Original Article

**Effect of Sedimentation and Aeration on Antibiotic Resistance Induction in the Activated Sludge Process**

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**ABSTRACT**

The influent of municipal wastewater treatment plants (WWTP) can contain micropollutants such as antibiotics, chlorine, detergents, and biocides. *In vitro* studies have shown that these micropollutants may induce antibiotic resistance in bacteria. Previous studies have reported increases or decreases of antibiotic-resistant bacteria between the influent and effluent of WWTP in an unpredictable manner. Thus, the triggers of resistance induction in WWTP are largely unknown. To investigate the effects of unit operations in WWTP on antibiotic resistance induction, we incubated sixteen strains of *Escherichia coli* susceptible to amoxicillin or norfloxacin under simulated conditions of the primary sedimentation tank, aeration tank and final sedimentation tank in sterilized and filtered wastewater from each tank at 25°C for 1, 6 and 2 hours, respectively, which are typical hydraulic retention time of each tank. The minimum inhibition concentration towards amoxicillin or norfloxacin was compared before and after incubation to evaluate the occurrence of induction. We found that resistance to both antibiotics was more likely to increase in the aeration tank than in the primary sedimentation tank or final sedimentation tank. The longer contact time with the wastewater and the aeration are factors that appeared to induce antibiotic resistance in an activated sludge process.

**Key words:** wastewater treatment plant, aeration, sedimentation, minimum inhibition concentration, antibiotic resistance induction

**INTRODUCTION**

Antibiotic resistance has become a major threat globally, especially in healthcare settings because it prevents curing once treatable infections that are becoming untreatable [1]. Globally, it is estimated that 700,000 deaths may be caused by resistance to antibiotics each year [2]. Recently, WHO released a priority list for development of new antibiotics against several antibiotic-resistant bacteria (ARB), where resistant *Escherichia coli* is a top priority [3]. *E. coli* may cause urinary tract infections, and fluoroquinolones which were used to treat this infection are now becoming ineffective [4].

Not all antibiotics that are administered therapeutically will be absorbed completely. About 10 – 90% of the antibiotics will be excreted out of the body unchanged or as metabolites through urine, feces, and perspiration [5]. For instance, about 30 – 40% of administered norfloxacin is absorbed, ~30% excreted unchanged through the urine, and 30% through feces [6, 7]. Eventually, the excreted antibiotics will enter a wastewater treatment plant (WWTP). Studies analyzing the concentration of antibiotics in WWTP have been summarized in Kummerer [5] and the concentration in the effluent has been shown to be in the range of low ng/L up to 6 µg/L. Quinolones were found at concentrations of 425 – 532 ng/L for levofloxacin and 155 – 514 ng/L for norfloxacin in two WWTPs in Kyoto, Japan [8], a concentration range that is found to induce resistance in bacteria [9–11].

Generally, wastewater from municipalities is high in nutrients, and may contain traces of antibiotics, chlorine,
detergents, household biocides and other pharmaceuticals. These characteristics of wastewater and processes applied in WWTP are suspected to help enhance the generation and replication of ARB. In vitro studies showed that detergent, antibiotics, chlorine and biocides may induce antibiotic resistance in bacteria and select for resistant strains [10, 12–15]. However, whether, how and which processes in WWTP may induce resistance need further investigation. Some studies showed that ARB and antibiotic-resistant genes (ARG) increased or decreased in an unpredictable manner between influent and effluent [16–18], or remained unchanged along the course of water treatment processes [19].

About 90% of municipal WWTP in the world uses the conventional activated sludge process especially in developing countries where the administration of antibiotics is somewhat loose. The conventional process consists of sedimentation and aeration steps, and depending on the necessity, may also include a disinfection step such as chlorination and ultraviolet light. The objective of this study is to investigate whether processes in conventional activated sludge WWTP induces resistance in bacteria. We investigated the effects of unit operations in WWTP on induction of antibiotic resistance against two different antibiotics in WWTP by simulating processes in an activated sludge process WWTP.

Wastewater sample collection

Influent of the primary sedimentation tank, mixed liquor from the aeration tank (activated sludge) and effluent of the final sedimentation tank were collected from a municipal WWTP located in Kanazawa City, Japan. The conventional activated sludge process is used in this WWTP, which consists of primary sedimentation, aeration, secondary sedimentation, and then the treated water is subjected to chlorination. The samples for antibiotic analysis were stored in Schott bottles which were prepared beforehand according to EPA Method 1694 [20], while samples for induction experiments were stored in Schott bottles pre-sterilized by autoclaving at 121°C for 20 min. All samples were stored in the dark all the time and iced during transportation to the laboratory, and kept at 4°C before being used.

Antibiotic analysis

Samples of the influent of the primary sedimentation tank and the activated sludge were autoclaved immediately after arrival at the laboratory. Aqueous and solid fractions were separated by filtration using GF/B Whatman paper (GE Life Sciences, Little Chalfont, UK) and then analyzed separately for fluoroquinolones. Extraction of antibiotics was done according to EPA Method 1694 [20]. SPE-HLB cartridges (Waters, Milford, USA) were used to extract the antibiotic fraction from the samples. The extracted antibiotics in the SPE cartridge were eluted with HPLC-grade methanol. The eluted component was then concentrated to near dryness and diluted with HPLC-grade acetonitrile:water (5:95, v/v) with 0.1% formic acid. The mixture was analyzed qualitatively using a 2-point calibration in LC-MS/MS (U3000 and TSQ Quantum Discovery Max, Thermo Fisher Scientific, San Jose, USA).

Materials and Methods

We exposed amoxicillin-susceptible strains to amoxicillin at sub-inhibitory concentrations, added to sterilized wastewater samples taken from the primary sedimentation tank, aeration tank, and final sedimentation tank in order to simulate the environments of WWTPs. We also investigated the possibility of resistance induction at ambient concentration of norfloxacin in the sterile wastewater for E. coli strains that are susceptible to norfloxacin.

E. coli strains

All strains used were previously isolated from the mixed-liquor samples of aeration tank collected seasonally from the WWTP [21]. After appropriate dilution of samples with saline water, the samples were filtered using 0.45-μm cellulose-acetate membrane filters (37 mm Monitors, Advantec Toyo, Niigata, Japan). The filters were then placed on Chromocult® Coliform Agar ES (Merck KGaA, Darmstadt, Germany), and incubated at 37°C for 24 h. Blue-colored colonies were isolated from the filter, incubated in Tryptic Soy Broth (Eiken Chemicals, Tokyo, Japan) at 37°C overnight, and then characterized for resistance to four classes of antibiotics: ampicillin (a type of β-lactams); ciprofloxacin and norfloxacin (types of fluoroquinolones); kanamycin and

streptomycin (types of aminoglycosides); and tetracycline (a type of tetracyclines) by disc diffusion test [22].

Two wild-type E. coli strains susceptible to amoxicillin and sixteen strains susceptible to norfloxacin were used for the induction experiment. The strains were randomly chosen out of 30 and 76 strains from strains susceptible for amoxicillin and norfloxacin, respectively.

**Induction experiment**

We simulated the conditions of (1) nutrition, (2) retention time, (3) aeration and mixing in the primary sedimentation tank, aeration tank and final sedimentation tank. We conducted two experiments: (1) induction of resistance to amoxicillin under simulated conditions of the primary sedimentation tank, aeration tank and final sedimentation tank; and (2) induction of resistance to norfloxacin under simulated conditions of the primary sedimentation tank and the aeration tank. In the first experiment, two strains labelled C1 and C2, susceptible to amoxicillin were used. The strains were incubated in test tubes filled with autoclaved unfiltered water taken from the primary sedimentation tank, aeration tank and final sedimentation tank, respectively. Amoxicillin was added to the cultivation water to create a series of sub-inhibitory concentrations of amoxicillin at 0.1, 1, 10, 100 and 1,000 ng/L. In the second experiment exploring norfloxacin resistance induction, 16 strains susceptible to norfloxacin were used. The strains were incubated in test tubes filled with wastewater taken from the primary sedimentation tank and the aeration tank, respectively. Before using the wastewater for cultivation, we autoclaved and then filtered the water with 0.2 µm cellulose-acetate membrane (Advantec Toyo). In this experiment, we did not add any antibiotics to the cultivation water. The tubes were incubated at 25°C, which was the mean summer temperature inside tanks in the WWTP. For both experiments, to simulate the process in the primary sedimentation tank and the final sedimentation tank, the tubes were let stand vertically for one and two hours, respectively, while to simulate the aeration tank, the tubes were placed at a 25° angle in a shaker and were shaken at 225 rpm for 6 h.

Before starting the above induction, the initial concentration of each strain was adjusted in the following manner. Frozen stocks of the strains were thawed and propagated in MH-broth (BD Co. Sparks, Grenoble, France) at 37°C for 18 h. The strain cultures were diluted and cultured on Mueller-Hinton-agar (BD Co. Sparks) plates at 37°C for 18 h. Morphologically uniform separated colonies were picked, suspended in saline water, and adjusted to match a 0.5 MacFarland turbidity standard which is equal to $1 - 2 \times 10^8$ cfu mL$^{-1}$ [23]. The adjusted culture was then diluted with cultivation water from WWTP to achieve $5 \times 10^6$ CFU mL$^{-1}$ in a total volume of 4 mL.

The minimum inhibitory concentration (MIC) towards amoxicillin or norfloxacin and the viable cell count were measured before and after the induction. We followed the cell counting protocol of Wiegand et al. [23], where before and after the induction, 10 µL of bacterial suspension was diluted in 990 µL of sterile saline, and then further diluted to 1:10. We plated 100 µL of the final dilution in MH-agar plate, and incubated the plates at 37°C for 18 h. The viable cells were counted afterwards. Note that for the induction experiment for amoxicillin, the viable cell counts were only measured for some randomly selected treatments. We calculated fold change in MIC and in viable cell count by taking the ratio of the measurement after induction ($X_1$) to that before induction ($X_0$, Eq. 1). We consider induction to occur when MIC increases by 2 or more levels from the MIC before induction, i.e. the fold change is ≥ 2, while for the viable cell count, a significant increase occurs when there is a change in cell count of ≥ 1 order of magnitude.

\[
\text{Fold Change in } X = \frac{X_1}{X_0} \tag{1}
\]

where: $X$ = MIC or viable cell count

$X_1 = $ MIC or viable cell count after induction

$X_0 = $ MIC or viable cell count before induction.

**MIC measurement**

MIC was determined by the broth micro-dilution method following Wiegand et al. [23] using geometrically increasing concentrations of antibiotics ranging from 0.0625 to 32 mg/L. Stocks of serially diluted norfloxacin and amoxicillin (Wako Pure Chemicals, Mie, Japan) solution were prepared in Mueller-Hinton-broth (BD Co. Sparks) in 96-well microplates and were stored at −20°C until use. The microplates were inoculated with bacterial solution (1:1) from the test tubes before and after induction experiments. After incubation, the MIC is defined as the lowest antibiotic concentration (in mg/L) where 90% of the bacterial growth is inhibited based on OD$_{405}$. This wavelength was chosen based on maximum absorbance readings after scanning the microplates (Model 680 microplate reader, Bio-Rad, Hercules, USA).
RESULTS AND DISCUSSION

Antibiotic concentration in the wastewater

Our analysis showed that the range of quinolone concentrations in wastewater is similar to other studies [8, 24, 25]. The concentration of fluoroquinolones was much higher in the solid fraction than in the aqueous fraction, and also was higher in activated sludge than in the influent of the primary sedimentation tank (Table 1). After sterilization by autoclaving, the fluoroquinolone concentrations tended to increase, particularly for activated sludge (up to 10 times) indicating a release of the antibiotics from the solid fraction to the liquid fraction. Lindberg et al. [26] and Wang et al. [27] also found the tendency of norfloxacin to be accumulated in sludge particles. While the negative values of the log octanol-water partitioning coefficient (logKow) of the examined quinolones in the literature (e.g. logKow of ciprofloxacin: −1.10, levofloxacin: −0.42, lomefloxacin: −1.13, nalidixic: 0.15, norfloxacin: −0.95, ofloxacin: −0.47) indicate that they are hydrophilic [27–29] our results showed that using logKow alone is inadequate to explain the higher concentration of quinolones in the solid than in the liquid fraction of the wastewater. Mechanisms other than hydrophilicity are a better explanation for the fluoroquinolone sorption to sludge particles.

When mechanisms other than hydrophilicity are dominant, pH and redox condition at which the process condition occur can influence the sorption [27, 30]. At neutral pH, which is similar to typical wastewater pH of 6.5 – 7.5, sludge particles are dominated by negatively charged carboxyl groups [31]. On the other hand, at this pH range, the fluoroquinolones are positively charged or neutral because the fluoroquinolones first pKa value and the isoelectric point are between this pH range [27]. In this pH range, zwitterions of the fluoroquinolones have been found to have the strongest affinity to sludge particles than either the cation or anion, thus contributing the largest portion of chemical species adsorbed in the sludge [27].

Antibiotic resistance induction in WWTP

We found that in both induction experiments, either for increasing concentrations of amoxicillin, or in ambient concentration of norfloxacin, the aeration tank conditions showed the highest MIC increase. During the induction to amoxicillin, an increase in MIC was observed from the initial concentration in both strains, and in all simulated processes the highest increases occurred (i.e. MIC ≥ 32 mg/L) in the aeration tank conditions, and no change in the simulated final sedimentation tank was observed (Fig. 1). Similar to the induction to amoxicillin, induction to norfloxacin showed that the simulated aeration tank conditions were more likely to result in an increase in MIC than in the simulated primary sedimentation tank conditions. In general, induction using the primary sedimentation tank condition did not change MIC, and in one strain, the level decreased from 2 to ≤ 0.0625 mg/L (Fig. 2a). On the other hand, induction using aeration tank conditions resulted in unchanged MIC levels in ten strains, but six strains showed an increased level of

Table 1 Quinolone concentrations in liquid (ng/L) and solid (µg/kg) fractions in wastewater samples and the limit of quantification.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sample fractions</th>
<th>Before sterilization</th>
<th>After sterilization</th>
<th>Limit of Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Influent of Primary sedimentation tank</td>
<td>Mixed liquor of aeration tank</td>
<td>Influent of Primary sedimentation tank</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>aqueous</td>
<td>&lt; 40</td>
<td>&lt; 40</td>
<td>&lt; 40</td>
</tr>
<tr>
<td></td>
<td>solid</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>nalidixic acid</td>
<td>aqueous</td>
<td>6</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>solid</td>
<td>8</td>
<td>9</td>
<td>40</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>aqueous</td>
<td>&lt; 40</td>
<td>&lt; 40</td>
<td>&lt; 40</td>
</tr>
<tr>
<td></td>
<td>solid</td>
<td>100</td>
<td>&lt; 100</td>
<td>500</td>
</tr>
<tr>
<td>lomefloxacin</td>
<td>aqueous</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
<tr>
<td></td>
<td>solid</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>ofloxacin+levofloxacin</td>
<td>aqueous</td>
<td>300</td>
<td>600</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>solid</td>
<td>&lt; 40</td>
<td>500</td>
<td>2,000</td>
</tr>
</tbody>
</table>
These results showed that resistance to both of the antibiotics we tested may be induced during process operations in WWTP, and the tendency of resistance to be expressed and/or to increase is more likely to happen in the aeration tank than in the primary sedimentation tank or in the final sedimentation tank. The conditions in the final sedimentation tank tend to cause no induction. However, further tests using more strains will be needed to generalize the results for the final sedimentation tank process, as we only tested two strains.

We propose that contact time and aeration might be the main factors explaining the tendency of induction in the aeration tank compared to the other tanks. For instance, in the induction to norfloxacin, although sub-inhibitory concentrations of the quinolones and fluoroquinolones in the water...
samples might induce resistance, we cannot be sure that the concentration levels of quinolones and fluoroquinolones in both types of water samples, where the highest concentration was 100 ng/L for norfloxacin, and 2,000 ng/L for others, was sufficient to promote induction within the time frame tested. To the best of our knowledge, the lowest antibiotic concentration employed for induction was 15,000 ng/L [10] and 230 ng/L to select for resistance [9], and 10,000 ng/L for plasmid transfer via plate mating [32]. In the first two studies, the incubation time applied was 24 h, and for the latter was 16 h.

Fig. 2 Fold change in MIC for induction experiment to norfloxacin in (a) primary sedimentation tank, (b) aeration tank. Fold change in MIC is calculated based on geometric mean of MIC determined after induction, divided by MIC determined before induction. n = 4, sd range = 1.0 – 1.2, sd mean = 1.0. Fold change in MIC ≥ 2, induction occurs.
It is likely that the longer contact time in activated sludge is one factor that produced significant change in MIC level in some strains after incubation.

We also notice that not all strains responded the same to the induction procedure, not even between strains that had the same initial MIC level. For example, in the induction experiment to amoxicillin, strain C2 showed a higher fold change increase in MIC than strain C1 (Fig. 1), meaning that C2 exhibited stronger resistance than C1. For the induction experiment without addition of antibiotics, strains B11 and C10 had the same initial MIC level of 1 mg/L. However, after induction in the activated sludge treatment, the MIC level of C10 increased to ≥32 mg/L, while strain B1 remained the same. Growth rate is thought to be one of the factors affecting horizontal gene transfer in *E. coli* [33], and it is generally observed that slow growth produces a more resistant bacterial strain [34]. In the induction to norfloxacin simulating aeration process the lower fold change in the viable cell count in C10 compared to B11 suggested that C10 had lower growth rate than B11.

The characteristics of wastewater and processes applied in WWTP may select for resistant bacteria and enhance their growth compared to non-resistant strains [10, 12–15]. We measured viable cell count of the strains before and after induction. In the first induction experiment, the viable cell count in the primary sedimentation tank and the final sedimentation tank did not change from the initial, in contrast to the aeration tank where besides the sharp increase in MIC in both strains, the viable cell count of both strains also increased up to two orders of magnitude (Fig. 3). However, we cannot make a firm generalization from the viable cell count data in this simulated aeration process, as this might be a coincidence. The viable cell count data we measured were the ones that showed high fold change in MIC (≥5) (Fig. 3b). Furthermore, the simulated aeration tank conditions used in the induction to norfloxacin where three strains, C1, C10 and D13, showed increases in MIC (Fig. 4b), only strain C1 showed a significant increase in the viable cell count (Fig. 4b). For the other two strains, the viable cell count was either constant, or reduced. Overall, there was no clear pattern that we can deduce for the viable cell count after induction in both simulated processes as the cell number increased or decreased in an unpredictable manner (Fig. 5a and 5b). No correlation was found between fold change in MIC and the viable cell count as can be seen in Fig. 4a and 4b.

Other triggers might affect induced resistance against antibiotics than the availability of antibiotics. In the induction experiment to amoxicillin, all control treatments without addition of amoxicillin behaved similarly to treatments with addition of amoxicillin in terms of MIC change (Fig. 1a and 1b). Thus, there are other factors than the added amoxicillin that might have a larger effect on MIC. The ambient concentration of amoxicillin or other β-lactam antibiotics which possibly cause cross-resistance might already be elevated in the wastewater. Thus, the added amoxicillin may have had little or no influence compared to the already available concentration of amoxicillin in the wastewater.

We consider that one of the possible mechanisms to ex-
plain the antibiotic resistance induction in our results is the presence of intrinsic resistance (e.g. efflux pumps). Bacteria may acquire resistance to antibiotics via two main pathways: (1) mutations, and (2) uptake of genes that encode resistance through horizontal gene transfer. Bacteria in WWTP face harsh conditions because many potential toxins (e.g. antibiotics, detergents, biocides) are present. Efflux pumps are ubiquitous in bacteria, and are intrinsic to Gram-negative bacteria. They can exude many kinds of substrates. An over expression of efflux pumps might be the cause of different levels of resistance. The efflux pumps encoded by AcrAB-TolC and EmrEF have been found to exude biocides and different classes of antibiotics, and have been related to multidrug resistance in E. coli [35–38]. Indeed, Sato et al. [39] found that an over-expression of AcrAB-TolC along with mutations of quinolone-resistant determinant regions was related to higher levels of fluoroquinolone resistance compared to strains with a low level of gene expression for efflux pumps. It could be that the strains that showed increased resistance to norfloxacin in our study experienced conditions leading to increased expression of efflux pump genes. Further investigation is needed to demonstrate if this mechanism was the case.

We do not think uptake of genes is a likely mechanism to explain our results. In natural settings, extracellular DNA exists in aquatic environment due to cell lysis or excretion [40] and WWTPs contain a plethora of mobile genetic elements, such as plasmids [41, 42] that increase the chance uptake of DNA for protection against e.g. antibiotics. E. coli is one of the Gram-negative bacteria that is naturally competent for transformation [43, 44], but the competence is time limited [45]. Again, a longer contact time will increase the probability of uptake. However, Zhu et al. [46] showed degradation of DNA to undetectable levels in 0.22 μm filtered-stereilized environmental water samples within 48 – 96 h. The wastewater samples that we used were autoclaved and filtered using 0.2 μm and then stored at 4°C until use, where the storage time exceeded 4 d. Thus, the potentially available resistance genes may already have degraded.

In the real-world setting of a WWTP, bacteria face a constantly changing set of environmental conditions (e.g. chemical fluctuations of the incoming influent, changes in the operational conditions as the bacteria are transported along the operational sequence) that would require them to adapt constantly. Phenotypic switching is another strategy for adaptation instead of costly genetic alterations [47, 48] and it has been suggested that short-term adaptations (minutes to hours) are purely phenotypic [34]. Thus, resistance level can increase in WWTP because of phenotypic change. Our finding that the strains showed a variable response upon induction may indicate the use of the phenotypic switch strategy. The existence of a sub-population that has multiple phenotypes that can be switched on and off in response to the changing environment helps guarantee a higher chance of survival for the entire population [49].

Another possible factor that could explain our result that the antibiotic resistance induction is more likely to happen in the aeration tank is oxidative stress. Oxygen radicals are produced naturally within bacterial cells as side products during cell respiration and are ubiquitous in the environment [50]. To reduce the deleterious effect (e.g. promote DNA and membrane damage) of these radicals, bacteria have defense mechanisms that change the species to become inactive or by reducing the radical concentrations. When the concentration of these radicals reaches the limit of this mechanism, this
could lead to cell damage and death. We speculate that the relatively high shaking speed that was used on tubes designated for the simulated aeration process provided significantly higher oxygen than the tubes designated for simulating the sedimentation process that were left sitting upright without any shaking, even though the duration of the experiment for

**Fig. 5** Fold change in viable cell count for induction experiment to norfloxacin in (a) primary sedimentation tank, (b) aeration tank. Fold change in viable cell count is calculated based on viable cell count determined after induction, divided by viable cell count determined before induction. The coefficient of variation (cv) for the viable cell count = 19 – 43% (n = 3). Note that not all of the viable cell count measurements were determined in triplicates.
the simulated sedimentation process was shorter. Further experiment is warranted to test our argument.

Furthermore, norfloxacin and ampicillin, a β-lactam antibiotic, have been found to induce the production of oxygen radicals in bacteria under aerobic conditions, but the effect was reduced to nearly background level when the condition was anaerobic [10]. However, the effect of oxygen concentrations depends on bacterial life state, i.e. active versus dormant/stationary state, and the antibiotic action mechanisms. Kim et al. [51] showed that cells of *P. aeruginosa* grown aerobically up to the stationary state were more tolerant to chlorine, a strong oxidizing antimicrobial agent, while dormant cells were more tolerant to the antibiotic tobramycin.

**CONCLUSIONS**

Our findings showed that induction for antibiotic resistance might happen during operation processes in WWTP, and the likelihood for this to occur is higher in the aeration tank than in the primary sedimentation tank. We suspect that the longer contact time and the aeration process are the main factors that facilitate the induction. The probability of individual strains of bacteria to be induced might be dependent on functional traits of each strain. We speculate that in a WWTP environment where multiple toxic substances to bacterial growth co-exist, expression of efflux pumps might also be an important factor for antibiotic resistance induction than uptake of genes. Further investigation is needed to confirm and elucidate these mechanisms.

**ACKNOWLEDGEMENTS**

This study is supported by Japan Society for the Promotion of Science (JSPS) through Grant-in-Aid for Scientific Research (B), (Grant Nos.: 26289180, 26281037). Sulfikar is financially supported by Indonesian Ministry of Higher Education and Research and Technology. Authors thank Kanazawa City Council for their cooperation and assistance during sampling, and Sen Yang, Ryo Aizawa, Xu Rui for their help in taking samples.

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