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Occurrence of tetracycline resistance genes in aquaculture facilities with varying use of oxytetracycline

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Abstract

The contribution of human activities to environmental reservoirs of antibiotic resistance is poorly understood. The purpose of this study was to determine if oxytetracycline (OTC) use in aquaculture facilities increased the detection frequency (i.e. prevalence) of tetracycline resistance genes relative to facilities with no recent OTC treatment. We used PCR to screen water and sediment from four non-commercial fish farms in northwestern Wisconsin for the presence of ten tetracycline resistance determinants (tet^R): *tet(A)*, *tet(B)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(M)*, *tet(O)*, *tet(Q)*, *tet(S)* and *tet(W)*. Water from farms with recent OTC use had significantly higher tet^R detection frequencies than did water from farms without recent OTC use, with prevalence in raceways and rearing ponds of farms with recent OTC use exceeded by more than two-fold that of farms not using OTC. Effluent from all farms, regardless of treatment regime, had higher tet^R detection frequencies than their corresponding influent for all genes, but the specific combinations of tet^R genes detected in a sample were not different from their corresponding influent. Although OTC use was associated with the increased occurrence and diversity of tet^R genes in water samples, it was not found to relate to tet^R gene occurrence in sediment samples. Sediment samples from facilities with no recent OTC use had significantly higher frequencies of tet^R gene detection than did samples from facilities with recent OTC use. All of the tet^R genes were detected in both the medicated and non-medicated feed samples analyzed in this study. These findings suggest that both OTC treatment in aquaculture facilities, and the farms themselves, may be sources of tet^R gene introduction to the environment. To our knowledge, this is the first study to use genotypic and cultivation-independent methods to examine tet^R gene occurrence associated with OTC use in aquaculture.

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Keywords

antibiotic resistance; antibiotics; PCR

INTRODUCTION

Bacterial communities in the environment can serve as reservoirs of antibiotic resistance. In antibiotic-impacted aquatic ecosystems, resistant bacteria as well as the genetic material conferring resistance, can be transported within and among systems and could potentially come into contact with human or animal populations. Antibiotics were detected at eighty percent of 139 river and stream sites across 30 states that were recently examined by the U.S. Geological Survey for a suite of organic contaminants [1]. Yang and Carlson [2] monitored the presence of various tetracycline compounds in pristine, agriculturally impacted, and urban impacted rivers. No tetracyclines were detected in samples from the pristine site; however, all targeted tetracycline antibiotics were detected in samples from the urban and agriculturally impacted site.

Much remains to be learned about the ecology of antibiotic resistance [3]. In particular, little is known about the impact of medical and agricultural antibiotic use on environmental reservoirs of resistance. A few recent studies focusing on antibiotic resistance genes as emerging contaminants measured their prevalence and abundance across human “impact” gradients. Pei et al. [4] surveyed sediments from a river in a mixed-use landscape for antibiotic resistance genes and observed higher resistance gene concentrations with increasing urban and agricultural impact. Auerbach et al. [5] demonstrated that municipal wastewater treatment plants disseminate many tetracycline resistance (tet^R) genes. Other potential sources of antibiotics and their associated resistance determinants include aquaculture [6, 7], animal feedlots [8], swine waste lagoons [9-11], runoff from manure-amended and effluent irrigated agricultural lands [12, 13], and fecal waste [14]. With so many routes through which antibiotics and antibiotic resistance genes can enter the aquatic environment, establishment of resistance reservoirs in natural microbial communities appears likely. A better understanding of the relative contributions of these sources to environmental resistance reservoirs is needed to prioritize targets for reducing antibiotic use or release.

Fish hatcheries and farms commonly use antibiotics (e.g., oxytetracycline, ormetoprim, sulfadiazine) therapeutically to treat ulcer diseases, furunculosis, bacterial hemorrhagic septicemia and *Pseudomonas* disease [15, 16]. Antibiotics are usually administered to fish in pelleted feed. Following application, antibiotics may dissolve into the water, settle to the bottom in uneaten feed or sorbed to particles, or be excreted by fish without metabolism. Previous studies found that 59% [17] to more than 90% [18] of OTC administered to sea bass or rainbow trout, respectively, was excreted in their feces.

Although previous studies have investigated the occurrence of specific culturable antibiotic resistant bacteria in hatcheries and fish farms [6, 19-25], none included a thorough genotypic and cultivation-independent examination of tet^R gene occurrence associated with OTC use. The goals of this study were to identify the types of tet^R genes present in aquaculture

facilities, and to evaluate whether aquacultural OTC use promotes increased frequency of detectable *tet*^R genes, which we took as a proxy for the presence of antibiotic resistant bacteria. We used a cultivation-independent PCR-based approach to detect ten types of *tet*^R determinants in four aquaculture facilities. The *tet*^R genes selected encompass both efflux pumps (*tet*(A), *tet*(B), *tet*(D), *tet*(E), and *tet*(G)) and ribosomal protection proteins (RPPs; *tet*(M), *tet*(O), *tet*(Q), *tet*(S), and *tet*(W)), representing the two primary tetracycline resistance mechanisms [26]. We compared differences between sediment and water samples across multiple sites within each farm. To our knowledge, this is the first study linking aquacultural OTC use to the cultivation-independent detection of *tet*^R both across and within facilities.

MATERIALS AND METHODS

Field sites and sample collection

Water, sediment and feed samples were collected on three occasions, between June and October during both 2005 and 2006, from multiple sites within four aquaculture facilities in northern Wisconsin, USA (Table 1). All four facilities engage in hatching and/or rearing and are managed by the state of Wisconsin Department of Natural Resources. The sampling regime corresponded to “before, during, and after” OTC use at treating facilities each year. Additional data related to measured OTC concentrations within the facilities are published elsewhere [27]. We refer to sample locations within a farm as *sample sites*. Table 1 lists the water and sediment samples collected from sites within each farm. Figure 1 depicts the typical flow process through an aquaculture facility. We note that some farms direct their effluent from raceways and/or rearing ponds to sedimentation ponds to settle solids and other particulates prior to releasing the water to the environment, while others discharge water directly to the environment and use sedimentation ponds only while cleaning raceways and rearing ponds.

To maintain confidentiality, we refer to the aquaculture facilities as 1, 2, 3, and 4. At the initiation of the study, Facility 1 had never used OTC. Facility 2 used OTC more than 20 years ago, but no longer administers the antibiotic. These farms are referred to as “controls”. Facilities 3 and 4 had recent histories of OTC use to mitigate furunculosis infections in salmonids and are referred to as “treating” facilities. Facility 3 administered OTC two years preceding our study (2003), while Facility 4 administered OTC during the late summer sampling events in both years of our study (2005 and 2006). Facility 1 treated with OTC during summer 2006 but only in the indoor rearing operations. The aquaculture facilities were selected to maximize our ability to discern differences between sites regularly administering OTC and those not generally using OTC. Furthermore, we sought four farms located in the same geographic area to minimize differences in water chemistry caused by differences in local geochemistry/geology.

General water quality parameters (pH, dissolved oxygen, and temperature) were measured on site, and prior to sampling, using the handheld Hach Model HG40d and Common Sensing TBODL6 meter or Yellow Springs YSI 58. Water was collected using sterile buckets from the center of each water sample site (Table 1) and transferred to sterile 2-L polycarbonate bottles. Bacteria in each of three replicate subsamples from a bucket were

concentrated by vacuum filtration onto 0.2- μ m filters (Supor-200; Gelman Sciences, Ann Arbor, MI). Sediment samples were collected using an Eckman Dredge and subsampled aliquots (40 mL) were stored in Corning Centrifuge Tubes (Corning Incorporated, Corning, NY). It should be noted that sediment samples were not available at each sampling point; several sites were either concrete structures or gravel lined. Feed samples were also collected and stored in Corning Centrifuge Tubes. Filters and sediment samples were stored at -80°C , and feed samples were stored at -20°C , until DNA extraction.

DNA extraction

We used the FastDNA kit (Qbiogene, Irvine, CA) to extract DNA from filtered water and the PowerSoil DNA kit (MoBio, Carlsbad, CA) to extract DNA from sediment and feed aliquots. Two filters per water sample site were extracted, however, when the yield per reaction was insufficient for downstream molecular analyses, triplicate extractions were performed. Three independent extractions were performed on sediment and feed subsamples. Concentrations of purified DNA were determined by spectrofluorometry (SPECTRAmax GEMINI XS®, Molecular Devices Corporation, Sunnyvale, CA) using the PicoGreen® dsDNA quantitation assay (Quant-it™ PicoGreen® dsDNA Assay Kit, Invitrogen Corporation, Carlsbad, CA). Extractions were diluted to 2 ng DNA/ μL in sterile, TE buffer (10mM Tris-HCl, 1mM EDTA [pH=8]).

PCR

Amplification was conducted in 25- μL reaction volumes containing 1 $\times$ PCR Buffer (Applied Biosystems, Foster City, CA), 3 mM MgCl_2 , 400 μM each deoxynucleoside triphosphate (dNTP), 400 nM of each forward and reverse primer, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) using an iCycler thermal cycler (BioRad, Hercules, CA). The thermal cycle was: initial denaturation at 95°C for 7 min; 40 cycles of 95°C for 45 s, annealing (various temperatures, see Table 2) for 45 s, and 72°C for 90 s; and a final extension of 72°C for 7 min. Reactions targeting ten tet^{R} genes were carried out using previously designed and validated primer sets (Table 2) and 2 ng of template DNA. Annealing temperatures were optimized for each primer set using target and non-target templates to verify specificity, to account for differences between our iCycler and the thermal cyclers previously used for validation. A DNA template mass of 2 ng per reaction was previously found to be optimal [5]. PCR product aliquots (4 μL) were visualized by electrophoresis on 1.5% (wt/vol) agarose gels stained with ethidium bromide and denoted as “+” when a clean and clear band of the expected size could be identified on the gel.

To assess reproducibility of end-point PCR, duplicate reactions were performed for each permutation of sample and primer set, with each assay including positive and negative controls. Positive controls were purified PCR products amplified from plasmids carrying the appropriate tet^{R} gene [10, 28]. PCR conditions were the same as described above. Products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and 2 ng of DNA was used per positive control reaction. Negative controls contained an additional 1 μL of sterile water. Detection limits were determined for each primer pair by a serial dilution of the positive control template. Detection limits for tet^{R} genes were determined by constructing titration curves of known gene copies per μL of template and running these

dilutions on an agarose gel until no product was visualized. Product for each gene typically disappeared below 10 copies/ μL . To determine whether there was matrix inhibition of PCR, 2 ng of DNA extract from sedimentation ponds and pooled feed samples were spiked with positive control template at 10^2 copies μL^{-1} . No inhibition was observed based on gel electrophoresis (data not shown).

Calculation of detection frequencies

Comparisons between the treatment groups (i.e., treated and control) were made for each tet^R gene using the frequency of detection of the gene in a treatment group (f_{gene}), the sum of detections of an individual gene across N samples (i.e., number of sites $\times$ number of sampling events):

$$\text{gene detection frequency } (f_{\text{gene}}) = \frac{1}{N} \sum_{j=1}^m \sum_{i=1}^n N_{r,ij} \quad (\text{Equation 1})$$

where

i = site

j = sampling event

m = number of sampling events

n = number of sample sites in the treatment group

N = number of samples per treatment group (i.e., $N = m \times n$)

$N_{r,ij}$ = individual gene detected at site j during sampling event i ($N_{r,ij}$ takes values of 0 or 1)

Comparisons among sites within a treatment group and between treatment groups for a given site were made using the gene composition frequency of a site (f_{comp}), defined as the sum of all tet^R genes detected in M samples from a specific site (i.e., number of sites of a given type $\times$ number of sampling events) within a treatment group:

$$\text{gene composition frequency } (f_{\text{comp}}) = \frac{1}{r \cdot M} \sum_{j=1}^m \sum_{l=1}^h \sum_{k=1}^r N_{d,klj} \quad (\text{Equation 2})$$

where

h = number of sites of a given type

k = tet^R gene

l = site of specific type

r = number of tet^R genes

M = number of samples per type of site within a treatment group (i.e., $M = h \times m$)

$N_{d,klj}$ = number of times tet^R gene k was detected at the site ($N_{d,klj}$ takes values of 0 or 1)

These equations were used to compare data classified by source (treatment and control facilities), and by matrix type (sediment and water).

Statistical analysis

Summary statistics were calculated to search for differences between groups of samples and among hatcheries with and without antibiotic use. Paired *t*-tests were conducted using the data analysis tool pack in Microsoft® Excel to test the null hypothesis that the frequency of *tet*^R gene occurrence obtained from qualitative PCR analysis did not differ significantly (1) between water and sediment samples, (2) between treatment and control facilities, and (3) between sample sites within facilities. The ten genes were treated as replicate measures of resistance prevalence in each sample group being compared. For example, a *t*-test between water and sediment would reveal if the two groups had a significant difference in their gene composition (f_{comp}). The null hypothesis was rejected when the *p* was < 0.05. Analysis of similarity (ANOSIM) tests [29, 30] were conducted using PRIMER 5 for Windows v. 5.2.7 (PRIMER-E Ltd., 2001) [31] to determine whether gene compositions differed between water and sediment, treatment and control, or influent and effluent samples. ANOSIM generates an *R* test statistic to indicate the degree of separation between groups, where an *R* value of 1 indicates complete separation and 0 indicates no separation. Monte-Carlo randomization of group labels (e.g. water v. sediment, or treatment v. control) was used to generate null distributions in order to test the hypothesis that gene composition measurements were more similar within a defined group than would be expected if gene composition measurements were grouped randomly. A *p* < 0.001 was considered significant to account for repeated significance tests [31]. Additionally, gene frequencies were compared between the following five data sets to determine if their detection frequency patterns were similar: (1) water and sediment from treatment facilities, (2) water and sediment from control facilities, (3) water from treatment and control facilities, (4) sediment from treatment and control facilities, and (5) influent and effluent water. A non-parametric Spearman's ρ was calculated using the data analysis tool pack in Microsoft® Excel, and α = 0.05 was used. Correlation values ≥ 0.55 were considered significant.

RESULTS

tet^R gene frequencies

The *tet*^R genes most commonly observed in water samples (detected in >50% of samples) were *tet*(A), *tet*(E), *tet*(G), and *tet*(M) (Figure 2A); *tet*(A), and *tet*(G) were also detected in more than 50% of sediment samples (Figure 2B). Detection frequencies of *tet*(B), *tet*(M), and *tet*(O) across both water and sediment samples were comparable, while *tet*(E) and *tet*(S) appeared unique to water samples, and *tet*(D) and *tet*(Q) were more prevalent in sediment samples. The detection frequency of *tet*^R genes (f_{comp} values) in water and sediment samples differed in both treatment and control facilities (both paired *t*-tests, *p* < 0.05). Gene composition frequency (f_{comp}) differed between water and sediment at treatment facilities (ANOSIM, *R* = 0.803, *p* = 0.001), but not at control facilities (ANOSIM, *R* = 0.294, *p* > 0.001). All of the *tet*^R genes were detected in the feed samples analyzed in this study. Gene frequencies (f_{gene}) for non-medicated feed were higher than 90%, except for *tet*(A), *tet*(D), *tet*(E), and *tet*(G), which were detected in 88, 49, 72, and 77% of the samples (Table S3). In

the medicated feed, all gene frequencies were greater than 90%, except for *tet*(D) and *tet*(G), which were detected in 71 and 86% of the samples. We note that individual feed samples were not analyzed for *tet*(O); DNA extracted from individual feed samples was pooled based on facility and sample date. All pools were found to contain *tet*(O).

tet^R gene frequencies: Treatment v. Control

OTC treatment was associated with a higher detection frequency of tet^R genes (f_{gene} values) in water samples (Figure 2A), but not in sediment samples (Figure 2B). With the exceptions of *tet*(B) and *tet*(Q), all tet^R genes in water samples from farms with recent OTC use had higher gene detection frequencies. The opposite trend was observed for sediments; most genes from treatment facilities had lower f_{gene} values than did control facilities. Pairwise *t*-tests comparing the f_{comp} detection frequencies between treatment and control facilities corroborated the trends observed in Figure 2: the detection frequency in water samples from treatment facilities and sediment samples from control facilities significantly exceeded those in control water samples and treatment sediment samples, respectively ($p < 0.05$). Despite the statistically significant difference in gene detection frequency (f_{gene}) between treatment and control samples, the gene composition frequencies (f_{comp}) between these samples were strongly correlated (Spearman $\rho = 0.88$ and 0.73 for water and sediment), and the actual gene composition did not differ (ANOSIM; $R = 0.17$ ($p > 0.001$) for water and $R = 0.294$ ($p > 0.001$) for sediment).

tet^R frequencies through a facility

Frequencies of tet^R detection in influent, raceway, and rearing pond water samples (f_{comp}) from treatment facilities exceeded those from control facilities (Figure 3A; *t*-test, $p < 0.05$ for all comparisons). Sedimentation pond and effluent sediment samples from control facilities had higher frequencies (f) of tet^R *comp* detection than did those from treatment facilities (*t*-test, $p < 0.05$), whereas rearing pond samples did not differ (Figure 3B; *t*-test, $p > 0.05$). Only Facility 3 had sediments at the influent point, preventing a comparison from being made for that site.

tet^R frequencies: Influent v. Effluent

The frequency of tet^R gene detection (f_{comp}) in effluent water was significantly higher than that in influent water (Figure 3A; *t*-test, $p < 0.05$), regardless of OTC use, and the gene composition frequencies at these two sites were moderately correlated (Spearman $\rho = 0.61$). All tet^R gene detection frequencies (f_{comp} values) at the other sites (viz., raceway, rearing pond, and sedimentation pond) downstream of the influent were significantly higher than the influent (*t*-test, $p < 0.05$). However, f_{comp} values at these sites were correlated to those of the influent samples (Spearman $\rho = 0.51, 0.77, 0.73$, respectively). Because only Facility 3 had sediment at the influent point, we examined the difference between that sample site and the effluent sediments from the same farm. The two sites were not different (*t*-test, $p > 0.05$).

Facility effect

To examine whether maintaining fish in an aquaculture facility, regardless of OTC treatment, is associated with the net release of tet^R genes to the environment, *t*-tests were

performed to compare frequencies of tet^R genes detected (f_{comp} values) in influent water from control facilities and subsequent downstream water sites within these facilities (raceway, rearing pond, sedimentation pond, and effluent). No significant difference was found between the influent water and the raceway and rearing pond water ($p > 0.05$), while a significant increase was noted from the influent to both the sedimentation pond and effluent ($p < 0.05$). Moreover, the influent gene composition (f_{comp}) correlated strongly with only the rearing pond and sedimentation pond sites (Spearman $\rho = 0.73$, and 0.60 respectively).

DISCUSSION

We hypothesized that tet^R reservoirs exist in aquaculture facilities using tetracycline antibiotics. Many fish farms are designed as flow-through systems with minimal to no effluent treatment. We sought to determine if such facilities represent a significant source of tet^R genes to aquatic environments.

In this study, we demonstrated that all ten studied tet^R genes could be detected in aquaculture facilities regardless of OTC treatment history. We detected all genes targeted in this study in water samples from all farms, although corresponding sediment samples contained no detectable levels of $\text{tet}(E)$ and $\text{tet}(S)$. The distribution of tet^R genes encoding efflux pumps and ribosomal protection proteins was comparable across all water and sediment samples, indicating that no perceivable selection process promoted the preservation of one resistance mechanism more so than the other.

We found that water and sediment contained different tet^R gene compositions. Although interactions between the water and sediment are possible in aquaculture facilities, we discuss them as separate ecosystems and assume that the water discharged from aquaculture drives the majority of tet^R gene dissemination to the environment.

Impact of OTC use on the frequency of tet^R gene detection

In this study, OTC use was associated with the increased occurrence and diversity of tet^R genes in water samples, but was not related to tet^R gene occurrence in sediment samples. However, for a given facility, the frequency of tet^R determinants detected (f_{gene}) in water samples from facilities using OTC was markedly higher than that in corresponding sediment samples, while the converse was observed in samples from facilities with no recent OTC use. Based on this observation, it could be hypothesized that OTC use reduces the number of tet^R gene types present in sediment samples. Additionally, it is conceivable that OTC use increases the *abundance* of particular tet^R genes through selection of unique populations, but a quantitative measurement of tet^R determinants would be required to adequately test this hypothesis.

Aquaculture facilities as sources of tet^R genes

Analyses comparing gene detection frequencies in influent and effluent water across all aquaculture facilities showed that detection frequency in influent water was significantly less than that in effluent water. Water samples from influent, raceway, and rearing pond sites in farms with a recent history of OTC use exhibited significantly higher frequencies of tet^R gene detection relative to the corresponding sample sites from farms with no recent OTC use.

($p < 0.05$). The observed differences in tet^R gene fingerprints in influent water from different aquaculture facilities may be attributable to influent water quality. Influent water to the facilities with recent OTC use was obtained from surface water sources, whereas groundwater serves as influent to facilities with no recent OTC. Other water quality parameters were comparable across sites and did not explain the observed differences (See Supplementary Table S4). Larger inputs of bacteria from surface water sources may contribute to the need for OTC treatment at a facility, further contributing to the relationship between surface water use and incidence of tet^R gene detection. The differences observed in raceway and rearing pond sites across farms could be a result of variations in fish populations (i.e., size, species), but differences in OTC use history is a more parsimonious explanation. The differences observed between influent and sites containing fish (raceway and rearing pond) may be attributable to the introduction of fish feed to the system. We were surprised to find that fish feed, regardless of whether or not it was medicated, contained all of the tet^R genes selected for the study; it likely contributed to the tet^R detection in water and sediment samples [32]. It is not clear why unmedicated feed contained antibiotic resistance genes, but it is conceivable that typical bacteria present in the production facilities carry many different resistance genes because antibiotics are used for production of some feed batches. This would be likely if both medicated and unmedicated feed batches are processed on the same equipment.

Interestingly, the frequency of tet^R gene detection in sedimentation pond water from all facilities, regardless of treatment, was approximately the same. However, sediment samples from facilities with no recent OTC use had significantly higher frequencies of tet^R gene detection (f_{comp}) than did samples from facilities with recent OTC use. Previous investigators showed that even in the absence of OTC, elevated levels of culturable resistant bacteria could be detected in sediments where fish feed had accumulated and decomposed [23, 33]. Aquaculture sedimentation ponds are designed to maximize gravitational settling of particulate matter, resulting in the accumulation of decomposing organic matter and thus providing optimal growth conditions for microorganisms. The mechanisms responsible for specifically increasing the abundance of OTC resistant culturable bacteria in this type of environment are unknown.

We compared the frequency of tet^R gene detection in downstream sample sites (within a facility) to their corresponding influent water, for all facilities, to determine which component of the aquaculture system may be associated with an increase in detection frequency. Although resistance genes were more frequently detected in influent derived from surface water than in influent with groundwater sources, the frequencies of resistance detected downstream of the influent throughout all farms was significantly higher. Thus, we propose that both facility unit processes (such as feed administration and general lack of effluent treatment processes) and OTC use were factors contributing to the presence of resistance genes. This could have implications for more advanced effluent treatment requirements for aquaculture facilities, regardless of antibiotic use.

In this study, we estimated the frequency and diversity of two groups and ten types of tet^R genes in four fish farms, where two recently used OTC and two had no recent history of OTC use. Although previous studies have addressed the occurrence of specific culturable

antibiotic resistant bacteria in hatcheries and fish farms, none included a thorough genotypic and cultivation-independent examination of the occurrence of tet^R genes as a result of OTC use in aquaculture. Our results suggest that aquaculture facilities may cause an increase in the number of tet^R genotypes present in aquatic bacterial communities regardless of antibiotic use history. Future studies should investigate the implications of releasing tet^R genes to the receiving aquatic environment (i.e. the subsequent impact on human pathogen resistance [16]), as well as the concentrations at which these genes are detected to better understand the affects of OTC treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A general schematic depicting water flow through an aquaculture facility. Stars represent sampling points (water and/or sediment) within a facility.

Figure 2.

The gene detection frequency of each tet^R gene (f_{gene}) in (A) water samples and (B) sediment samples, from treatment and control facilities. The diagonal line represents the situation in which the detection frequency does not differ between facilities treating or not treating with OTC.

Figure 3.

Frequency of tet^R gene detection in (A) water samples and (B) sediment samples from treatment (black bars) and control (white bars) facilities. The values are reported as the frequency of tet^R gene detection by sites within the facilities, f_{comp} . We note that only Facility 3 had sediment at the influent site, while one treatment (Facility 3) and one control facility (Facility 1) had sediment at the effluent sample points. Letters indicate significant differences between frequency values following pairwise comparisons with Bonferroni corrections ($\alpha = 0.05/28$). The number of samples per type of site within a treatment group that were used to calculate f_{comp} (M, see Equation 2) are presented below the x-axis label

Table 1

Aquaculture facilities sampled (“w” and “s” indicate water and sediment samples).

FACILITY	OTC USE	SAMPLE SITES	FISH PRODUCTION
Facility 1	Never ¹	Influent <i>Groundwater</i> (w) Rearing Pond <i>Walleye or Perch</i> (w, s) Sedimentation Pond (w, s)	4.9×10^5 fish/year 1) <i>Walleye</i> ^c 2) <i>Yellow perch</i> ^d 3) <i>Splake</i> ^a
Facility 2	Not in past 20 years	Influent <i>Groundwater</i> (w) Raceway <i>Brown trout</i> (w) Sedimentation Pond (w, s) Effluent (w)	9.0×10^5 fish/year 1) <i>Brook trout</i> ^e 2) <i>Brown trout</i> ^f
Facility 3	Not Since 2003	Influent <i>River Water</i> (w, s) Raceway <i>Brook trout</i> (w) Rearing Pond <i>Rainbow trout</i> (w) Sedimentation Pond (w, s) Effluent (w, s)	4.0×10^5 fish/year 1) <i>Brook trout</i> ^e 2) <i>Brown trout</i> ^f 3) <i>Rainbow trout</i> ^g
Facility 4	Annual	Influent <i>Lake Water</i> (w) <i>Groundwater</i> (w) Raceway <i>Splake</i> (w) <i>Coho salmon</i> (w) Rearing Pond (no fish) (w, s) Sedimentation Pond (w, s) Effluent (w) WWTP 2° <i>Clarifier</i> (w)	1.2×10^6 fish/year 1) <i>Lake trout</i> ^h 2) <i>Brook trout</i> ^e 3) <i>Brown trout</i> ^f 4) <i>Splake</i> ^a 5) <i>Steelhead trout</i> ^b 6) <i>Chinook salmon</i> ⁱ 7) <i>Coho salmon</i> ^j

¹ OTC was the only antibiotic administered at any of the facilities within this study.

² Facility 1 treated their indoor recirculation tanks in the 2005/2006 winter, however, these indoor tanks were not hydrologically connected to the outdoor rearing ponds that were sampled.

^a Splake (*Salvelinus namaycush* × *Salvelinus fontinalis*) are a hybrid trout resulting from the cross between brook and lake trout.

^b Steelhead trout (*Oncorhynchus mykiss*) are anadromous rainbow trout.

^c *Stizostedion vitreum*,

^d *Perca flavescens*,

^e *Salvelinus fontinalis*,

^f *Salmo trutta*,

^g *Salmo gairdneri*,

^h *Salvelinus namaycush*,

ⁱ *Oncorhynchus tshawytscha*,

^j *Oncorhynchus kisutch*

Table 2

Primers used to detect tetracycline resistance genes

Targeted tet ^R Class		Sequence (5'→3')	Annealing Temperature (°C, T_A)	Amplicon Size (bp)	Reference
<i>tet(A)</i>	FW RV	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	61.3	210	[34]
<i>tet(B)</i>	FW RV	TAC GTG AAT TTA TTG CTT CGG ATA CAG CAT CCA AAG CGC AC	58	206	[10]
<i>tet(D)</i>	FW RV	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	63.1	787	[34]
<i>tet(E)</i>	FW RV	GGT ATT ACG GGA GTT TGT TGG AAT ACA ACA CCC ACA CTA CGC	61.2	199	[10]
<i>tet(G)</i>	FW RV	GCA GAG CAG GTC GCT GG CCY GCA GAG GAA GCC AGA AG	61.3	134	[10]
<i>tet(M)</i>	FW RV	ACA GAA AGC TTA TTA TAT AAC TGG CGT GTC TAT GAT GTT CAC	61.2	406	[34]
<i>tet(O)</i>	FW RV	ACG GAR AGT TTA TTG TAT ACC TGG CGT ATC TAT AAT GTT GAC	60	171	[28]
<i>tet(Q)</i>	FW RV	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	61.2	904	[34]
<i>tet(S)</i>	FW RV	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	65	667	[34]
<i>tet(W)</i>	FW RV	GAG AGC CTG CTA TAT GCC AGC GGG CGT ATC CAC AAT GTT AAC	64	168	[28]