

# EFFECT OF NORFLOXACIN ON ANTIBIOTIC RESISTANCE OF ESCHERICHIA 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, sulfamethoxazole 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 norfloxacin (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 2<sup>nd</sup> 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<sub>4</sub>EDTA 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<sup>TM</sup> 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, 3µm. 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 Fig. 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 polyvinylidene fluoride (PVDF) (Mitsubishi Rayon, Japan) with pore size of 0.45 µm and filtration area of 0.07 m<sup>2</sup>. 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),  $\text{KH}_2\text{PO}_4$  (80 mg/L),  $\text{NH}_4\text{Cl}$  (60 mg/L), and a mineral solution containing  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.57 mg/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (7 mg/L),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (0.206 mg/L),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.25 mg/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.25 mg/L), and  $\text{NaHCO}_3$  (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,  $\text{NH}_4$ , 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°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 °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  $35\pm 2$  °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  $35\pm 2$  °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.

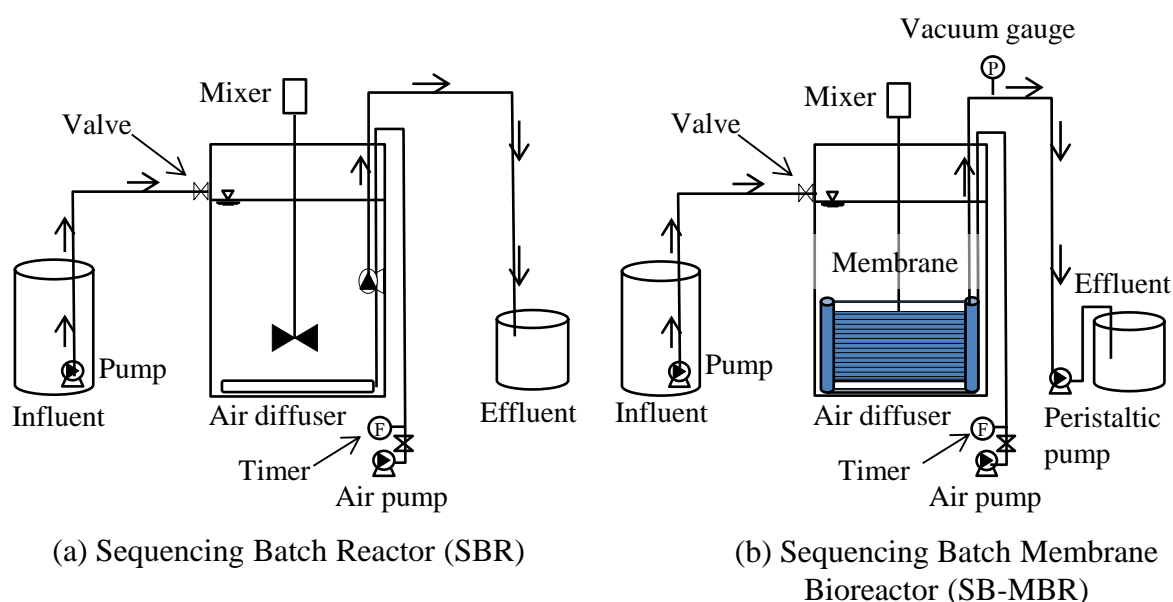


Figure 1. Schematic diagram of SBR and SB-MBR

## 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

Fig. 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.

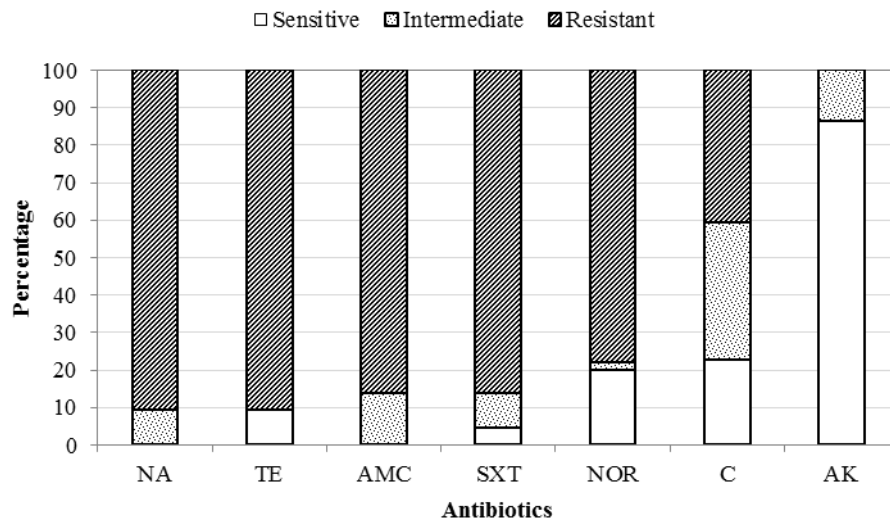


Figure 2. Resistances to various antibiotics of E. coli isolates from MLSS in aeration tank of CAS wastewater treatment plant in Bangkok

### Effect of NOR on Growth of E.coli

Fig. 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.

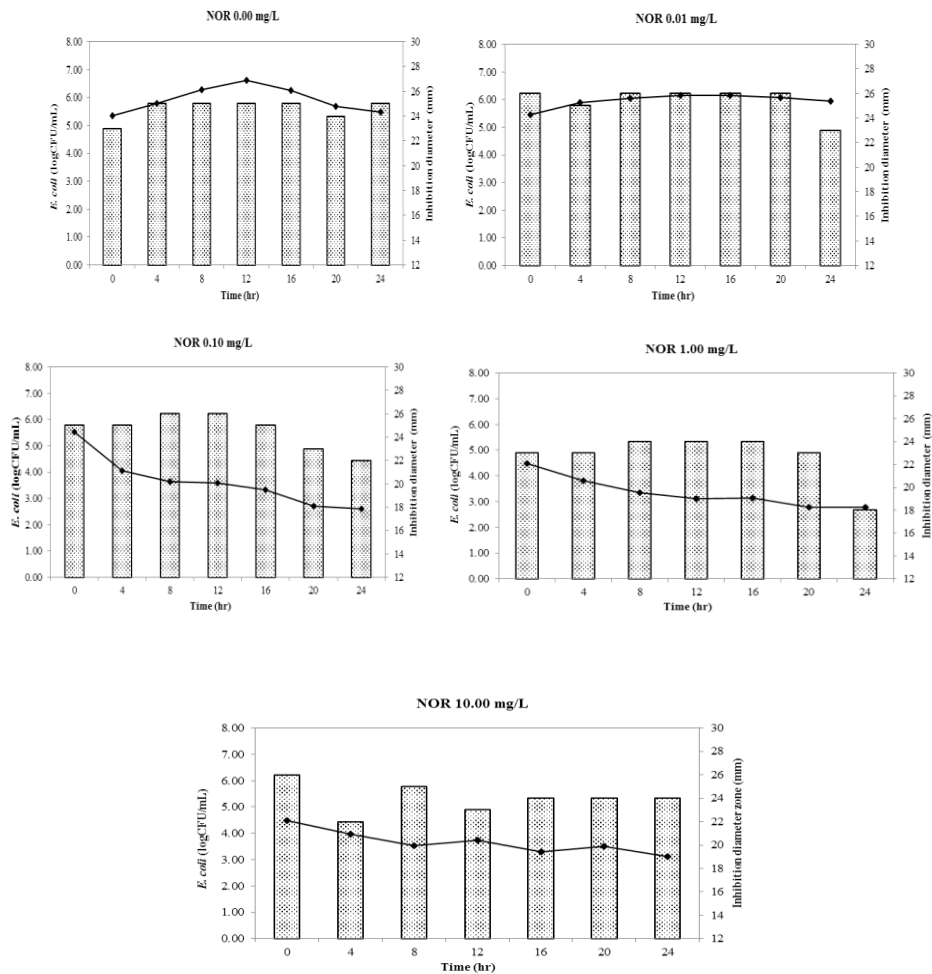


Figure 3. Effect of various norfloxacin doses on E.coli growth (line) in batch experiment test in relation to inhibition diameter (bar)

Note: the inhibition threshold diameter =12 mm

### Treatment Performance of SBR and SB-MBR

Fig. 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<sub>2</sub>/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 <sub>5</sub>	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 <sub>4</sub> <sup>+</sup> -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

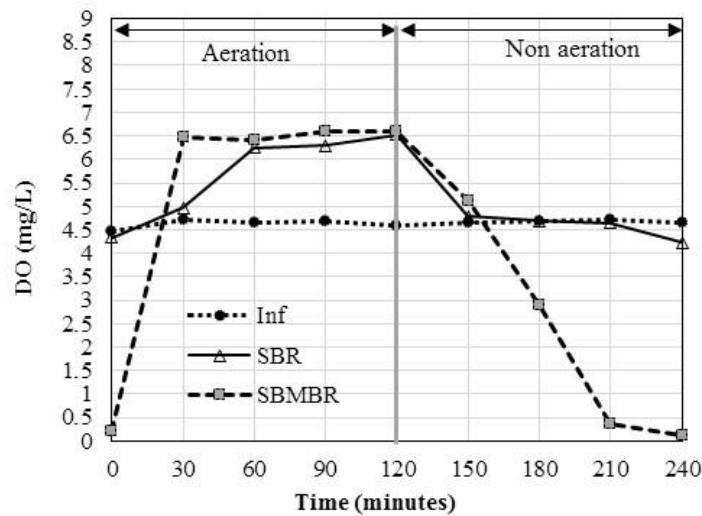


Figure 4. DO changes patterns in the SBR and SB-MBR

### 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 (Fig.5a) and compared to that in the MLSS initially seeded (Fig. 2). The percentages of *E. coli* resistant to AMC, NA, TE, SXT, C, and NOR decreased from those initial values. Especially, the resistance to











