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Occurrence of *sul* and *tet*(M) genes in bacterial community in Japanese marine aquaculture environment throughout the year: profile comparison with Taiwanese and Finnish aquaculture waters

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Short title: *sul* and *tet*(M) in marine aquaculture

Highlights:

1. *sul1, sul2, sul3*, and *tet*(M) were detected in Japanese mariculture environment.
2. *sul2* was constant, whereas others varied monthly.
3. *sul3* was present in non-culturable bacteria in seawater, but not in cultured bacteria.
4. Four resistance gene profiles among Japan, Taiwan, and Finland were different.

ABSTRACT

The use of antibiotics in aquaculture causes selection pressure for antibiotic-resistant bacteria (ARB). Antibiotic resistance genes (ARGs) may persist in ARB and the environment for long time even after stopping drug administration. Here we show monthly differences in the occurrences of genes conferring resistance to sulfonamides (i.e. *sul1, sul2*, *sul3*), and tetracyclines (*tet*(M)) in Japanese aquaculture seawater accompanied by records of drug administration. *sul2* was found to persist throughout the year, whereas the occurrences of *sul1, sul3*, and *tet*(M) changed month-to-month. *sul3* and *tet*(M) were detected in natural bacterial assemblages in May and July, but not in colony-forming bacteria, thus suggesting that the *sul3* was harbored by the non-culturable fraction of the bacterial community. Comparison of results from Taiwanese, Japanese, and Finnish aquaculture waters reveals that the profile of *sul* genes and *tet*(M) in Taiwan resembles that in Japan, but is distinct from that in Finland. To our knowledge, this work represents the first report to use the same method to compare the dynamics of *sul* genes and *tet*(M) in aquaculture seawater in different countries.

1. Introduction

Exposure of bacteria to antibiotics selects for antibiotic-resistant bacteria (ARB). High concentrations of antibiotics are used in human and veterinary medicine, which flow into the environment, selecting for ARB and ARGs (Zhang et al., 2009). Aquatic environments, especially coastal seas, are readily contaminated with both antibiotics and ARB from human and animal farm wastewater effluent (Suzuki et al., 2017). Even at low concentrations, the antibiotic contaminants originating from land discharges are expected to have effects on the selection and dissemination of ARGs among bacterial communities in the seawater environment (Al-Bahry et al., 2009; Gullberg et al., 2011). Additionally, antibiotics are used for marine aquaculture, resulting in the direct spread of these compounds into the sea. Thus, aquaculture is expected to be another source of the contamination of seawater with antibiotics, ARB, and ARGs (Cabello et al., 2013).

Fish species grown in Japanese aquaculture differ from those cultivated in Europe and the Americas. For instance, red seabream (*Pagrus major*) and yellowtails (*Seriola quinqueradiata* and related species), which belong to the order Perciformes, are major aquaculture products in the southern region of Japan. Fish feed for these species consists

of “moist-pellet”, which is an artificial dry pellet that is softened by mixing with seawater immediately before feeding; this softening step provides a feed that is superior to the dry pellet in terms of digestability and nutritional effects. If needed, antibiotics are mixed in with the pellets at the time of feeding.

The antibiotics in the feed directly impose selective pressure on the environmental bacterial community, as mentioned above. Additionally, the coastal sea receives land-derived microbes harboring ARGs, making the coastal sea an environment in which a number of ARGs are mixed together (Suzuki and Hoa, 2012). However, it remains unclear as to whether this pooling of ARGs in the coastal sea represents a risk to humans. Risk assessment would require quantitative estimates of human exposure to ARGs and of the frequency of transmission of ARGs to humans.

Leonard et al. (2015) suggested that human recreation in marine environments raises the risk of human exposure to ARB. ARB and ARG transmission also are suspected between the aquaculture environment and fishermen, who frequently perform antibiotic administration and fish harvesting. Fish and the aquaculture environments are candidate sources of ARB exposure. Various transmission cases may be the result of interactions between humans and the environment (Huijbers et al., 2015). Horizontal gene transfer (HGT) of ARGs between marine bacteria and enteric bacteria (Neela et al., 2009) also might be a risk factor.

A specific feature of marine bacteria is that a high percentage of bacteria are “non-culturable and/or yet-to-be cultured” (Kogure et al., 1979; Staley and Konopka, 1985; Lloyd et al., 2018). In the work presented here, we consider the ARG dynamics among this silent majority community of uncultured bacteria. Recent metagenomic studies have revealed a new trait of ARGs in the sea (Hatosy and Martiny, 2015), but the “culture-independent approach” is “database dependent”. Recent comprehensive ARG databases incorporate accessions derived from various origins, including sequences representing the culture-independent metagenome (McArthur et al., 2013). However, ARGs have classically been identified based on their phenotypic effects in culturable bacteria. Our approach has been to perform a quantitative analysis of selected four ARGs detected in both culturable and non-culturable bacteria; this strategy is expected to be a highly useful approach for evaluating the abundance of ARGs and ARB across the entire bacterial community (Suzuki et al., 2013; 2015).

Sulfamonomethoxine and sulfadimethoxine (sulfonamides) were used in Japan until the 1980s, when these compounds were replaced by oxytetracycline (OTC). OTC is the most frequently used antibiotic for aquaculture in the world (EPA, 2013). In the 1990s, multidrug-resistant *Photobacterium damselae* ssp. *piscicida* was found in red seabream and yellowtails in several parts of Japan (Kim and Aoki, 1993; Kim et al., 2008); these bacteria were resistant not only to sulfonamides and OTC, but also to florfenicol, bicozamycin, and fosfomycin. Fish-pathogenic bacteria with OTC resistance frequently have been isolated, and the corresponding resistance genes have been characterized (Furushita et al., 2003; Kim et al., 2004). Kim et al. (2008) isolated bacteria from the southern region of Japan that carried a plasmid harboring a sulfonamide-resistance gene (*sul2*) in combination with tetracycline-resistance *tet* genes. Nonaka et al. (2012) reported a new multi-drug resistance plasmid (pAQU1) from seawater bacteria in the Seto Inland Sea, where net-pen aquaculture of red seabream is performed. The plasmid pAQU1 carries multiple resistance elements, including *bla*$_{\mathrm{CARB}}$-9-like, *floR*, *mph*(G), *mef*(C), *sul2*, *tet*(M), and *tet*(B) (Nonaka et al., 2012; 2014). These findings suggest that Japanese aquaculture sites in the coastal seas are highly contaminated by multidrug-resistance genes. Among the ARGs, *sul2* and *tet* genes are ubiquitously distributed along the coast of southern Japan (Kim et al., 2008; Nonaka et al., 2012).

We hypothesized that if plasmids harboring *sul2* and *tet* are widely distributed across seawater bacterial communities, despite the rapid exchange of water that occurs in the open sea, then seawater serves as a pandemic reservoir of ARGs. Knowledge of month-to-month changes in the levels of ARGs in seawater will be important for estimating the retention of ARGs in the coastal seas, which would represent a possible risk factor for human exposure. The quantitative monitoring of *sul2* and *tet* will be a factor for estimating the present distribution of ARGs in aquaculture areas in the open sea.

The present study sought to reveal seasonal dynamics of selected marker ARGs in aquaculture areas, examining the total assemblage including both non-culturable and culturable bacteria. Three *sul* genes and forty-five *tet* genes (Roberts et al., 2012) have been reported to date, including the recently reported *sul4* gene (Razavi et al., 2017). Among these *sul* genes, *sul2* and *sul1* have been detected frequently in clinical strains isolated from European (Blahna et al., 2006) and Asian waters (Hoa et al., 2008), respectively. *sul3* is found infrequently in bacterial isolates (Grape et al., 2003). Our

129 recent monitoring revealed that *sul3* is abundant in the non-culturable community in
130 seawater from Manila Bay (Suzuki et al., 2013). As for the *tet* series, *tet*(M) has well been
131 studied due to the broad host range (Roberts et al., 2012). Additionally, the evolutionary
132 history of *tet*(M) has been described (Kobayashi et al., 2007).
133 In the Uwa Sea, the focus of the present research, *tet*(M) has been detected ubiquitously
134 in Gram-positive and -negative bacteria, including *Vibrio, Photobacterium, Lactococcus*,
135 and unknown Gram-positives (Kim et al., 2004; Neela et al., 2007). We here chose *sul1,*
136 *sul2, sul3*, and *tet*(M) as markers due to the fact that these ARGs are well characterized
137 and widespread, and that the history of the use of the corresponding compounds is readily
138 available. Recent resitome analysis reported that *tet*(M) is the most prevalent and
139 abundant (Muziasari et al., 2017). We also monitored for the presence of these ARGs in
140 Taiwanese and Finnish aquaculture sites, providing comparators to the environmental
141 ARG profiles in Japan. All sampling sites in Japan, Taiwan and Finland are set every year
142 stably, and rearing fish are different. The same sampling and analysis methods were used
143 in all three countries. The resulting data were subjected to cluster analysis using the copy
144 numbers of the ARGs in natural assemblages.
145
146 **2. Materials and Methods**
147 **2.1. Sampling**
148 Surface water was sampled using an alcohol-washed bucket, and the resulting water
149 samples were stored in sterilized polypropylene bottles. All samples were held on ice and
150 employed for filtration and colony counting within several hours of collection. Sampling
151 was performed from April 2013 to January 2014 in the Uwa Sea, Japan. Sampling sites
152 are shown in Fig. 1. Three sampling sites were located in a net-pen area where red
153 seabream (*Pugrus major*) was reared; a separate sampling site located 600 m from the
154 net-pen was used as a control. The water depth in the area is 30−50 m. Individual net-
155 pens have dimensions of 11 m × 11 m × 7.5−10 m (depth), as shown in Fig. 1. Water
156 temperature, cumulative administered feed amount, and the oxytetracycline (OTC)
157 administration schedule are indicated in Fig. 2. Water samples were obtained in 2012 and
158 2013 from both Finland and Taiwan; samples were collected by the same procedure as
159 that used in Japan, although monthly sampling was not performed in these other countries.
160 The reasons of choosing these countries are; 1) all are seawater coastal aquaculture having

different fish species, 2) all sampling sites are set every year stably, and we could obtain rearing condition information, and 3) laboratory facilities are appropriate to do experiment as soon as possible after sampling campaign. Japanese and Finnish aquaculture uses net-pens located in the open sea, whereas Taiwanese aquaculture employs concrete ponds maintained with a continuous supply of seawater. Cultured fish in Finland was whitefish (*Coregonus lavaretus*) and that in Taiwan was groupers (*Epinephelus* sp.) and shrimp (*Penaeus monodon* and *Litopenaeus vannamei*). In all surface water samples, salinity, pH, and temperature were measured using a pH/conductivity meter (Horiba D-54, Horiba, Kyoto, Japan). Site in Taiwan (N24˚48'13.42", E121˚48'55.11") was coast in Yilan city and those in Finland (site K, N60˚09'49.22", E21˚41'29.96"; site M, N60˚16'3.28", E21˚24'30.73"; site P, N60˚16'08.47", E21˚25'34.92") were coast around Turk city.

2.2. Enumeration of bacteria

The colony-forming units (CFUs) were counted on 1.5% agar plates with marine broth 2216 (Difco). Each water sample (0.5 mL) was subjected to 10-fold serial dilution with 4.5 mL phosphate-buffered saline (PBS, pH 7.4). Aliquots (100 μL) of each dilution were spread on individual plates, and plates (generated in duplicate) were incubated at 25˚C for 2 days. Agar plates containing 60 μg/mL of sulfamethoxazole (SMX) or oxytetracycline (OTC) were used to enumerate SMX-resistant (SMX^r) and OTC-resistant (OTC^r) bacteria (Hoa et al., 2011; Kim et al., 2003). For total cell counts, 1-mL water samples were supplemented with neutral buffered formalin to a final concentration of 1% to fix the cells. After filtration over a black polycarbonate filter (0.2-μm pore size; Millipore, Billerica, MA, USA), bacterial cells were stained with 4', 6-diamidino-2-phenylindole (DAPI). The bacteria were enumerated by epifluorescence microscopy (BX60, Olympus Co., Tokyo, Japan). More than 300 cells were enumerated from each sample, examining a minimum of 10-15 randomly selected microscopic fields per sample.

2.3. DNA extraction

Extraction of DNA was performed from filters as "natural assemblage DNA" and from mixtures of colonies as "cultured bacterial DNA". To obtain DNA from the natural assemblage, an appropriate volume (30−200 mL) of each water sample was filtered through 47-mm polycarbonate filters (0.2-μm pore size, Millipore); the resulting filters then were stored at −20˚C and transported to the laboratory. Triplicate filters were used

for DNA extraction. To obtain DNA from pooled colonies, all colonies that appeared on agar plates with or without antibiotics were picked up and suspended in PBS; the cells were then harvested by centrifugation. The resulting cell pellets were stored at −80˚C until DNA extraction. The extraction of DNA from filters and pooled colonies was performed using the cetyl-trimethyl-ammonium bromide (CTAB) method (Dempster et al., 1999) with some modification. Briefly, filters were dipped in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing sodium dodecylsulfate (SDS, 0.5%), Proteinase K (0.1 mg/mL, TaKaRa, Otsu, Japan), and RNase A (0.05 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). The filters then were incubated at 37˚C for 1 h. To remove polysaccharides, a CTAB/NaCl solution (10% CTAB, 0.7 M NaCl) was added, and the samples were incubated at 65˚C for 10 min. Freeze-thawing was repeated with three cycles of freezing at −80˚C and thawing at 65˚C to increase the recovery of DNA from bacterial cells. Subsequently, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the tubes were inverted and centrifuged at 2100×g at 4˚C for 10 min. The upper (aqueous) phase was divided between two 1.5-mL tubes, and an equal volume of chloroform-isoamyl alcohol (24:1) was added. The tubes were inverted and centrifuged at 21,600 ×g for 10 min at 4˚C, and upper (aqueous) phase was transferred to a fresh 1.5-mL tube. The samples were precipitated by the sequential addition of 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The resulting pellets were dried under vacuum and each dissolved in 50 μL of sterilized Milli-Q water. The recovered DNA was quantified by ultraviolet absorption using a DU640 meter (Beckman Coulter, Orange County, CA, USA), and the quality of the DNA was checked by electrophoresis on a 1.0% agarose gel stained with Gel Red (Fujifilm, Tokyo, Japan).

2.4. qPCR quantification of antibiotic resistance gene (ARG) copy number

Quantitative PCR (qPCR) was performed using a CFX96 Real-Time system (BioRad Laboratories, Hercules, CA, USA); an increase of double-stranded DNA was detected as an increase in fluorescence. Each PCR amplification was performed in a 20-μL reaction volume containing 1× Sso Fast Eva Green Supermix (BioRad), 500 nM of each primer, and 1 μL of sample DNA. qPCR was performed using previously designed primers for the *sul* genes (Suzuki et al., 2013) and *tet*(M) (Suzuki et al., 2018). Performance conditions of qPCR were as described previously (Suzuki et al., 2013; 2018). Each sample was tested in triplicate. The copy numbers of *sul1, sul2*, *sul3*, and *tet*(M) were normalized

by dividing by the 16S rRNA gene copy number at the respective time points to account for any temporal variation in bacterial cell numbers. Throughout the text, copy number is presented in units of copies/16S.

2.5. Bacterial class analysis by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH)

The abundances of Alpha-, Beta-, and Gammaproteobacteria, and of the class Flavobacteria of the phylum Bacteroidetes, were determined using CARD-FISH with phylogeny-specific oligonucleotide probes (Teira et al., 2004). Sample water was collected with 5L-Kiskin sampler from 1 m and 20 m depths at the same sites as indicated in Fig. 1 from December 2012 to October 2013. Although the ARGs were analyzed surface water because of net-pen depth, samples from 20 m depth were analyzed for profiling class structure in water column. Water samples (10 mL each) were fixed by overnight incubation in the dark in the presence of 0.2-μm-filtered neutral formalin (1% final concentration). The fixed samples then were filtered through 0.2-μm-pore-size polycarbonate filters (Millipore, GTTP, 25-mm filter diameter) with 0.45-μm cellulose nitrate support filters (Millipore, HAWP), washed with Milli-Q water, air dried, and stored at −20°C until further processing. Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme. Phylogeny-specific probes were used to detect Alpha- (Alf968) (Glöckner et al., 1999), Beta- (Bet42a) (Manz et al., 1992), and Gamma- (Gam42a) (Manz et al., 1996) proteobacteria, and to detect the class Flavobacteria of the phylum Bacteroidetes (CF319a) (Manz et al., 1996). To avoid the unspecific binding of probes between Bet42a and Gam42a, we included the same concentration of unlabeled competitor probes. The probes were added to a final concentration of 0.28 ng/μL (0.05 μM), and hybridization was performed at 35°C for 15 h. Thereafter, the fluorescence signal was amplified by incubating the filters with H_2O_2 (0.0015%) and tyramide-Alexa488 at 37°C for 30 min. The filters were stained and mounted on slides with a DAPI mix (5.5 parts Citifluor, 1 part Vectashield, and 0.5 parts PBS with DAPI at a final concentration of 1 μg/mL). Cells were detected under an Olympus BX 50 epifluorescence microscope with a 100 W Hg lamp and corresponding filters for Alexa488 and DAPI.

2.6. Cluster analysis of samples from each country by NMDS

The resistance gene copy numbers were normalized by dividing by the 16S rRNA gene copy numbers, averaged over the biological replicates of each sampling site. Nonmetric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity distance between the normalized resistance gene numbers was performed using the metaMDS function provided by the Vegan 2.5-3 library (Oksanen et al., 2018) in R version 3.5.1, using a maximum of 1000 random starts. The position of the weighted average of the ARGs within the ordination space of the NMDS was calculated using the wascores function provided by Vegan. The results were plotted using the ggplot2 3.1.0 library in R (Wickham, 2016).

Significant differences between month-to-month gene copy numbers were assessed by one-tailed Student's t-tests. p values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. ARB occurrence in Japan

Total cell number counting by DAPI staining in the aquaculture area was 10^6 cells/mL throughout the year (Fig. 3), whereas total-CFU/mL varied over the course of the year, ranging from 10^3 -10^4 in April to July, and 10^2 in December and January. These values are consistent with the previous observation that the culturable cell number is less than 0.1% of the total cell number in sea water (Kogure et al., 1979).

Through the period, OTC^r was 11-72%, and SMX^r was 7-24%. OTC-resistant (OTC^r) bacterial number was 10^3 (72% of the total-CFU) in April, just after first administration of OTC. OTC was administered three times (in April, June, and July), as shown in Fig. 2; these applications should impose selective pressure. However, the OTC^r bacterial number remained at 10^3 from April to July. The OTC^r number decreased in December (to 10^2) and January (to 10^1–10^2) synchronously with total-CFU, although total cell number (10^6) was unchanged. These observations suggested that the culturable OTC^r bacteria are distributed among the viable bacterial community throughout the year, and do not respond quickly to OTC administration. This result is consistent with that of Harnisz et al. (2015), which reported that the abundance of OTC^r bacteria was constant from the upstream to the downstream in a river where aquaculture impact was present.

The time course change of SMX-resistant (SMX^r) bacteria exhibited a similar trend as

that seen with OTC^r bacteria. SMX^r numbers fluctuated with total CFU, exhibiting abundances ranging from 7.4−18.5%. All of the total-CFU, OTC^r, and SMX^r numbers declined in December and January. It is known that *sul* and *tet* genes are occasionally conveyed together on the same plasmid (Kim et al., 2008; Nonaka et al., 2014); thus, the trend of similar occurrence of SMX^r and OTC^r may reflect the distribution of a resistance plasmid carrying resistance genes for both drugs.

The occurrences of SMX^r- and OTC^r-bacteria in Taiwan and Finland were summarized in Table S1 and S2. SMX^r in Taiwan was 32.9-87.0% in aquaculture pond and OTC^r was detection limit to 46.9%, showing OTC^r was lower than SMX^r. Finnish case was similar to Taiwan. SMX^r was 17.0-65.2%, whereas OTC^r was almost lower than detection limit. These were different profiles to Japan. It is reported when culture dependent method is used that SMX^r bacterial rate is higher than OTC^r in aquaculture settings in Pakistan and Tanzania (Shah et al., 2012) and Chile (Shah et al., 2014); however, OTC^r is higher in the Philippines (Tendencia and de la Pena, 2011). Japanese case (Fig. 3) showed that OTC^r rate was higher than SMX^r, suggesting OTC administration selects culturable bacteria.

The distribution of bacterial taxa in this area was examined by CARD-FISH analysis (Fig. S1). The results showed that the abundance of Gammaproteobacteria was higher in the 1 and 20 m water than were other classes ($p < 0.05$), both at the net-pen sites and the far site. In all classes, cell numbers in summer (June and July) tended to be high in 1 m depth than 20 m. This is due to stratification of the water column. It has been reported that members of the Gammaproteobacteria are typical copiotrophs, adapted to high nutrient concentrations (Eilers et al., 2000); thus, Gammaproteobacteria are abundant in coastal sea areas and comprise culturable species and Bacteroidetes (synonym Cytophaga-Flavobacterium) are ubiquitously abundant in the ocean (Glöckner et al., 1999). The area monitored in Japan in the present work received large amounts of fish feed (Fig. 2 bar graph), which should make the seawater eutrophic. Majority of ARB among marine bacteria have been reported to consist primarily of Gammaproteobacteria (Cabello et al., 2013). However, non-culturable organisms will be revealed to be ARB in future work by advanced resistome for example, by using epic-PCR (Hultman et al., 2018; Spencer et al., 2016) .

3.2. ARG dynamics throughout the year in Japan

Quantitative analysis of the four tested ARGs (*sul1, sul2, sul3*, and *tet*(M)) was performed for natural assemblages and pooled colonies (Fig. 4). No difference was found between the four sampling sites, which consisted of three aquaculture sites and one non-aquaculture site. This result suggests that the ARGs were ubiquitously present in natural assemblages in this area (Fig. 4A). Data on natural assemblages (Fig. 4A) showed that *sul2* was detected consistently at levels of around 10^{-3} copies/16S from April to June, increasing in December to 10^{-2}, before subsequently decreasing in January to 10^{-4}. The *sul1* gene was detected in May, but was not detected in any other month. Similarly, the *sul3* gene was detected in April, May, and July at levels similar to those seen for *sul2* and *sul1*. Interestingly, *sul3* was detected at high copy number (10^{-3}-10^{-2}) in July. *sul3* has been reported to constitute a very minor *sul* among culturable enteric and environmental bacteria (Hoa et al., 2008; Su et al., 2011). Our previous research revealed that *sul3* was abundant in the total assemblage in Manila Bay seawater, but was not abundant in colony-forming bacteria in that environment (Suzuki et al., 2013). Since the occurrence profiles of *sul1, sul2*, and *sul3* do not match in the present study, the reservoir bacteria of these *sul* genes might be different. Notably, *sul1* and *sul3* were not detected in the colony-forming community through May to January (Fig. 4B), suggesting that the reservoirs of the *sul1* and *sul3* genes are non-culturable bacteria. The occurrence of *sul1* and *sul3* in the Japanese waters investigated in the present study may reflect the dynamics of the sea current in this area, since this area receives abrupt Kyucho (warm and oligotrophic water mass) and bottom intrusion (cold and nutritional water mass) currents from the Pacific Ocean during the summer season (Takeoka et al., 1993; Kaneda et al., 2002). These water movements yield rapid mixing and replacement of the seawater mass. Although the monitoring performed in the present study did not examine the Kyucho currents and bottom intrusions occurring during this research, such changes in the current structure are expected to have influenced the microbial community. The abundance of *sul2* through the year suggests that various bacterial communities possess this gene even if the sudden current occurs.

Occurrences of *tet*(M) in the natural assemblage were observed in almost all of the sites (Fig. 4A). The colony-forming bacterial communities also showed different profiles among sites and from the natural assemblages (Fig. 4B). These observations suggest that *tet*(M)-possessing bacteria are distinct from those possessing *sul* genes. In spite of the

fact that OTC was administered at sites 1-3, the detected *tet*(M) copy number did not differ greatly from that of the *sul* genes, suggesting that *tet*(M) was only partly responsible for OTC resistance; other *tet* genes are presumed to be contributing to the OTC^r. *tet*(M) is known to be a wide-host-range *tet* (Roberts et al., 2012), but other efflux genes also have been detected in bacteria isolated from this area (Kim et al., 2008). *tet*(M) decreased in December. This might due to that two months was long enough to reduce the effect of OTC in seawater for *tet*(M) possessing community. Similarly, reduction of resistant bacteria in December was reported in another aquaculture site (Nonaka et al., 2007).

As shown in Fig. S2, culturable OTC^r and SMX^r bacteria were negative for *sul3*, suggesting that the culturable bacteria did not possess *sul3*. This finding is similar to that obtained in the Philippines, where results showed that the non-culturable assemblage serves as a reservoir for *sul3* (Suzuki et al., 2013). Taking into consideration the current evidence, we infer that the *sul3* gene is abundant in the non-culturable community but rare among culturable bacteria in the coastal sea. Newly found *sul4* is reported in deep sea bacteria (Razavi, et al., 2017), which reminds us that *sul3* and *sul4* are marine bacteria origins.

3.3. ARG profiles in Taiwan and Finland

In the aquaculture environment, *tet*(M) has been detected frequently, not only in Japan (Nonaka et al., 2007) but also in freshwater aquaculture water in the USA (Seyfried et al., 2010). The *sul1* and *sul2* genes are ubiquitous in sediment from Finnish aquaculture areas (Muziasari et al., 2014). The *sul* and *tet*(M) genes targeted in the present study appear to already be ubiquitous in the Japanese aquaculture area due to the long history of use of the corresponding antibiotics. To test the presence of these genes at other geographic areas, we collected water samples from Taiwan and Finland aquaculture sites, although time course sampling throughout the year was not performed for these other sites. ARG profiles in both Taiwan and Finland aquaculture sites varied, and results differed between 2012 and 2013 (Figs. S3 and S4). In the Taiwanese case, since the fish are exchanged every year and seawater flowed constantly through the ponds, the occurrences of ARGs were expected to change over time. However, Taiwanese aquaculture water in 2013 showed that *sul3* was present in the natural assemblage but not among colony-forming bacteria. This pattern was the same as that detected in Japan in the present study (Figs. 4 and S2) and in the Philippines seawater in a previous study (Suzuki et al., 2013). In

384 contrast, the Finnish natural assemblage showed lower detection rates for all of the genes
385 that were observed in Japan and Taiwan (Fig. S4), whereas in Finland the culturable
386 bacteria were positive for *sul2* (10^{-4}) and SMX^r bacteria possessed higher copy numbers
387 (10^{-3}–10^{-1}) of *sul2* and *sul1* in 2012, but not in 2013 (Fig. S4 B and D). These results
388 suggested that the targeted ARGs are not abundant in the Baltic aquaculture waters and
389 that the Finnish ARG profile is changing with time. Research at the same sites in Finland
390 has detected *tet* and *sul* genes in the sediment (Tamminen et al., 2011; Muziasari et al.,
391 2014). These observations suggest that the ARGs in the Baltic Sea coast persist in the
392 sediment community but not in the water community. However, this persistence may be
393 caused by a constant influx of the genes via fish feces, meaning that these genes are
394 present via quasi-persistence rather than by true persistence in the sediment (Muziasari et
395 al., 2017). Comparison among countries reveals that the profile of *sul* genes and *tet*(M)
396 in Taiwan is similar to that in Japan, but differs from that in Finland.
397 Nonmetric multidimensional scaling (NMDS) was performed to visualize and visually
398 compare the resistance gene composition among different locations. NMDS reached a
399 two-dimensional low-stress solution (stress 0.0684), indicating a good representation of
400 the normalized resistance gene copy numbers in two dimensions. While the samples from
401 Japan, Taiwan, and Finland mostly clustered in separate regions of the graph, the NMDS
402 analysis also identified certain samples where the Taiwan or Finland samples plotted to
403 positions overlapping with the samples from Japan. However, the overlapping positions
404 had no obvious shared characteristics (Fig. 5), although Finnish samples typically plotted
405 to positions that were separated from those for Japan and Taiwan. Notably, the samples
406 from Finland and Taiwan plotted to widely different positions on the graph. Finland
407 samples had overall lower levels of antibiotic resistance genes than did the samples from
408 Japan and Taiwan. Most Taiwan samples were characterized by an elevated number of
409 *sul2* genes, while a considerable proportion of the Japanese samples had an elevated
410 number of *sul3* genes. Thus, the present study suggested that the seawater in aquaculture
411 areas in the three countries have distinct individual characteristics. Notably, European
412 countries and Chile are performing salmonid aquaculture in the coastal open sea, whereas
413 most aquaculture in Japan and Taiwan employs fishes of the order Perciformes. Thus,
414 both environment and fish diversity may play a role in the selective retention of specific
415 classes of ARG.

4. Conclusions

Year-long monitoring in a Japanese marine aquaculture environment showed that *sul2* was present in the water throughout the year, suggesting ubiquitous persistence of *sul2* in this area. *sul1* and *tet*(M) were detected only intermittently in various months, possibly due to water mass exchange by the Kyucho current. *sul3* was detected in the seawater assemblage but not in cultured bacteria. This last finding is similar to results obtained in Taiwan and the Philippines (Suzuki et al., 2013), where *sul3* appeared to be associated primarily with the non-culturable community in coastal seas. Sulfonamides and OTC have been used widely in aquaculture in Japan, allowed wide and constant persistence of *sul* genes and *tet*(M) in seawater. The ARG profiles from Japan, Taiwan, and Finland showed distinct patterns, with that in Finland differing more than did those in Japan and Taiwan. The occurrence of ARGs in aquaculture sites may be affected by the history of drug usage, the cultured fish species, and seawater exchange.

Competing interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at xxxx

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621
622
623 **Figure legends**
624 **Fig. 1.** Map of sampling sites in the Uwa Sea, Japan. Numbers with squares indicate the
625 sites, where 1-3 were net-pen sites, and 4 was the far (non-aquaculture) site. Net-pen size,
626 water depth, and distances are indicated in the figure.
627
628 **Fig. 2.** Water temperature at the sampling time (10:00 AM), history of antibiotic
629 administration, and cumulative amounts of feed administered in net-pens 1-3. Symbols:
630 square, water temperature at net-pen site; circle, water temperature at far site; bar graph,
631 cumulative feeding amount (kg); arrow, administered amount of oxytetracycline (OTC)
632 (kg) and period. Waters in September and October were used for the CARD-FISH
633 experiment, but not for ARG detection.
634
635 **Fig. 3.** Bacterial cell numbers in each month. Symbols: dotted bar, total cell number by
636 DAPI counting; open bar, total colony-forming number; hatched bar, OTC^r colony
637 number; shaded bar, SMX^r colony number. Data are plotted as mean ± standard deviation
638 across sites 1-4. Percentage at each bar is OTC^r and SMX^r colony % of total colony-
639 forming number.

640

641 **Fig. 4.** Copy numbers of antibiotic resistance genes in each month in total seawater
642 assemblage (A) and pooled colonies (B). Samples were collected in net-pen sites 1, 2,
643 and 3, and at the far site. Symbols: open circle, *sul1*; closed circle, *sul2*; square, *sul3*;
644 triangle, *tet*(M). Vertical bar in each symbol shows standard deviation within triplicate
645 samples collected at each site. Comparisons of *sul2* copy number by t-test revealed
646 significant ($p < 0.05$) increases from June to July and from July to December, and
647 significant ($p < 0.05$) decreases from December to January in panel A. Significant
648 different ($p < 0.05$) decreases were observed from July to January in panel B. Date in
649 each month is same as in Fig. 3.

650

651 **Fig. 5.** MDS analysis of total data from Japan, Taiwan, and Finland. Samples are
652 represented by dots and genes by arrows. Symbols: red dot, Japan; blue dot, Taiwan;
653 green dot, Finland. The arrows point in the direction of maximal variation in the gene
654 abundances, and arrow lengths are proportional to their maximal rate of change.

655

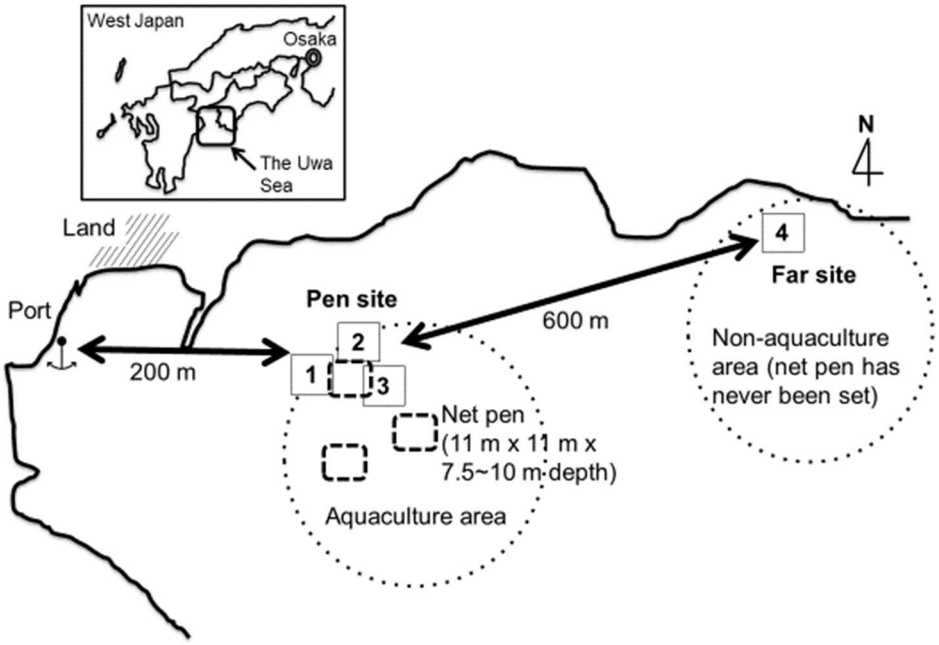


Fig. 1. (Suzuki et al)

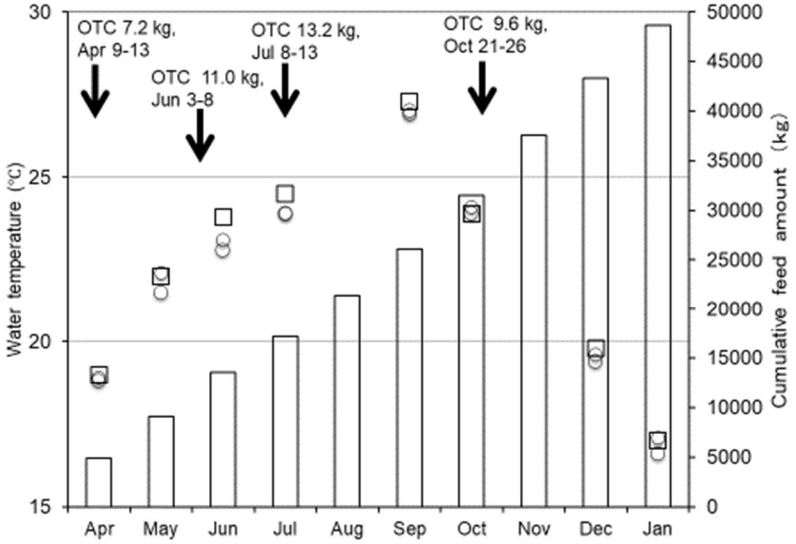
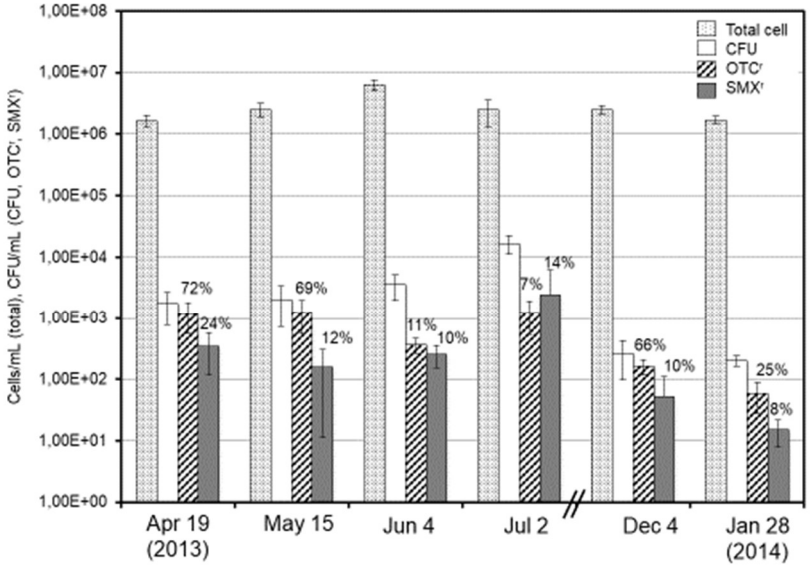
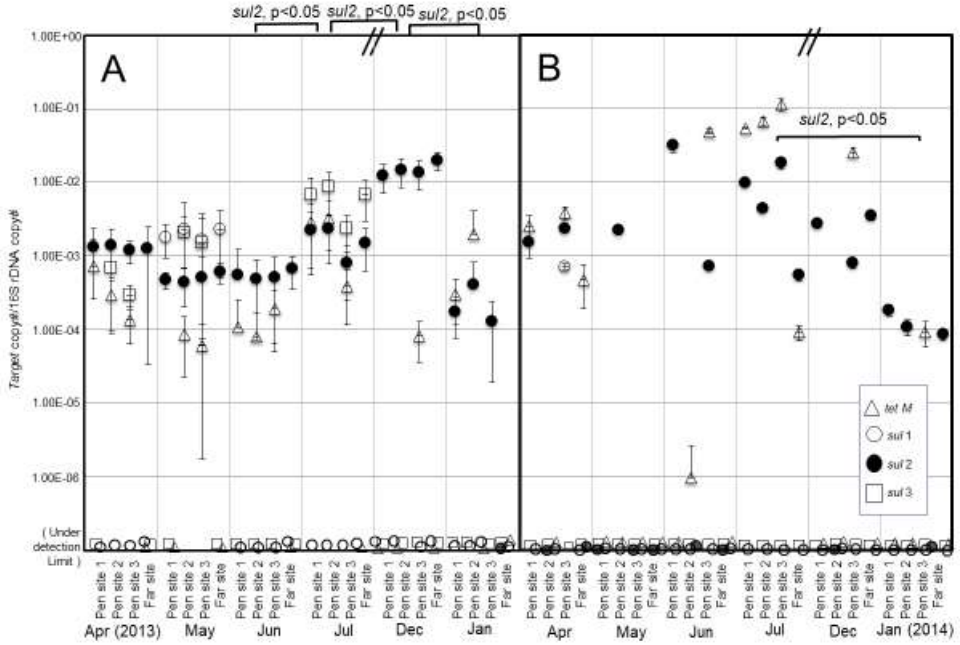


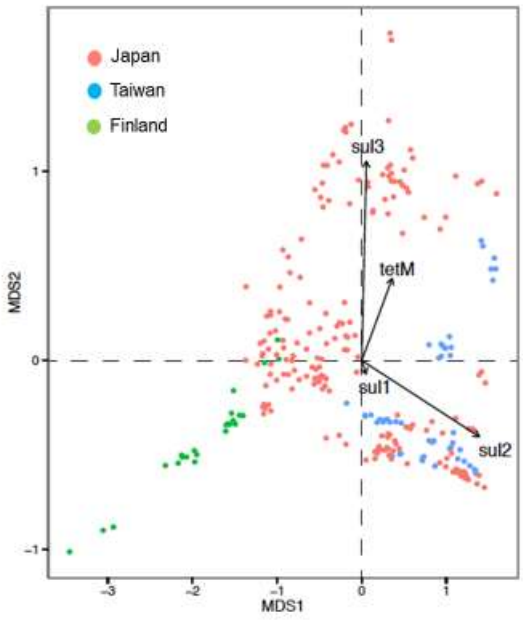
Fig. 2. (Suzuki et al)



658 Fig. 3. (Suzuki et al)



659 Fig. 4. (Suzuki et al)



660 Fig. 5. (Suzuki et al)