GASTROINTESTINAL MICROBIOME OF CRITICALLY ENDANGERED WHITE ABALONE (*Haliotis sorenseni*) TREATED FOR WITHERING SYNDROME

Christine A. Parker-Graham[a,1,2], Ameen Eetemadi[b,1], Zeinab Yazdi[a], Blythe C. Marshman[c], Malina Loeher[c], Christine A. Richey[d], Samantha Barnum[e], James D. Moore[c], and Esteban Soto[a*]

[a] Department of Medicine & Epidemiology, School of Veterinary Medicine, University of California, Davis, CA, USA.

[b] Department of Computer Science, University of California, Davis, CA, USA.

[c] Shellfish Health Lab, California Department of Fish and Wildlife, Bodega Bay, CA, USA.

[d] Fish Health Lab, California Department of Fish and Wildlife, Rancho Cordova, CA, USA.

[e] Real-time PCR Research and Diagnostic Core Facility, School of Veterinary Medicine, University of California, Davis, CA, USA.

[1] These authors contributed equally to this work

[2] Present address: US Fish and Wildlife Service, Lacey, WA, USA.

* Corresponding author at: Department of Medicine & Epidemiology

School of Veterinary Medicine
University of California-Davis
2108 Tupper Hall
Davis, CA 95616-5270
Office number: +1 (530)752-2440
sotomartinez@ucdavis.edu

Abstract

White abalone (*Haliotis sorenseni*) are critically endangered marine gastropods that are native to kelp forests in the northeastern Pacific. White abalone are highly susceptible to withering syndrome, a fatal bacterial disease caused by *Candidatus* Xenohaliotis californiensis (*Ca*Xc), an intracellular, order Rickettsiales prokaryote that is endemic throughout the white abalone's range in California and Mexico. Oxytetracycline (OTC) baths at a dose of 500 mg/L are successful in clearing *Ca*Xc infections from the gastrointestinal tract of infected abalone. The impact of OTC treatment on the diversity and stability of the gut microbiome in white abalone is unknown. The objectives of this study were two-fold: (1) to characterize the gastrointestinal microbiome of clinically-normal white abalone and (2) to compare the gastrointestinal microbiomes of OTC-treated white abalone to those of control animals. Gastrointestinal tracts from five OTC-treated individuals and five untreated controls were sampled at each time point: day 0, one day after the 21-day OTC treatment (day 22), and at 203 days post-treatment. Gastrointestinal tract microbiomes were analyzed after amplification and sequence of the 16S rRNA. Gastrointestinal microbiomes of untreated animals were dominated by three core bacterial phyla: *Proteobacteria*, *Fusobacteria*, and *Bacteroidetes*. Reduced Shannon α-diversity and absence of various phyla in the microbiome of OTC-treated animals were observed in samples at day 22. Bacterial profiles were improved in terms of α-diversity at 203 days but some bacterial phyla, mainly *Fusobacteria,* remained absent. All animals remained clinically normal throughout the study period and there was no significant difference in a condition index between the two groups. OTC treatment for withering syndrome appears to be clinically safe in white abalone.

Keywords: White abalone, Withering syndrome, Oxytetracycline, Microbiome, Metagenomics, *Candidatus* Xenohaliotis californiensis

1. Introduction[1]

Numerous populations of *Haliotidae* abalone, including white abalone (*Haliotis sorenseni*), are in decline worldwide (Cook, 2016; Stierhoff et al., 2012). White abalone are herbivorous grazing marine snails native to rocky-bottomed kelp forests in the northeastern Pacific. Prior to the 1970s white abalone numbered in the millions throughout their native range from Point Conception, California to Baja California, Mexico. Today, the species is at critical risk of extinction in the wild due to overfishing (NOAA Fisheries, 2020; Catton et al., 2016; Hobday and Tegner, 2000a). Recent surveys estimate that the extant wild white abalone population may be comprised of as few as 1,600 individuals (1,600-2,500), which is less than 0.1% of baseline historical abundance (Rogers-Bennett et al., 2002; NOAA Fisheries, 2020). The California fishery for white abalone closed in 1997 and in 2001 as the species earned the dubious honor of being the first marine invertebrate listed under the Endangered Species Act (Catton et al., 2016). Despite these protections, the species has continued in precipitous decline. The White Abalone Recovery Program includes a captive-rearing program located at Bodega Marine Lab in Bodega Bay, California, which has successfully cultured white abalone with the intent to re-establish wild populations throughout the species' native range (Rogers-Bennett et al., 2016). The captive breeding program started with twenty-one adult white abalone collected from the Channel Islands, California, between 1999 and 2004. In November 2019, the program released the first

[1] Abbreviations in text: WS- withering syndrome; OTC- oxytetracycline; *Ca*Xc- *Candidatus* Xenohaliotis californiensis

group of approximately 3,000 captive-cultured white abalone back into the wild off the coasts of Los Angeles and San Diego, California.

White abalone face several impediments to survival and recovery, including recruitment failure, disease, and climate change. Like all members of *Haliotidae*, they are broadcast spawners; as members of the extant population become increasingly geographically separated, transmission of gametes and successful recruitment becomes increasingly unlikely (Hobday et al., 2000b). Most of the remaining white abalone in the wild are separated by long distances from other members of the species, making them functionally sterile (Stierhoff et al., 2012). Disease also poses a significant threat to wild abalone populations and recovery efforts (Moore et al., 2000; Tan et al., 2008; Travers et al., 2008). Withering syndrome (WS), in particular, is a fatal disease caused by colonization of the abalone host's gastrointestinal tract by an intracellular, order Rickettsiales prokaryote, identified as *Candidatus* Xenohaliotis californiensis (*Ca*Xc; Crosson et al., 2014; Friedman et al., 2000; Moore et al., 2001). *Ca*Xc appears to compromise the host's ability to extract nutrients from feed, leading to a fatal wasting syndrome. In white abalone, WS manifests as profound lethargy, cachexia, and atrophy of the foot muscle as muscle tissue is catabolized for energy. Subsequent loss of muscle mass and body condition renders the abalone unable to adhere to substrate and feed properly. Abalone in the end stage of WS are much more easily dislodged from habitat and preyed upon than their unaffected counterparts. Induction of disease following infection with *Ca*Xc and eventual mortality are significantly accelerated in increased water temperatures, making this disease of special interest with regards to climate change and ocean warming (Moore et al., 2000). Abalone species such as white (*H. sorenseni*), green (*H. fulgens*), red (*H. rufescens),* and black (*H. cracherodii*) abalone are susceptible to *Ca*Xc infection but clinical expression of WS varies between species and with environmental conditions (Altstatt et

al., 1996; Crosson and Friedman, 2018; Davis at al., 1998; Moore et al., 2009; Vater et al., 2018; Vilchis et al., 2005). White abalone have the highest susceptibility and the lowest intrinsic resistance to WS of all Pacific abalone species (Crosson and Friedman, 2018; Vater et al., 2018). Mortality associated with WS has yet to be observed in wild white abalone populations although *Ca*Xc is present; they may be protected by relatively cold water microenvironments (CDFW unpublished observations; NOAA Fisheries, 2020); in contrast, cultured white abalone have experienced WS mortalities. The disease poses a considerable threat to captive culture operations and wild restoration efforts for white abalone (Moore et al., 2002; Friedman et al., 2007; Vater et al., 2018).

Traditionally, antimicrobials have been used in aquaculture facilities worldwide to prevent and treat bacterial diseases (Romero et al., 2012). Oxytetracycline (OTC), a broad-spectrum, naturally-occurring tetracycline antimicrobial, is effective in reducing or eliminating *Ca*Xc from the gastrointestinal tract of infected red abalone and white abalone (Winkler et al., 2018; Friedman et al., 2007; Moore et al., 2019). OTC concentrates in the digestive gland of treated abalone and provides protection against reinfection with *Ca*Xc for numerous months following completion of treatment (Friedman et al., 2007; Moore et al., 2019; Rosenblum et al., 2008). Bath immersions in OTC are used to treat and protect captive culture populations from WS. Clinically, OTC treatment is well-tolerated by all abalone examined and there are no significant differences in growth rates between treated and untreated red abalone (Moore et al., 2019).

Microbiome homeostasis is critical for abalone's ability to utilize their primary food source, kelp, effectively (Nel et al., 2017). It is important to evaluate the potential impact that treatments rendered during the captive-raising period may have on the microbiome prior to release into the wild. The impact of antimicrobials on the gut microbiome of treated individuals is an emerging

field of study in human and veterinary medicine. Several studies have shown that antibiotic treatment reduces host microbiome diversity and can cause increased colonization of the gastrointestinal tracts in human, mouse, marine mammal, and fish patients with pathogenic bacteria (Theriot et al., 2014; Langdon et al., 2016; Schmidt et al., 2017; Carlson et al., 2017). Nothing is known about the influence of antibiotic treatment on the gastrointestinal microbiome of abalone. The goal of this study is to characterize the gastrointestinal microbiome of clinically healthy white abalone in a captive-culture setting using 16S metagenomics and to compare the microbiomes of animals undergoing routine OTC-treatment for WS with untreated abalone to evaluate the impact that OTC treatment has on the gut microbiota.

2. Materials and Methods

2. 1. Animals

Thirty-one juvenile to young adult white abalone from the 2017 spawning at Bodega Marine Lab were enrolled in this study; the animals ranged in weight from 0.27 g to 7.55 g and had shell lengths of 12.2 mm to 38.8 mm on day 0. The abalone enrolled in this study were not used for any other research purpose prior. Prior to inclusion in the study abalone were considered clinically healthy based on visual examination and known to be free of *Ca*Xc infection by periodic tank feces testing with a qPCR protocol. Abalone were housed communally in a flow-through system containing natural seawater sourced from Bodega Bay, California, and passed through a gravel filter, 21 µm paper cartridge filter, and ultraviolet sterilizer prior to reaching the housing tanks. The abalone were fed a mixture of wild kelp *Macrocystis pyrifera* and cultured *Palmaria mollis* that was immersed in freshwater for five minutes to reduce the chance of exposing animals to endemic *Ca*Xc. Animals were identified numerically by plastic tags attached to their shells with a methacrylate glue.

The general sampling plan was to process five animals immediately prior to treatment (pre-sample, day 0); then to process five animals from each of the OTC and mock (control) treatments just after the treatment regimen (day 22), and to process five animals from the OTC and mock-treatments upon termination at day 203. Three additional animals were added to each of the OTC and mock treatment groups in case any mortality occurred prior to termination. Animals were randomly assigned to the Pre-treatment (n=5), OTC treatment (n=13), and mock treatment (n=13) groups. The OTC and mock treatment groups were housed in two separate containers throughout the study.

Animals in the treated groups were exposed to the standard OTC bath treatment used to eliminate *Ca*Xc in abalone (Moore et al., 2019). This treatment consists of eight 24-hour immersions in an OTC bath (500 mg/L) over a period of twenty-one days. The sampling days were selected to correspond with the end of OTC treatment (day 22 sampling) and six months following treatment (day 203). Mock-treated animals were handled exactly the same way as OTC treated animals, except that oxytetracyline was not added to their holding tank.

2. 2. Experimental Methods

On day 0 the five animals randomly assigned to the Pre-treatment group were processed. Animals were weighed and measured (maximum shell length) and body condition index (c.i.) was calculated for each animal (c.i. = total shell length, cm/[total weight, g]^3). The animals were removed from the shell and the head (including the mouth and distal esophagus) was sharply incised from the body using a scalpel blade. The gastrointestinal tract was isolated from surrounding tissue by dissecting away the epipodium, gonads, gills, and as much of the foot and shell muscles and surrounding connective tissue as possible. The resultant gut tissue bloc was weighed, placed in a 50 ml centrifuge tube containing 0.1 % Tween80 (Sigma-Aldrich Corp, St.

Louis, MO, USA) in 0.22 µm filtered seawater and rocked back and forth ten times to remove bacteria on external surfaces. The rinse was repeated with a new tube and Tween80 solution and the tissue was immediately frozen at -80 °C in sterile cryovials labeled with the animal's identification number, date, and study group. On day 22 and day 203, five animals from each of the OTC and mock treatment groups were randomly selected from their holding tanks and an identical dissection and sample preparation protocol was used, except that the Tween80 rinse solution volume used on day 203 was 20 ml because the animals had grown significantly. All samples were held frozen at -80 °C until processing as described below.

2.3. Library Preparation and Template Preparation/Enrichment.

DNA of abalone tissues was extracted following manufacturer's guidelines for the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). The Ion 16S™ Metagenomics Kit, (ThermoFisher Scientific, Carlsbad, CA, USA) which uses two primer pools to amplify seven hypervariable regions (V2, V3, V4, V6, V7, V8, and V9) of bacterial 16S rRNA and enables detection of a broad-range of bacteria from complex mixed populations, was used to detect bacterial phyla in this study. Briefly, 20 ng of DNA was amplified through 25 cycles with the Ion 16S™ Metagenomics Kit. After purification using the Agencourt AMPure XP beads (Beckman Coulter, Pasadena, CA, USA) according to the manufacturer's procedure, 1 µl of each PCR was run on a 2100 Bioanalyzer® (Agilent, Santa Clara, CA, USA) to determine concentration and to confirm successful PCR. The entire PCR product underwent end repair and was purified with XP beads. Adapter and Ion Xpress Barcodes were ligated to allow pooling of all the samples for sequencing and each sample received a unique barcode. The samples were purified again with the XP beads and 7 cycles of PCR were performed to increase the number of amplicons and to select for amplicons with adapters. Samples were purified with XP beads and 1 µl was run on a

2100 Bioanalyzer® to determine a final library concentration. The library was diluted to 100 pM prior to template preparation on the Ion OneTouch™ using the Ion PGM™ Hi-Q™ View OT2 kit (ThermoFisher Scientific, Carlsbad, CA, USA) according to manufacturer's procedure. The template preparation is required to form template-positive Ion Spear™ particles (IPS), which contain clonally amplified DNA. IPS were then enriched on the Ion OneTouch™ ES Instrument (ThermoFisher Scientific, Carlsbad, CA, USA) to select IPS with only one amplified DNA amplicon.

2.3.1. Sequencing with Person Genome Machine (PGM™)

The PGM™ (ThermoFisher Scientific, Carlsbad, CA, USA) was set up for initialization using the Ion PGM™ Hi-Q™ View Sequencing Kit (ThermoFisher Scientific, Carlsbad, CA, USA) according to manufacturer's procedure. An Ion 314™ Chip was loaded with half of the IPS and run on the PGM™ with the Torrent Suite™ System software (ThermoFisher Scientific, Carlsbad, CA, USA).

2.4. Microbiome Data Analysis

Raw ThermoFisher Ion Xpress ".bam" files were converted to fastq format using samtools-1.9 (Li et al., 2009). For metagenomic analysis, DADA2 pipeline (Callahan et al., 2016) version 1.10 implemented in R version 3.5.2 was used as described online (benjjneb.github.io/dada2/bigdata.html). First, quality control was performed by removing 16S rRNA reads that were chimeric, shorter than 240 bp, or had at least two expected errors. In addition, longer reads were truncated at 240bp since read qualities decreased sharply afterward. Approximately 24% of the total reads were marked as high quality. Next, *de novo* sequence assembly was performed. Then SILVA database (Quast et al., 2012) version 32 was used to identify bacterial taxonomies associated with 16S rRNA assembled sequences. The

phylogenetic tree was constructed next using phangorn (Schliep, 2011) R library. Taxa that were only observed in a single sample were filtered and taxa counts were transformed to relative abundances using PhILR library (Silverman et al., 2017). Finally, phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2016), and ggpubr (Kassambara, 2017) libraries were used for data visualization and statistical analysis.

3. Results

Microbiome Shannon α-diversity (i.e. diversity of microbial species within each sample) was significantly reduced in the digestive tracts of OTC-treated white abalone between day 0 and day 22, but recovered by day 203 (Figure 1). α-diversity differences among other treatment groups and time points were not significant ($p > 0.05$) suggesting that OTC treatment is an important factor influencing intestinal microbiome diversity of OTC-treated white abalone. β-diversity analysis (Figure 2) shows that microbiome profiles are similar in each group across different timepoints. Furthermore, there is a consistent shift between day 22 and day 203 in the microbime profiles of OTC-treated white abalone which is not observed in controls across different time points. Phylogenetic trees relating microbiome populations of control and treated samples at various time points is presented in supplementary Figure 1.

Despite recovering α-diversity over the course of the study period, animals in the OTC-treated group showed a notable absence of bacteria within the phylum *Fusobacteria* at day 22 and day 203 (Figure 3). The absence of the *Fusobacteria* phylum in the OTC-treated group may explain the distinguishable difference in microbiome profiles between OTC-treated and mock samples at day 203 (Figure 2).

From a clinical perspective, animals in both the OTC-treated and mock groups remained normal throughout the duration of the study. Individuals in both groups continued to eat and ambulate normally throughout the study period. A one-way ANOVA on ranks comparing the condition indexes of five pre-treatment animals and the five treated and mock-treated groups at days 22 and 203 showed no significant differences (p= 0.149). There was no mortality in either group during the study period. Adverse side effects such as anorexia and lethargy have been documented in other veterinary species in association with OTC therapy but, notably, no adverse side effects (ie: anorexia or lethargy) were observed in white abalone in this study.

4. Discussion

This study identified three core bacterial phyla that made up the majority of the gut microbiome in the untreated white abalone: *Proteobacteria*, *Fusobacteria,* and *Bacteroidetes*. There was no significant difference in this composition of the microbiome in untreated animals over the course of the study. The predominance of *Proteobacteria* is consistent with what has been documented in numerous species of marine invertebrates, such as Eastern oysters (*Crassostrea virginica*), blue-rayed limpets (*Patella pellucida*), and green sea urchins (*Lytechinus variegatus*) (King et al., 2012; Chauhan et al., 2014; Dudek et al., 2014; Hakin et al., 2016). More specifically, *Proteobacteria* was also the dominant bacterial phylum identified in the gut in studies of several *Haliotis* species, including variously colored abalone (*H. diversicolor*), European abalone (*H. tuberculata*), and green lip abalone (*H. laevigata*) (Zhao et al., 2018; Huang et al., 2018; Gobet et al., 2018; Danckert, 2020). Surprisingly, bacteria within the phylum *Tenericutes* were found in very small numbers in the control animals, which is contrary to what has been seen in green abalone (*H. fulgens*) and pink abalone (*H. corrugata*) (Cicala et al., 2018).

The putatively robust population of *Fusobacteria* in the microbiome of *Haliotis* species is an interesting finding and it may correlate to an aquatic lifestyle. *Fusobacteria* has been found in high abundances in the gastrointestinal tracts of sea squirts (*Ciona intestinalis*) and several fish species, such as channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), and zebrafish (*Danio rerio*) (Dishaw et al., 2014; Larsen et al., 2014; Roeseler et al., 2011). Several mammalian, avian, and reptilian species that are associated with aquatic or semi-aquatic life histories have been shown to have *Fusobacteria* as a dominant bacteria in their gastrointestinal tracts (Sun et al. 2018; Hird, 2017; Nelson et al., 2012; Keenan et al., 2013). Indeed, marine mammals have a significantly greater average relative abundance of *Fusobacteria* in their intestinal tracts than terrestrial mammals (Nelson et al., 2012; Nelson et al., 2013). *Fusobacteria* was the most commonly identified bacterial phylum in the lower gastrointestinal tracts of American alligators (*Alligator mississippiensis*), which was a novel finding as *Firmicutes* and *Bacteroidetes* are the dominant bacterial phyla in the intestinal tracts of most other species of reptiles (Keenan et al., 2013). Interestingly, of the species known to harbor large populations of *Fusobacteria* in their gastrointestinal tracts normally, the white abalone appears to be the only strictly herbivorous species represented.

It is possible that bacteria within the phylum *Fusobacteria* play an important role in digestion and energy production. In humans *Fusobacterium varium* is a minor, but important, component of the normal gastrointestinal microbiome because of its ability to ferment amino acids and glucose and produce butyrate (Potrykus et al., 2008; Potrykus et al., 2007). It is also an important competitor for pathogenic bacteria like *Shigella* and *Salmonella* (Potrykus et al., 2008). *Fusobacteria* is a minor component of the gastrointestinal microflora in oscar cichlids (*Astronotus ocellatus*) and angelfish (*Pterophyllum scalare*), but in both host species

Fusobacteria produces important digestive enzymes, including alkaline and acid phosphatases, esterase, lipase, and α-glucuronidase (Ramirez and Dixon, 2003). These digestive enzymes play important roles in digestion, such as absorption of lipid, glucose, and calcium, and the breakdown of proteins and carbohydrates. When transfaunated into gnotobiotic mice, human strains of *Fusobacteria* produce polyamines from pectin, a soluble indigestible polysaccharide found in plant cell walls, and these polyamines can be used by the host (Noack et al., 2000). This may be a key to why *Fusobacteria* is so prevalent in the gastrointestinal tract of abalone. As grazers, abalone exploit a wide range of green, red, and brown algae as food resources. White abalone rely heavily on giant kelp (*Macrocystis pyrifera*), which is a large, perennial species of brown algae. Giant kelp contains algin, an anionic heteropolysaccharide abundant in the cell walls of brown algae. Unlike other seaweed hydrocolloids, such as carrageenan, that owe their ionic characteristics to sulfate groups, algin is anionic because of its carboxyl groups, which makes it more similar to pectin than to other seaweed hydrocolloids (Barbaroux). *Fusobacteria* may catabolize algin similarly to the manner in which it acts on pectin and enables the host to produce amino acids through bacterial synthesis, thus allowing abalone to exploit a wider range of marine vegetation.

Microbiome resilience is critical for abalone because the gut microbiome plays an important role in their overall health and ability to digest marine vegetation (Nel et al., 2017; Cicala et al., 2018). While studies involving other marine invertebrates have shown that location, season, diet, and water temperature all profoundly affect the composition of the host's microbiome (Lokmer et al., 2016a; Pierce et al., 2015), the microbiome of *Haliotis* species changes seasonally but appears to remain fairly stable despite changes in diet (Gobet et al., 2018). In ruminants, volatile fatty acids in acidic pH are toxic to some bacterial phyla, so the rumen environment selects for

specialized bacterial species that can tolerate these extreme conditions. The gastrointestinal environment of *Haliotis* species is microaerophilic/anaerobic and acidic; like in ruminants, this environment may lend itself to developing a specialized and stable bacterial profile (Gobet et al., 2018).

A decrease in bacterial diversity and quantity was expected after treatment with oxytetracyline, as it is a broad-spectrum antimicrobial. This study showed a decrease in gut microbiome α-diversity of OTC-treated abalone on day 22, which corresponded to the completion of a full treatment course with OTC; α-diversity was restored by day 203 in OTC-treated animals, but with notable differences in the bacterial composition. Studies evaluating the effect of antimicrobials on the gut microbiome are generally lacking in aquatic veterinary medicine, but a study of Pacific oysters (*Crassostrea gigas*) yielded similar decreased microbiome α-diversity after a cohort of oysters was treated with a combination of unspecified antibiotics (Lokmer et al., 2016b). As Gram-negative bacteria, *Fusobacteria* are within the antimicrobial spectrum of natural tetracyclines like oxytetracycline; indeed, oxytetracycline is used as a therapeutic against pathogenic strains of *Fusobacteria*, like *F. necrophorum*, in veterinary species (Lechtenberg et al., 1997). It appears that the decline in *Fusobacteria* observed in this study correlated to oxytetracycline therapy. Bacteria within the phylum *Fusobacteria*, however, are found only rarely in seawater and in association with marine vegetation (Gobet et al., 2018), which may explain why they did not repopulate the gut of treated animals as readily as the other bacterial phyla.

In this study it was not immediately clear whether the change in gut microbiota composition would compromise a white abalone's ability to compete in the wild, whether by compromising their ability to digest food or by compromising their immunity to disease. Antibiotics disturb the

gut microbiome community and may decrease colonization resistance which leads to increased downstream disease susceptibility and mortality in the host, and microbiome profiles have been identified as potentially important factors in shellfish mortality events (Schmidt et al., 2017; King et al., 2019a; King et al., 2019b). While the OTC bath treatment we used was reported to cause no adverse effects on growth or condition index in red abalone (Moore et al. 2019) a separate study with red abalone using an alternate OTC bath protocol reported slower growth over eleven months in treated animals versus untreated controls (Winkler et al. 2018).

Significantly, Lokmer and colleagues found that antimicrobial treatment of Pacific oysters (*Crassostrea gigas*) actually increased survival after animals were translocated; in this study, oysters that were not treated with an antibiotic prior to translocation experienced a significantly higher mortality rate than oysters treated with an antibiotic prior to translocation (Lokmer et al., 2016b). The authors speculate that part of this increased survival in treated animals was due to the decreased diversity and "reset" of the gut microbiome following antimicrobial therapy. Because the diversity of bacteria within the gastrointestinal tract was reduced in antimicrobial-treated oysters there were fewer negative interactions within the microbiome as novel bacteria were introduced to the gut at the new location. In one study examining the resilience of the microbiome in South African abalone (*H. midae*) gnotobiotic abalone still showed digestive enzymatic activity, suggesting that there is a baseline level of digestive enzymatic activity within the digestive gland (Erasmus et al., 1997). This suggests that while the microbiome is important for digestion, there is a measure of intrinsic enzyme activity within the digestive tract. Whether this intrinsic digestive capacity is present in white abalone, and to what extent, is unknown.

No wild white abalone specimens were available for inclusion in this project so it remains unknown whether, and to what extent, the gut microbiome of wild abalone differs from those

that are raised in the culture setting. Green sea urchins (*Lytechinus variegatus*), for example, maintained remarkably similar microbiome profiles between wild and captive-cultured individuals, despite the putative differences in feed items consumed and environment (Hakim et al., 2016). While the seawater supplied to the animals in our study was sterilized prior to reaching the housing tanks and thus an unlikely source of microbes, the macroalgal food items that were fed to the study abalone were lightly sanitized by immersion in fresh water for five minutes prior to feeding. It is likely that the wild vegetation included in the diet also introduced a natural algal holobiont to the study animals' gastrointestinal tract. A significant difference in the makeup of captive-cultured white abalone and wild counterparts is not expected.

The OTC bath concentration was 500 mg/L, which follows the protocol that Bodega Marine Lab currently uses for their white abalone culture operation. In this study OTC appeared to reach effective concentrations in the gastrointestinal tracts of treated abalone, given the reduction of *Fusobacteria* in the gut microbiomes of treated animals. Previous work on the pharmacokinetics of oxytetracyline in red abalone showed that OTC persisted in the digestive gland for significantly longer than in the foot muscle (Rosenblum et al., 2008). This study also found that there was a significantly higher presence of cations (iron, zinc, and manganese) present in the digestive gland versus the foot muscle, leading the authors to speculate that cations may be important to retention of OTC. A more recent study showed that the concentrations of cations, particularly calcium and magnesium, in seawater can bind OTC and reduce bioavailability of the drug in immersion treatments (Vorbach et al., 2019). Cation concentrations in the water were not measured in this study, but such measurements would be an important consideration for any future studies examining pharmacokinetics of oxytetracycline in abalone.

Further study to investigate the role that *Ca*Xc plays on the intestinal microbiota of infected abalone would further characterize the disease and its effect on infected abalone. A study of Sydney rock oysters (*Saccostrea glomerata*) showed that infection with a protozoal parasite (*Marteilia sydneyi*) drastically changed the composition of the microbiota of infected animals (Green and Barnes, 2010). Probiotics are of increasing interest within aquaculture for their purported ability to improve feed conversion rates and growth. Probiotics may be of interest to wild translocation projects, such as the one for white abalone. Multiple studies have shown benefits to giant abalone (*H. gigantean)*, South African abalone (*H. midae)*, and disk abalone (*H. discus hannai*) in terms of growth and immunity with the administration of probiotics with feed (Iehata et al., 2009; Macey and Coyne, 2005; Jiang et al., 2013; Iehata et al., 2014; Lee et al., 2016). Further study is necessary to quantify the effects of probiotics on white abalone and the optimal probiotic combination for this species.

This study suggests that oxytetracyline is safe for white abalone. While there were changes in the composition of the microbiome of OTC-treated abalone there were no significant changes in growth and weight gain between the treated and untreated control animals. Further study to evaluate the impact of the loss of certain bacterial phyla, notably *Fusobacteria*, is necessary to fully characterize the long-term impact of OTC-treatment on white abalone.

5. Acknowledgements

This work was funded by the University of California, Davis Aquatic Animal Health fellowship and the Fisheries Branch, California Department of Fish and Wildlife. The authors would also like to thank Dr. Daniel Ben-Aderet for his assistance in sample collection and Patrick Graham for his editorial input on this manuscript.

6. References:

Altstatt, JM, Ambrose RF, Engle JM, Haaker PL, Lafferty KD, Raimondi, PT. 1996. Recent declines of black abalone *Haliotis cracherodii* on the mainland coast of Central California. Mar. Ecol. Prog. Ser. 142, 185–192.

Barbaroux O. Production, properties, and uses of alginate, carrageenan and agar. http://www.fao.org/3/ab728e/AB728E09.htm (accessed 19 January 2020).

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods. 13(7), 581.

Carlson JM, Leonard AB, Hyde ER, Petrosino JF, Primm TP. 2017. Microbiome disruption and recovery in the fish *Gambusia affinis* following exposure to broad-spectrum antibiotic. Infect. Drug Resist. 10, 143.

Catton CA, Stierhoff KL, Rogers-Bennett L. 2016. Population status assessment and restoration modeling of white abalone *Haliotis Sorenseni* in California. J. Shellfish Res. 35(3), 593–600.

Chauhan A, Wafula D, Lewis DE, Pathak A. 2014. Metagenomic assessment of the Eastern oyster-associated microbiome. Genome Announc. 2(5), e01083-14. https://doi.org/10.1128/genomeA.01083-14.

Cicala F, Cisterna-Céliz JA, Moore JD, Rocha-Olivares A. 2018. Structure, dynamics and predicted functional role of the gut microbiota of the blue (*Haliotis fulgens)* and yellow (*H. corrugata*) abalone from Baja California Sur, Mexico." Peer J. 6, e5830. http://doi.org/10.7717/peerj.5830.

Cook P. 2016. Recent trends in worldwide abalone production. J. Shellf. Res. 35(3), 581-583.

Crosson LM, Friedman CS. 2018. Withering Syndrome susceptibility of northeastern Pacific abalones: A complex relationship with phylogeny and thermal experience. J. Invertebr. Pathol. 151, 91–101.

Crosson LM, Wight N, VanBlaricom GR, Kiryu I, Moore JD, Friedman CS. 2014. Abalone Withering Syndrome: Distribution, impacts, current diagnostic methods and new findings. Dis. Aquat. Org. 108(3), 261–270.

Danckert NP. 2020. Characterising the intestinal microbiome of Australian aquacultured abalone. PhD thesis University of Sydney.

Davis GE, Haaker PL, Richards DV. 1998. FISHERIES- the perilous condition of white abalone *Haliotis sorenseni*, Bartsch, 1940. J. of Shellfish Res. 17, 871–876.

Dishaw LJ, Flores-Torres J, Lax S, Gemayel K, Leigh B, Melillio D, Mueller MG, Natale L, Zucchetti I, de Santis R, Pinto MR, Litman GW, Gilbert JA. 2014. The gut of geographically disparate *Ciona intestinalis* harbors a core microbiota. PLoS. 9, e93380. https://doi:10.1371/journal.pone.0093386.

Dudek M, Adams J, Swain M, Hegarty M, Huws S, Gallagher J. 2014. Metaphylogenomic and potential functionality of the limpet *Patella pellucida*'s gastrointestinal tract microbiome. International J of Molecular Sci. 15(10), 18819-18839.

Erasmus JH, Cook PA, Coyne VE. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. Aquaculture. 155, 377-386.

Friedman CS, Scott BB, Strenge RE, Vadopalas B, McCormick TB. 2007. Oxytetracycline as a tool to manage and prevent losses of the endangered white abalone, *Haliotis sorenseni*, caused by Withering Syndrome. J. of Shellfish Res. 26(3), 877–886.

Gobet A, Mest L, Perrenou M, Dittami SM, Caralp C, Coulombet C, Huchette S, Roussel S, Michel G, Leblanc C. 2018. Seasonal and algal diet-driven patterns of the digestive microbiota of the European abalone *Haliotis tuberculata*, a generalist marine herbivore. Microbiome. 6(60). https://doi.org/10.1186/s40168-018-0430-7.

Green TJ, Barnes AC. 2010. Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. J of Appl. Microbiol. 109(2), 613-622.

Hakim JA, Koo H, Kumar R, Lefkowitz EJ, Morrow CD, Powell ML, Watts SA, Bej AK. 2016. The gut microbiome of the sea urchin, *Lytechinus variegatus*, from its natural habitat demonstrates selective attributes of microbial taxa and predictive metabolic profiles. FEMS Mirobiol. Ecol. 92(9), fiw146. https://doi.org/10.1093/femsec/fiw146.

Hird SM. 2017. Evolutionary biology need wild microbiomes. Front in Microbiol. 8(725). http://doi.org/10.3389/fmicb.2017.00725.

Hobday AJ, Tegner MJ, Haaker PL. 2000a. Over-exploitation of a broadcast spawning marine invertebrate: Decline of the white abalone. Rev. Fish Biol. Fish. 10(4), 493–514.

Hobday AJ, Tegner MJ. 2000b. Status review of white abalone (*Haliotis sorenseni*) throughout its range in California and Mexico.

Huang ZB, Guo F, Zhao J, Li WD, Ke C. 2018. Molecular analysis of the intestinal bacterial flora in cage-cultured adult small abalone, *Haliotis diversicolor*. Aquaculture Res. 41(11), 760-769.

Iehata S, Nakano M, Tanaka R, Maeda H. 2014. Modulation of gut microbiota associated with abalone *Haliotis gigantea* by dietary administration of host-derived *Pediococcus* sp. Ab1. Fisheries Res. 80, 323-331.

Iehata S, Inagaki T, Okunishi S, Nakano M, Tanaka R, Maeda H. 2009. Colonization and probiotic effects of lactic acid bacteria in the gut of the abalone *Haliotis gigantea*. Aquacul. 75, 1285-1293.

Jiang HF, Liu XL, Chang YQ, Liu MT, Wang GX. 2013. Effects of dietary supplementation of probiotic *Shewanella colweilliana* WA64, *Shewanella olleyana* WA65 on the innate immunity

and disease resistance of abalone, *Haliotis discus hannai Ino*. Fish and Shellfish Immunol. 35(1), 86-91.

Kassambara, A. 2017. Ggpubr:'Ggplot2' based publication ready plots. R Package Version 0.1.6.

Keenan SW, Engel AS, Elsey RM. 2013. The alligator gut microbiome and implications for archosaur symbiosis. Sci Rep. 3, e2877. https://doi.org.10.1038/srep02877.

King WL, Jenkins C, Go J, Siboni N, Seymour JR, Labbate M. 2019a. Characterisation of the Pacific oyster microbiome during a summer mortality event. Microbial Ecol. 77(2), 502-512.

King WL, Jenkins C, Seymour JR, Labbate M. 2019b. Oyster disease in a changing environment: decrypting the link between pathogen, microbiome and environment. Mar. Environ. Res. 143, 124-140.

King GM, Judd C, Kuske CR, Smith C. 2012. Analysis of stomach and gut microbiomes of the Eastern oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. PLoS One 7(12), e51475. https://doi.org/10.1371/journal.pone.0051475.

Langdon A, Crook N, Dantas G. 2016. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Med. 8(1), 39.

Larsen AM, Mohammed HH, Arias CR. 2014. Characterization of the gut microbiota of three commercially valuable warm water fish species. J. Appl. Microbiol. 116(6), 1396–1404.

Lechtenberg KF, Nagaraja TG, Chengappa MM. 1997. Antimicrobial susceptibility of *Fusobacterium necrophorum* isolated from bovine hepatic abscesses. AJVR. 59(1), 44-47.

Lee MJ, Lee JJ, Chung HY, Choi SH, Kim BS. 2016. Analysis of microbiota on abalone (*Haliotis discus hannai*) in South Korea for improved product management. Int. J. for Food Micrbiol. 234, 45-52.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The sequence alignment/map format and SAMtools. Bioinformatics. 25(16), 2078–2079.

Lokmer A, Goedkneght MA, Thieltges DW, Fiorentino D, Kuenzel S, Baines JF, Wegner KM. 2016a. Spatial and temporal dynamics of Pacific oyster hemolymph microbiota across multiple scales. Front. Microbiol. 7, 1367. https://doi.org/10.3389/fmicb.2016.01367.

Lokmer A, Keunzel S, Baines JF, Wegener KM. 2016b. The role of tissue-specific microbiota in initial establishment success of Pacific oysters. Environ. Microbiol. 18(3), 970-987.

Macey BM, Coyne VE. 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquacul. 245(1-4), 249-261.

McMurdie PJ, Holmes S. 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PloS One. 8(4), e61217. https://doi.org/10.1371/journal.pone.0061217.

Moore JD, Finley CA, Robbins TT, Friedman CS. 2002. Withering Syndrome and restoration of Southern California abalone populations. Reports of California Cooperative Oceanic Fisheries Investigations. 43, 112–119.

Moore JD, Juhasz CI, Robbins TT, Vilchis LI. 2009. Green abalone, *Haliotis fulgens* infected with the agent of Withering Syndrome do not express disease signs under a temperature regime permissive for red abalone, *Haliotis rufescens.* Mar. Biol. 156(11), 2325–2330.

Moore JD, Robbins TT, Friedman CS. 2000. Withering Syndrome in farmed red abalone *Haliotis rufescens*: Thermal induction and association with a gastrointestinal Rickettsiales-like prokaryote. J. of Aquat. Anim. Health. 12(1), 26–34.

Moore JD, Robbins TT, Hedrick RP, Friedman CS. 2001. Transmission of the Rickettsiales-like Prokaryote *Candidatus xenohaliotis californiensis* and its role in Withering Syndrome of California abalone, *Haliotis spp*. J. Shellfish Res. 20(2), 867–874.

Nel A, Pletschke BI, Jones CLW, Kemp J, Robinson G, Britz PJ. 2017. Effects of kelp *Ecklonia maxima* inclusion in formulated feed on the growth, feed utilisation and gut microbiota of South African abalone *Haliotis midae*. Afr. J. Mar. Sci. 39(2), 183–192.

Nelson TM, Rogers TL, Brown MV. 2013. The gut bacterial community of mammals from marine and terrestrial habitats. PLoS One. 8(12), e83655. https://doi.org/10/1371/journal.pone.0083655.

Nelson TM, Rogers TL, Carlini AR, Brown MV. 2012. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ. Microbiol. 15(4), 1132-1145.

NOAA Fisheries Online at: https://www.fisheries.noaa.gov (accessed 13 January 2020).

Noack J, Dongowski G, Hartmann L, Blaut M. 2000. The human gut bacteria *Bacteroides thetaiotaomicron* and *Fusobacterium varium* produce putrescine and spermidine in cecum of pectin-fed gnotobiotic rats. J of Nutrit. 130(5), 1225-1231.

Potrykus J, White RL, Bearne SL. 2008. Proteomic investigation of amino acid catabolism in the indigenous gut anaerobe *Fusobacterium varium*. Proteomics. 8, 2691-2703.

Potrykus J, Mahaney B, White RL, Bearne SL. 2007. Proteomic investigation of glucose metabolism in the butyrate-producing gut anaerobe Fusobacterium varium. Proteomics. 7, 1839-1853.

Pierce ML, Ward, JE, Holohan BA, Zhao X, Hicks RE. 2015. The influence of site and season on the gut and pallial fluid microbial communities of the eastern oyster *Crassostrea virginica* (Bivalvia, Ostreidea): community-level physiological profiling and genetic structure. Hydrobiologica. 756, 97-113.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO, Walker BJ, Ashlock LW, Marshman BC. 2016. Implementing a restoration program for the endangered white abalone (*Haliotis sorenseni*) in California. J. Shellfish Res. 35(3), 611–619.

Ramirez, RF, Dixon BA. 2003. Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). Aquacul. 227(1-4), 417-426.

Rogers-Bennett L, Aquilino KM, Catton CA, Kawana SK, Walker BJ, Ashlock LW, Marshman BC, Moore JD, Taniguchi IK, Gilardi KV, Cherr, GN. 2016. Implementing a restoration program for the endangered white abalone (*Haliotis sorenseni*) in California. Journal of Shellfish Research 35:611-618.

Rogers-Bennett L, Haaker PL, Huff TO, Dayton PK. 2002. Estimating baseline abundances of abalone in California for restoration. CalCOFI Rep. 43, 97-111.

Romero J, Feijoó CG, Navarrete P. 2012. Antibiotics in aquaculture–use, abuse and alternatives, in: Carvalho E, David GS, Silva RJ (eds), Health and Environment in Aquaculture. InTech, Rijeka, Croatia, p. 159.

Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, Rawls JF. 2011. Evidence for a core gut microbiota in the zebrafish. ISME J. 5, 1595-1608.

Rosenblum, ES, Robbins, TT, Scott, B. B., Nelson S, Juhasz C, Craigmill A, Tjeerdema, RS, Moore, JD, Friedman, CS. 2008. Efficacy, tissue distribution, and residue depletion of oxytetracycline in WS-RLP infected California red abalone *Haliotis rufescens*. Aquaculture 277:138-148.

Schliep KP. 2011. Phangorn: Phylogenetic analysis in R. Bioinformatics. 27(4), 592.

Schmidt V, Gomez-Chiarri M, Roy C, Smith K, Amaral-Zettler L. 2017. Subtle microbiome manipulation using probiotics reduces antibiotic-associated mortality in fish. *Msystems* 2 (6), e00133–17.

Silverman JD, Washburne AD, Mukherjee S, David LA. 2017. A phylogenetic transform enhances analysis of compositional microbiota data. eLife. 6, e21887. https://doi.org/10.7554/eLife.21887.

Stierhoff KL, Neuman M, Butler JL. 2012. On the road to extinction? Population declines of the endangered white abalone, *Haliotis sorenseni*. Biol. Conserv. 152, 46-52. https://doi.org/10/1016/j.biocon.2012.03.013.

Sun C, Liu H, Zhang Y, Lu C. 2018. Comparative analysis of the gut microbiota of hornbill and toucan in captivity. Microbiol. Open. 8, e00786. https://doi.org/10.1002/mbo3.786.

Tan J, Lancaster M, Hyatt A, van Driel R, Wong F, Warner S. 2008. Purification of a Herpes-like virus from abalone (*Haliotis spp.*) with ganglioneuritis and detection by transmission electron microscopy. J. Virol. Methods. 149(2), 338–341.

Theriot CM, Koenigsknecht MJ, Carlson Jr PE, Hatton GE, Nelson AM, Li B, Huffnagle GB, Li JZ, Young VB. 2014. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium Difficile* infection. Nature Commun. 5, 3114.

Travers MA, Goïc NL, Huchette S, Koken M, Paillard C. 2008. Summer immune depression associated with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection. Fish and Shellfish Immunol. 25(6), 800–808.

Vater A, Byrne BA, Marshman BC, Ashlock LW, Moore JD 2018. Differing responses of red abalone (*Haliotis rufescens*) and white abalone (*H. sorenseni*) to infection with phage-associated *Candidatus* Xenohaliotis californiensis. PeerJ 6:e5104; DOI 10.7717/peerj.5104.

Vilchis LI, Tegner MJ, Moore JD, Friedman CS, Riser KL, Robbins TT, Dayton PK. 2005. Ocean warming effects on growth, reproduction, and survivorship of southern California abalone. Ecological Applications 15:469-480.

Vorbach BS, Chandasana H, Derendorf H, Yanong RPE. 2019. Pharmacokinetics of oxytetracyline in the great danio (Devario aequipinnatus) following bath immersion. Aquacul. 498, 12-16.

Wickham H. 2016. Ggplot2: Elegant Graphics for Data Analysis, second ed. Springer-Verlag, New York.

Winkler FM, García R, Valdivia MV, Lohrmann KB. 2018. Assessment of oxytetracycline baths as therapeutic treatment for the control of the agent of Withering Syndrome (WS) in red abalone (*Haliotis rufescens*). J. of Invertebr. Pathol. 153, 109–116.

Zhao J, Ling Y, Zhang R, Ke C, Hong G. 2018. Effects of dietary supplementation of probiotics on growth, immune responses, and gut microbiome of the abalone *Haliotis diversicolor*. Aquacul. 493, 289-295.

7. Figures:

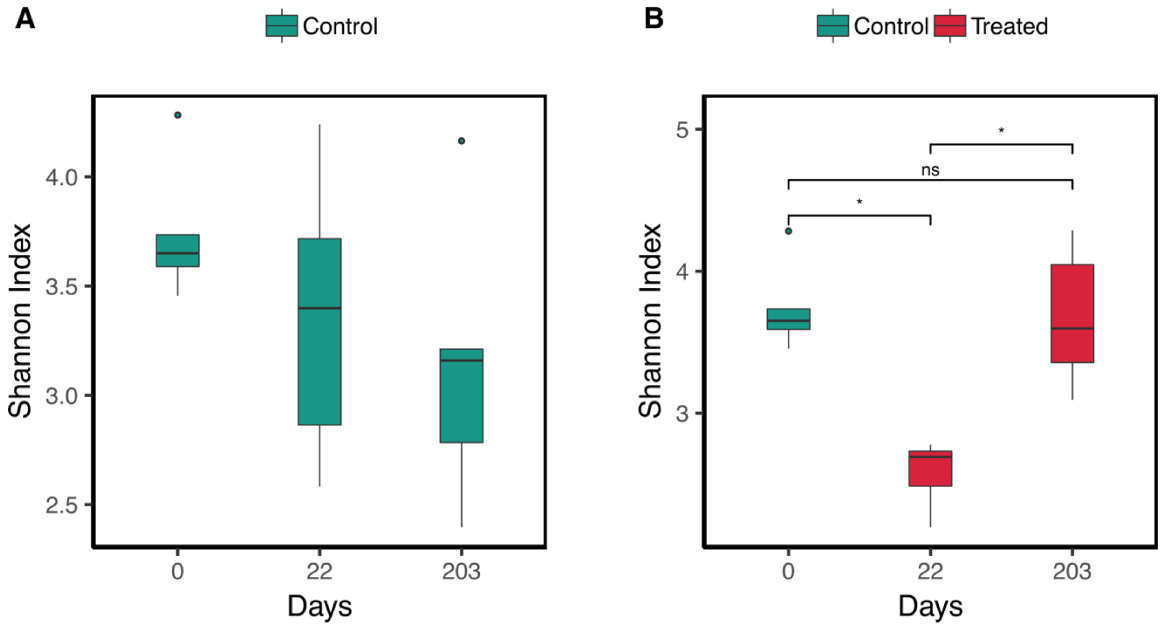


Figure 1. Shannon α-diversities for control and treated samples. Each box represents five samples including the outliers. **A)** No significant differences between controls at different time points with p-value threshold of 0.05. **B)** Microbiome diversity is significantly reduced on day 22 in oxytetracycline treated samples when compared to controls at day 0 ($p < 0.008$) and reconstituted treated microbiomes at day 203 ($p < 0.008$) based on two-sided Wilcoxon rank-sum test.

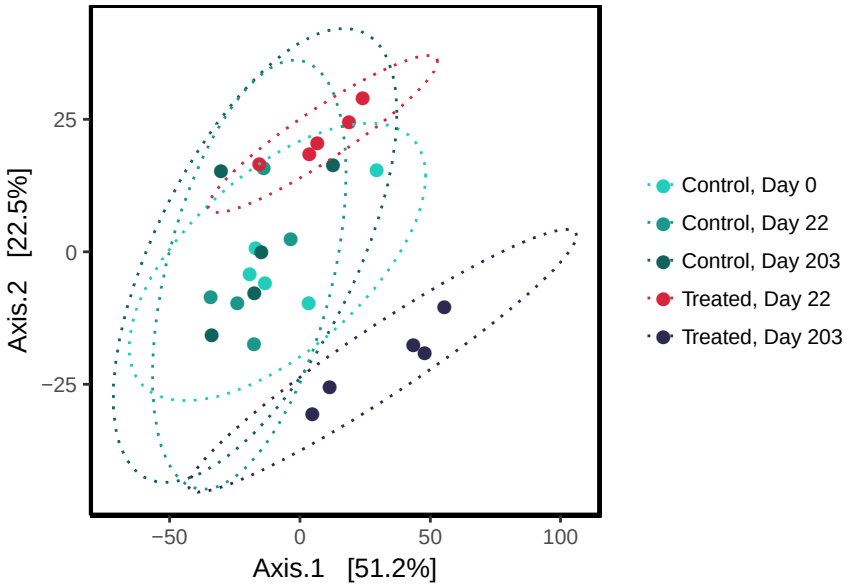


Figure 2. Principal coordinate analysis (PCoA) plot based on Euclidean distances after PhILR transform for all samples (β-diversity analysis). The components explain 73.7% of the variance. The microbiome profile of oxytetracycline treated samples at day 22 exhibits differences when it is compared to controls and treated samples at day 203, which corroborates α-diversity analysis results.

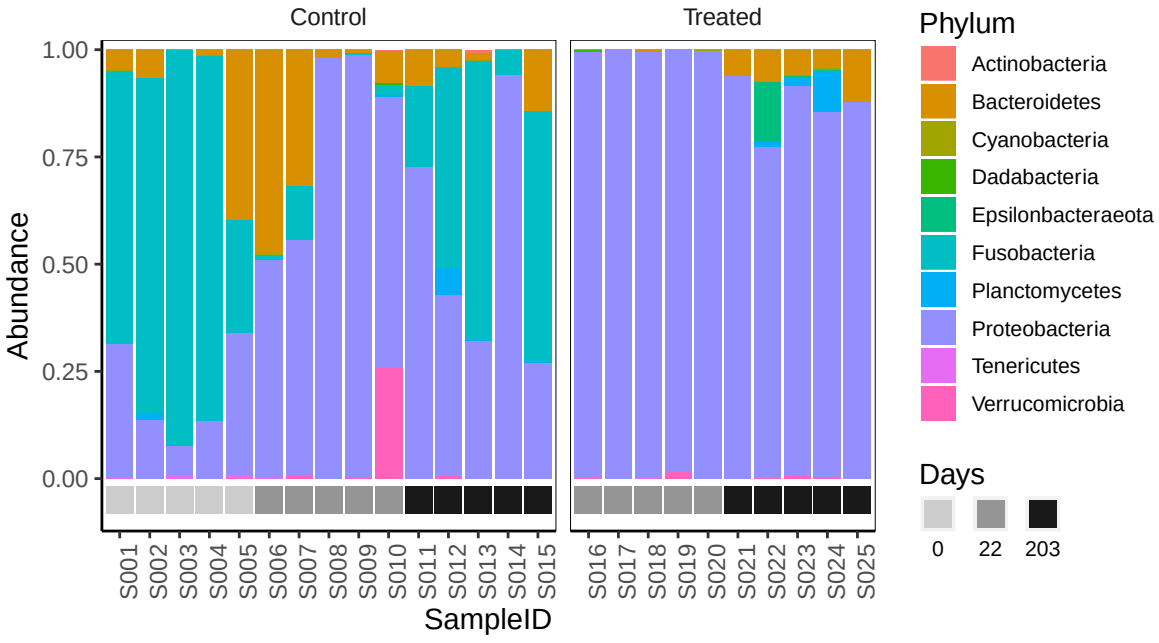
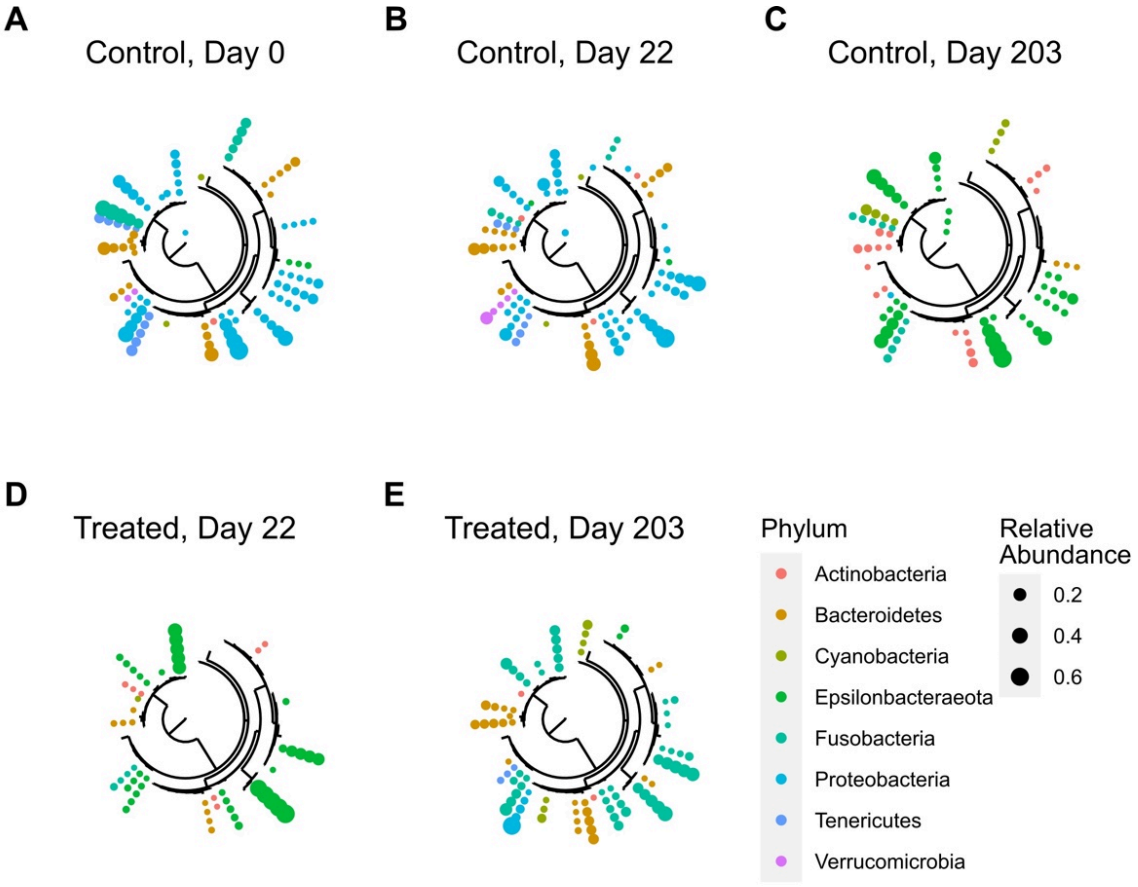


Figure 3. Relative abundances of bacterial taxa for all samples at phylum level. Taxa diversity is reduced after treatment but is reconstituted at day 203, albeit with differences.



Supplementary Figure 1. Phylogenetic trees relating microbiome populations of control (A-C) and treated (D-E) samples at various time points. Each point represents the relative abundance (between 0 and 1) of closely related OTUs in one sample, colored based on its taxonomy at the phylum level. Note the considerable difference between control and treated samples at day 22.