EFFECT OF NORFLOXACIN ON ANTIBIOTIC RESISTANCE OF ESCHERICIA COLI: COMPARISON BETWEEN SEQUENCING BATCH REACTOR AND SEQUENCING BATCH MEMBRANE BIOREACTOR

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Abstract

Seven antibiotics including norfloxacin (NOR) were tested via disk susceptibility test on E. coli culture isolated from the MLSS of the two types of lab-scale sequencing batch reactors (SBR): a common SBR and an SBR with microfiltration membrane (SB-MBR) for treatment of synthetic municipal wastewater. The same experiment treating the NOR-added wastewater to examine the possible induction of resistance to itself and the other antibiotics. The MLSS from Bangkok's municipal wastewater treatment plant was employed as an initial seed and the susceptible E. coli culture (TISTR780) were spiked daily into both reactors. The reactors were continuously operated under 2hr/2hr of aeration/non-aeration cycle and resistances to antibiotics of E. coli in MLSS were monitored. When NOR was not added, the SB-MBR showed lower percentages of resistant E. coli than the SBR did to amoxicillin/clavulanic acid, amikacin, nalidixic acid, tetracycline and chloramphenicol. Oppositely, the SB-MBR treating the NOR-added wastewater appeared to promote resistances of E. coli to nalidixic acid, sulfamethoxasole and tetracycline probably due to a long SRT and low DO compared to that of SBR. Although its mechanism should be analyzed with molecular techniques in further studies, this NOR-induced expression of resistance resulted in a higher occurrence of multidrug resistant E. coli in the SB-MBR than that in the SBR.

Keywords: Antibiotic resistance, Escherichia coli, Membrane bioreactor, Sequential batch reactor, Wastewater treatment

Introduction

Wastewater treatment plant is the most important facility to remove impurities and eliminate pathogens from human excreta before releasing into natural water sources [1]. Treated wastewater is usually discharged into surface water in most countries, ubiquitous distribution of human enteric microorganisms in water environment has not been avoided. With increase in utilization of antibiotics worldwide, recently, domestic wastewater has been claimed to be one of the important source of surface water contamination with

antibiotic resistant bacteria [2]. For example, increase in antibiotic resistance of E.coli was reported in a river receiving wastewater [3]. As researches in many countries [2, 4, 5, 6] including Thailand [7] demonstrated the existence of E. coli resistant to one or more antibiotics in municipal wastewater and its treatment systems, behavior of antibiotic resistant bacteria in the treatment processes is critical in discussing about its prevalence and distribution in water environment. Although researchers had conducted such investigations based on the monitoring at the real treatment plants [2,4], there are a limited number of reports [9] which tried to find significant factors for prevalence of antibiotic resistant bacteria under a well-controlled condition in lab-scale reactor and operational condition.

This study has two objectives. One objective is to observe changes in antibiotic resistance of E. coli in activated sludge during municipal wastewater treatment by sequencing batch reactor (SBR) and sequencing batch membrane bioreactor (SB-MBR) under periodical aeration. Aeration was controlled as a factor expected to affect resistance of E. coli because it must influence selection of microbial species and activities. The other objective is to examine whether or not norfloxaxine (NOR) induces occurrence of resistance to itself and other antibiotics in the wastewater treatment process. The reasons why we selected NOR in quinolone group antibiotics among many groups found in municipal wastewater [10, 11] are that quinolone group is of special concern due to their low biodegradability in environment and in wastewater treatment plants [11] and that NOR is often used to treat certain bacterial infections of the genitals, bladder and the digestive system [12].

Methodology

Determination of NOR in Bangkok's Sewage Treatment Process

At a representative municipal wastewater treatment plant in Bangkok, NOR concentrations in the influent, the MLSS (solids and soluble) and the 2nd sedimentation tank effluent of conventional activated sludge (CAS) process were analyzed. The water sample was centrifuged (4000 rpm, 20 min) to separate from solids. The supernatant was filtered through a GF/C filter and acidified to pH 2.0 using the concentrated hydrochloric acid (HCl). Na_4EDTA was added into the filtrate in order to prevent a complex of antibiotics and residual metal ions. The mixture of 500 mL was filtered via a 200 mg Vertipak™ HCP in a 6 ml SPE cartridge [13]. For the solids of MLSS, they were extracted as described by EPA method 1694 [13]. The final extract was analyzed by the liquid chromatography/tandem mass spectrometry (LC/MS/MS). The mobile phase contained 1mM Ammonium acetate and acetonitrile. Separation was achieved using an Atlantis T3 2.1x100mm, 3um. The LC was coupled to the MS using electrospray in positive ion mode.

Setup of SBR and SB-MBR and their Operation

The schematic diagrams of the SBR and SB-MBR reactors are illustrated in Figure 1. Both reactors have the same rectangular reaction tank of 12.1 cm (width) × 30 cm (length) × 50 cm (depth) with an effective working volume of 16 L. The membrane module used in the SB-MBR was a hollow fiber micro-filtration made of polyvinylidenefluoride (PVDF) (Mitsubishi Rayon, Japan) with pore size of 0.45 μm and filtration area of 0.07 m^2. Prior to start-up the bioreactors, the MLSS from the activated sludge process at a municipal wastewater treatment plant in Bangkok, Thailand was aerated for about 1 week in the synthetic wastewater for acclimation to the wastewater substrates and then equally seeded to the SBR and SB-MBR reactors. The initial MLSS concentration of liquid and solid mixture in the reaction tank was controlled approximately 1,500-2,000 mg/L. The SBR and

SB-MBR were fed with the synthetic wastewater with a flow rate of 48 L/d and operated under 2 hr-aeration (mixing phase) and 2 hr-non aeration (settling phase) continuously. The hydraulic retention time (HRT) and sludge retention time (SRT) of SBR were 8 hr and 10 days, respectively, while the SB-MBR had the same HRT and a much longer SRT as it was operated under no sludge wastage condition. The SBR and SB-MBR were continuously operated in parallel for three months, conducting the batch treatment of the synthetic wastewater, to which NOR (0.01 mg/L) was added or not (as control), at the interval of four hours.

Synthetic Domestic Wastewater and Water Analysis

The synthetic wastewater components were: sucrose (226 mg/L), KH_2PO_4 (80 mg/L), NH_4Cl (60 mg/L), and a mineral solution containing $CaCl_2 \cdot 2H_2O$ (0.57 mg/L), $MgSO_4 \cdot 7H_2O$ (7 mg/L), $MnSO_4 \cdot H_2O$ (0.206 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.25 mg/L), $FeSO_4 \cdot 7H_2O$ (0.25 mg/L), and $NaHCO_3$ (100 mg/L) which simulated common domestic wastewater, was treated independently in the two reactors. In order to maintain the E.coli consistency in the bioreactors, the E. coli cells (TISTR780, Microbiological Resources Center, Thailand Institute of Scientific and Technological Research, Thailand) were spiked into the bioreactors to make up the E. coli concentrations to 3.5-5.5 log cell density (CFU/ml) of MLSS. This E. coli was tested for susceptibility and it expressed the sensitive to all 7 tested antibiotic disks in this study. For chemical properties, the water samples of both reactors, DO, pH, BOD, COD, TKN, NH_4, MLSS and MLVSS of the influent and effluent were analyzed at an interval according to the standard methods [14]. Besides, EPS (extracellular polymers) as protein and carbohydrate were analyzed for MLSS samples [15] of the SBR and SB-MBR.

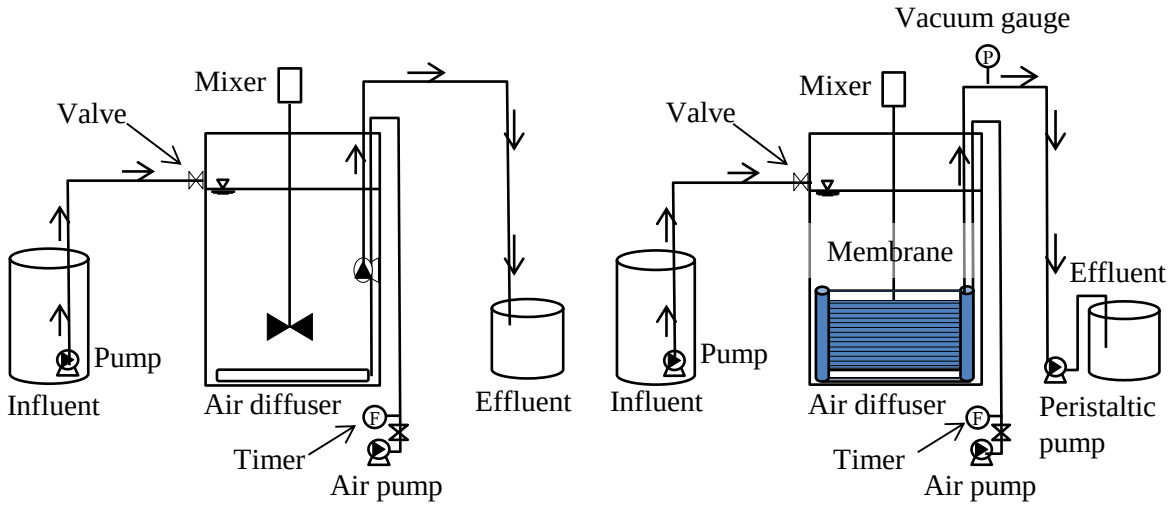
Batch Study of Norfloxacin Effecting on E.coli Growth and Resistance

The pure strain of E. coli TISTR780 was used to study the effect of NOR on growth of E. coli. The E. coli was re-grown in Tryptic soy broth (TSB) in a set of 200 mL flask at 35^{o}C for 24 hr to obtain about 4.5-5.0 log cell density (CFU/mL). Then the cultures were spiked with NOR solution to obtain different concentrations of 0.0, 0.01, 0.10, 1.0 and 10 mg/L. The standard microbial inhibition concentration (MIC) of NOR are in range of 0.03-0.12 mg/L for E. coli (ATCC 25922) [16]. The NOR solution was prepared by dissolving norfloxacin 200 mg/tablet (Siam Bheasach®) in the laboratory reverse osmosis water. The cultures were continuously and gently stirred on shaker for 24 hours. A small amount of the culture was pipetted every 4 hours in order to enumerate the vital cells by membrane filtration technique on Coliform Agar (Merck KGaA®). Finally, 20 colonies of E. coli was isolated and further examined for susceptibility to NOR (10 µg) by disk-diffusion method described in next section.

Isolation of E.coli and Antibiotic Resistance Test

E. coli was isolated from the MLSS of the CAS-WWTP at which seed sludge was taken in order to reveal its original antibiotic resistances. The 22 strains were tested for susceptibility to seven antibiotics as described below. Similarly, the samples of MLSS were taken from the reaction tanks of the SBR and SB-MBR twice a month and were diluted with 0.85 % sterilized NaCl solution to a desired level. The diluted samples were filtrated by 0.45 µm membrane filter (Millipore) and then the filter was placed on ChromoCult® Coliform Agar (Merck KGaA®). After incubation of the plates at 35 ^{o}C for overnight, 20 colonies of E. coli with dark-blue/violet color were identified, picked up and inoculated to Tryptic Soy

Broth (TSB) (HIMEDIA®). The broth was incubated at 35±2 ^{o}C until the turbidity reaches 0.5 McFarland standard. The broth after incubation was swabbed on Muller-Hinton (BBLTM BD) plate and then the Antimicrobial Susceptibility Testing Disks for the following six antibiotics were put on the plate: trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), amikacin (AK, 30 µg) and nalidixic acid (NA, 30 µg) for both experimental conditions. In addition, cephalothin (KF, 30 µg) and NOR (10 µg) were tested in the conditions without antibiotic addition and with NOR addition, respectively. For this susceptibility test to antibiotics except NA, BD BBL Sensi-Disc™ was used, while another disk (Oxoid™) was used for NA. After incubation of the plate at 35±2 ^{o}C for 18-22 hours, diameters of inhibition zone around the disks were measured using a ruler. Based on the measured diameters, susceptibility of E. coli isolates to each antibiotic was determined following the leaflets provided by the companies. A susceptible strain of E. coli TISTR780 (Microbiological Resources Center, Thailand Institute of Scientific and Technological Research, Thailand) were used for quality control.



(a) Sequencing Batch Reactor (SBR)

(b) Sequencing Batch Membrane Bioreactor (SB-MBR)

Figure 1. Schematic diagram of SBR and SB-MBR

Results and Discussion

Occurrence of NOR at CAS-WWTP in Bangkok

The LC/MS/MS analysis demonstrated that the NOR concentrations in influent, MLSS solids, MLSS liquid and effluent at the CAS-WWTP in Bangkok were 0.03 µg/L, 0.87 µg/kgTS, 0.05 µg/L and 0.02 µg/L, respectively. NOR concentrations in domestic wastewaters are reported in ranges of 0.22-0.54 µg/L which are treated in six WWTPs in China [11], indicating the very low concentration of NOR in Bangkok sewage. During the treatment process, NOR was mostly accumulated in the MLSS solids in the aeration tank. Adsorption onto the sludge is the dominant pathway of treatment for fluoroquinolones, including NOR, in the CAS process [17]. The adsorption of fluoroquinolones onto particles occurs mainly through electrostatic interaction, which is more effective with

activated sludge than with suspended solids in raw sewage [12]. However, an amount of NOR was desorbed from the MLSS solids as indicated by the higher concentration of NOR in the MLSS liquid than in the influent. This implies that NOR was rarely biodegraded in the CAS in Bangkok. In conclusion, NOR was slightly removed in sedimentation tank, resulted in its lower concentration in effluent than in influent. Overall removal efficiency of NOR at the CAS-WWTP was only 33.33 %. This efficiency is comparable to those (30 and 45%) reported in CAS processes treating saline sewage and freshwater sewage, respectively, at WWTPs in Hong Kong [18].

Antibiotic Resistance of E.coli Isolates in Seed Sludge

Figure 2 illustrates the resistance prevalence to 7 antibiotics of 22 E. coli strains isolated from the MLSS prior to be used as seed sludge for the lab-scale experiment. It appeared that the E. coli isolates highly resisted to NA, TE, AMC, SXT and NOR with resistance percentages from 79% up to 93%. Only 2% of E. coli isolates were sensitive to SXT and TE. Around 20% of E. coli isolates were sensitive to NOR and C and most isolates (83%) were sensitive to AK.

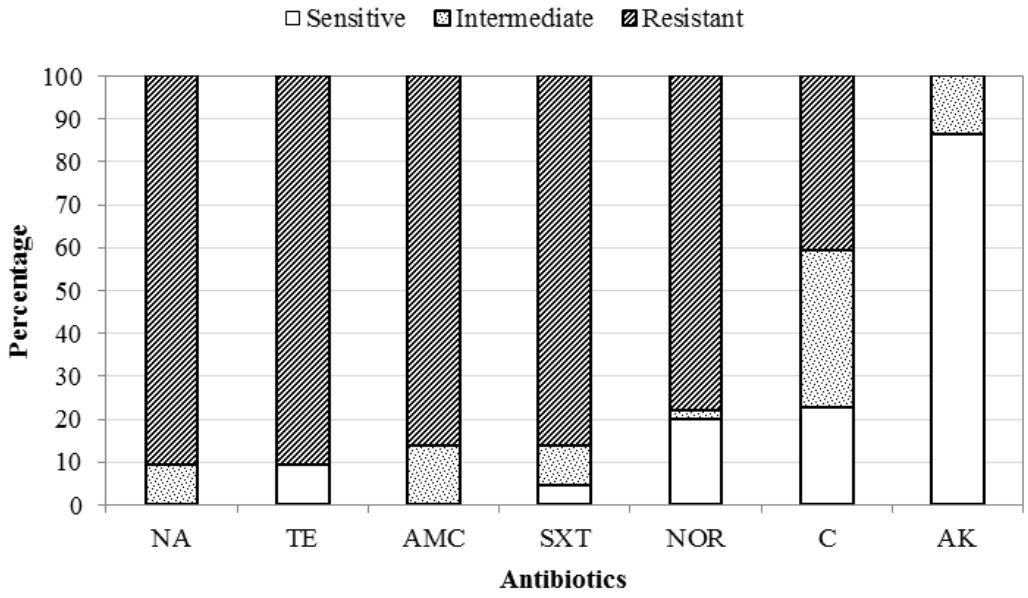


Figure 2. Resistances to various antibiotics of E. coli isolates from MLSS in aeration tank of CAS wastewater treatment plant in Bangkok

Effect of NOR on Growth of E.coli

Figure 3 illustrates growth of pure E. coli (TISTR 780) under different doses of NOR in batch experiment. Dose of 0.01 mg/L did not show any effects on E. coli growth. The growth curve of 0.01 mg/L which gave about 5.5-6.0 log cell density (CFU/mL), was little different to the control. The growth curves at other higher doses (0.1, 1.0 and 10 mg/L) showed the trend of decrease with time and the E. coli concentration reduced from 4.5-5.4 to 2.6-3.1 log cell density (CFU/mL) within 24 hours of the culture period. Thus, in bioreactor experiment, we selected the NOR dose of 0.01 mg/L to investigate the changes in antibiotic resistance of E. coli in the SBR and SB-MBR. All doses, we did not find any clear trends in inhibition diameter within the short time of 24 hours.

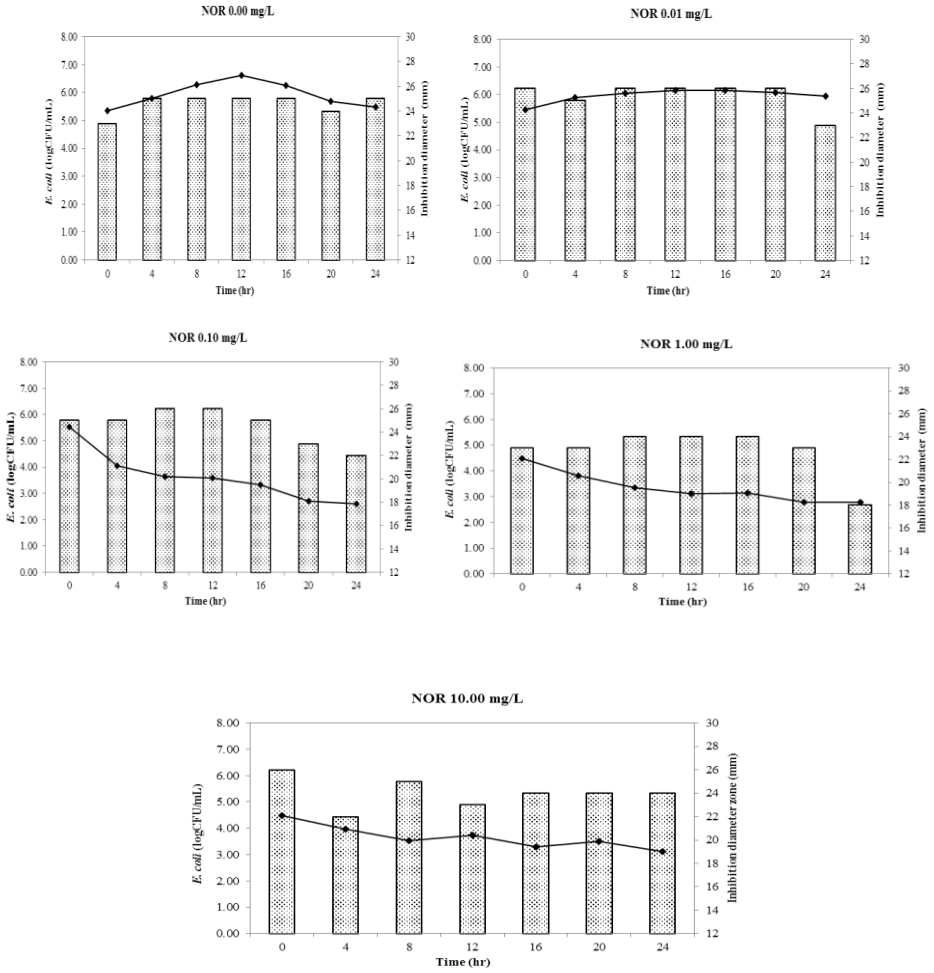


Figure 3. Effect of various norfloxacin doses on E.coli growth (line) in batch experiment test in relation to inhibition diameter (bar)

Note: the inhibition threshold diameter =12 mm

Treatment Performance of SBR and SB-MBR

Figure 4 illustrates changes of DO concentrations in the SBR and SB-MBR. The DO level was measured at the middle of water depths in the tank from the beginning of aeration until the end of non-aeration period. The measured DO concentrations in the reaction tank fluctuated correspondingly to the aeration. After 60 minutes of aeration, the DO concentrations in both reactors could reach to 6.3-6.5 mgO_2/L and keep this consistency till the end of aeration period (120 min). After aeration was stopped, the DO level between SBR and SB-MBR was obviously different. The DO concentration in SB-MBR sharply decreased from 6.5 mg/L to near zero during the last 30 minutes of the 4 hours cycle. This is because oxygen was rapidly consumed in SB-MBR due to biomass re-suspended throughout the reaction tank from a continuous mixing to avoid the membrane fouling even in the non-aeration period. On the other hand, the lowest DO level in SBR could be maintained at 4.0 mg/L in the non-aeration period.

Although the DO levels were different, the treatment performances based on general chemical parameters in both reactors had not shown a significant difference in both cases with and without NOR addition (Table 1). This indicates that most of microbial activity in MLSS was not affected by NOR, which is

supported by the result of batch study that 0.01 mg/L of NOR did not influence the growth of E. coli. Other microorganisms in MLSS also seemed to be tolerant to this NOR level. Since NOR concentration at the wastewater treatment plant in Bangkok is lower than 0.01 mg/L, as above described, the effect of NOR on microbial activity would be negligible.

Table 1. Average of Water Characteristics of Effluents from the Bioreactors

Parameters (mg/L)	Influent	SBR Effluent		SB-MBR Effluent	
		No NOR	With NOR	No NOR	With NOR
BOD_5	82-158	5.08 (95.4)	3.59 (96.7)	3.80 (96.5)	1.63 (98.5)
COD	125-172	32.71 (76.2)	28.33 (80.3)	31.08 (77.4)	29.59 (79.5)
TKN	11.9-27.1	0	3.0	0.6	0.5
NH_4^+-N	7.7-22.1	0.1	0.6	0.3	1.2
pH**	7.00	7.51	NA	7.42	NA
MLSS*	-	1,451	1929	1,807	2,607
MLVSS*	-	1,041	1525	1,414	2,205

NA: Not available.
Number in parentheses means removal efficiency (%) of BOD, COD in each run.
* Concentration in the bioreactors; ** Unitless

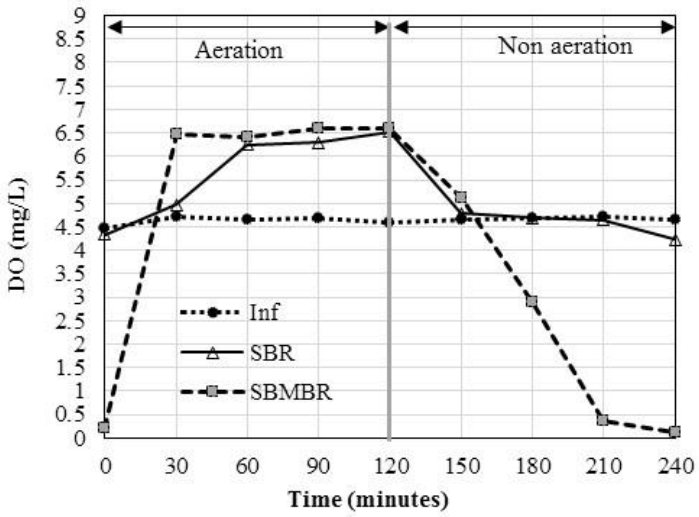


Figure 4. DO changes patterns in the SBR and SB-MBR

Antibiotic Resistances of E. coli in the Bioreactors

Without NOR Condition

The concentrations of E. coli in the reaction tanks of SBR and SB-MBR were in ranges of 3.4-4.3 and 3.2-4.8 log cell density (CFU/ml), respectively. These comparable concentrations suggest that the operation of both system did not give any effects on the E. coli survival in the bioreactor. The total 103/119 isolates of E. coli from the SBR/SB-MBR tanks were tested for their susceptibility to seven antibiotics (Figure 5a) and compared to that in the MLSS initially seeded (Figure 2). The percentages of E. coli resistant to AMC, NA, TE, SXT, C, and NOR decreased from those initial values. Especially, the resistance to

NOR obviously reduced from 78% to only 2% within 3 months of operation. The reduction of resistant E. coli was consistent with the report that the wild resistant E. coli from the seed sludge was reduced with time [1]. In our experiment, continuous addition of E. coli TISTR780 to both bioreactors may have contributed to relative increase in the percentages of the sensitive E. coli.

On the other hand, the resistance to AK increased from none to 5-23%, suggesting the possible occurrence of resistance gene transfers from wild E. coli with intermediate expression (Figure 2) or other bacteria to E. coli TISTR780 (Figure 3a) during the wastewater treatment in both systems. It is reported that E. coli and other bacteria such as K. puemoniae and S. typhi were all conjugally proficient. The conjugation is a gene transfer mediated by cell-to-cell contact which happens between the donor and recipient strains in the same environment [19]. Resistance markers technique revealed that the genes were transferred into E. coli (K-12) at a frequency of approximately $7x10^{-3}$ [19]. Moreover, it is reported that a low oxygen condition is responsible for the development of tolerance to antibiotics because bacterial plasmids of antibiotic resistance are able to transfer between E. coli strains or between different species of microorganisms under anaerobic conditions [20].

Compared to SBR, the SB-MBR showed lower percentages of resistant E. coli to AMC, NA, C and AK. This suggests that the SB-MBR provides some condition better for reduction of resistant E. coli to these antibiotics than SBR. As shown in Figure 4, much lower DO levels during settling period in SB-MBR is the most probable reason for this reduction. The DO level of SB-MBR suddenly dropped to zero during settling period might have gave a significant influence on E. coli survivals. Another study reported higher microbial concentration in aerated waste compared to that in anaerobic condition [21]. At this moment, the effect of oxygen available on resistance prevalence in SBR/SB-MBR systems has not been clearly understood. Further study using molecular technique will help more understanding of the mechanisms of resistance reduction due to the oxygen limitation.

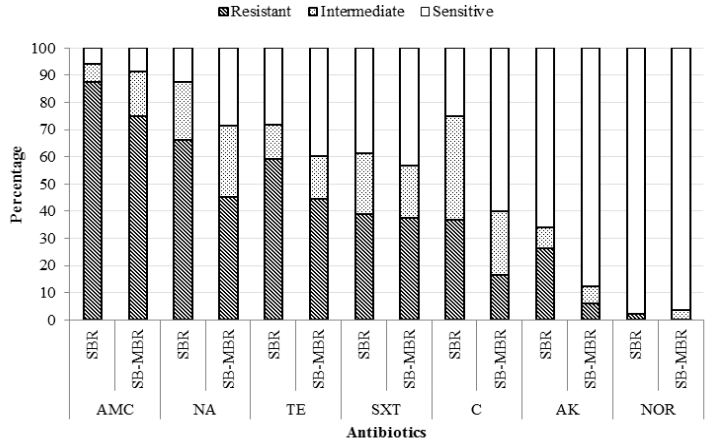
With NOR Addition

When NOR presented in the wastewater at 0.01 mg/L, the lower concentrations of E. coli in NOR addition were found during the first month of operation. However, they gradually increased with time due to acclimatization to NOR (data not shown). The result was consistent with our batch test demonstrating that E. coli could survive in 0.01 mg/L. During the 3 months of the experiment, the E. coli were found in ranges of 1.9-4.4 log cell density (CFU/ml) and 2.0-3.9 log cell density (CFU/ml) for SBR and SB-MBR, respectively. The total 74/70 isolates of E. coli from the SBR/SB-MBR tanks, respectively, were tested for their susceptibility to seven antibiotics (Figure 5b). Resistances to NA sharply increased in both bioreactors from initial values (Figure 2). This is probably because NA is first generation of quinolone, which has a main chemical structure similar to NOR in the 2^{nd} generation called fluoroquinolone, and NOR could induce E. coli resistance to the 1^{st} generation antibiotics in the same group. This is well known as "cross resistance" occurrence of same group of antibiotic [22].

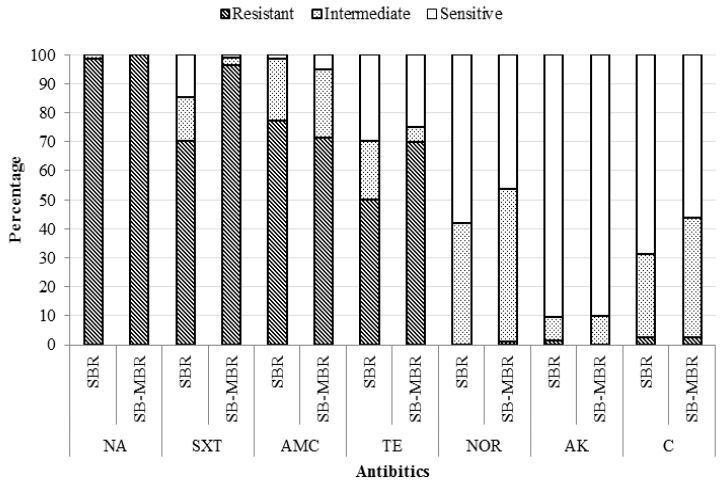
On the other hand, the occurrence of resistance to NOR itself was hardly found in both reactors. Peltier et al. (2010) mentioned that influent antibiotic alone not enough to induce bacterial resistance, which subsequently resulted in cross resistances to ciprofloxacin, tetracycline and tylosin, in activate sludge process except when Zn at sub-toxic level had presented concurrently with the antibiotics [23]. In addition to NOR, lower resistances to AK (30s initiation inhibitor) and C (50s peptidyl transferase inhibition), which share protein synthesis inhibition as resistance mechanism, also appeared. [22]. The reason for

this sudden drop in resistance to these antibiotics in this group caused by addition of NOR is still unclear and of interest in future studies.

Oppositely, the resistances of E. coli to SXT and TE, known as antibiotics of nucleic acids and protein synthesis inhibitors [22], increased in both bioreactor sludge but particularly in SB-MBR as compared to the non-antibiotic condition. In SB-MBR, the resistance percentages (70-90%) were over the initial seed sludge (Figure 5b). The result of concurrent high resistances to NA, SXT and TE was consistent with those observed at the initial seed MLSS from the CAS in this study (Figure 2). Another CAS-WWTP in Bangkok also demonstrated that most of E. coli isolated were sensitive to AK, while high resistances of E.coli (44%-54%) to NA, SXT and TE were found [7].



(a)



(b)

Figure 5. Antibiotic resistances of E.coli (a) without antibiotics addition, (b) with NOR addition at concentration of 0.01 mg/L

Developments of multidrug resistance (MAR) in E. coli isolates from the SBR and SBMBR tanks are illustrated in Figure 6. NOF clearly promoted the occurrence of MAR to 3-4 antibiotics in both reactors, while MAR to more than 4 antibiotics

were reduced. Without NOR addition, the resistance pattern of MAR found most frequently was NA-SXT-TE-AMC in both reactors: 12 of 63 isolates in SB-MBR and 5 of 48 in SBR. With NOR addition, the frequencies became higher in both reactors; 18 of 80 in SB-MBR, 10 of 65 in SBR. As shown in Figure 5b, addition of NOR induced resistance to some antibiotics, resulted in more frequent occurrence of this pattern. This implies that NOR added to the reactors have contributed to selection of E. coli resistant to such antibiotics. On the other hand, E. coli resistant to other antibiotics such as AK and C, which were not induced by NOR, disappeared and this is the reason for reduction of MAR of 5-6 drugs.

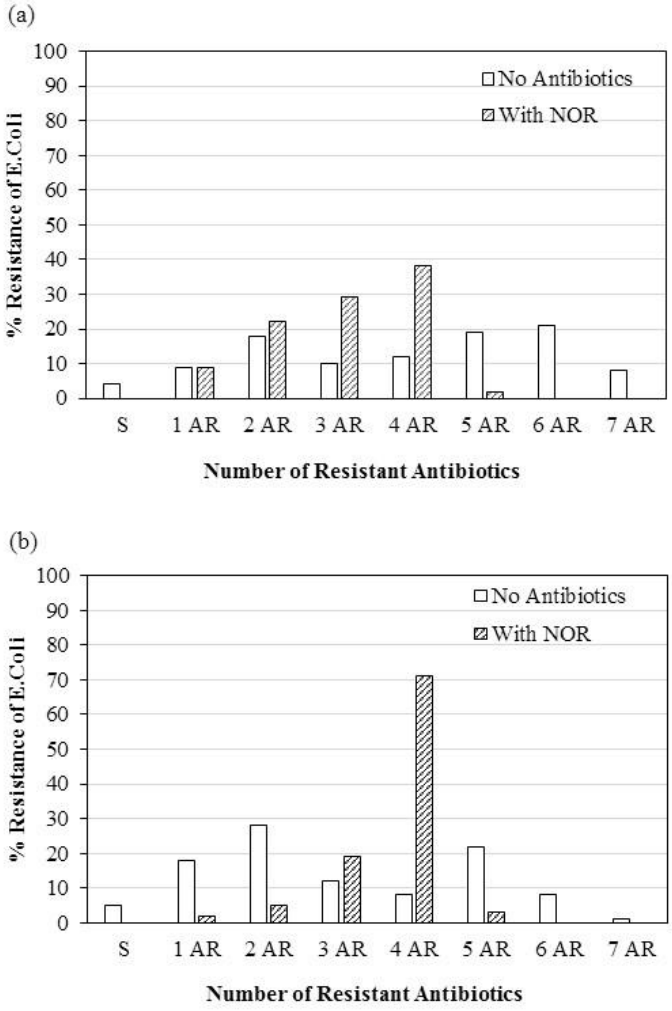


Figure 6. Number of sensitive and multidrug resistance antibiotics of E. coli (a) SBR, (b) SB-MBR

Conclusions

Without NOR addition, the E. coli isolates in the SB-MBR showed lower percentages of resistant E. coli, than those in the SBR, to amoxicillin/clavulanic acid, amikacin, nalidixic acid, tetracycline and chloramphenicol. Oppositely, in the SB-MBR to which NOR was added, it appeared to promote resistance of E. coli to nalidixic acid, sulfamethoxasole and tetracycline probably due to longer SRT and low DO compared to SBR. This expression of cross resistance induced by NOR resulted in a higher occurrence of multidrug resistant E. coli in SB-MBR than in SBR.

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