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Effects of Oxolinic Acid on Microbial Community under Simulated Marine Fish Farm Environment

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해산어 양식환경하의 미생물군집에 대한 옥소린산의 영향

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ABSTRACT : The microbial response after treatment of antibiotics was studied for investigating the resistance pattern under simulated aquaculture environment. A marine microcosm was developed for marine fish farm environment using artificial seawater and sediment. Oxolinic acid, which has been commonly used in aquaculture, was employed for the experiment. Resistance patterns and the changes of microbial community were monitored before, during and after use of oxolinic acid. Vibrionaceae was the dominant bacterial species throughout the experiment, consisting 65-75% of total bacterial number in fish farm environment. However, some gram-positive bacteria, *Micrococcos* sp. and *Bacillus* sp. strains in marine farm environment were increased in proportion to their number during the treatment. ETS activity of the bacterial communities in aquaculture environment was reduced to 42-67 % during the treatment of oxolinic acid. But recovering trends of bacterial number were also detected immediately after cease of oxolinic acid treatment. Frequent treatment of oxolinic acid under the simulated fish farm environment showed bacterial resistance to increase sharply.

KEY WORDS : Aquaculture, Resistance, Microcosm, Oxolinic acid, Antibiotics.

요 약: 이 연구는 해산어 양식장 환경을 재현한 해양 microcosm을 이용하여, 양식장에서 빈번히 사용하고 있는 옥소린산에 대한 미생물이 나타내는 항생제 내성획득에 관해 알아보고자 하였다. 옥소린산 처리 전과 후의 세균상을 비교한 결과, 비브리오 과 세균은 실험기간 전반에 걸쳐 65-75% 정도로 우점하였으며, 그람양성세균인 *Micrococcos* sp. 와 *Bacillus* sp. 는 옥소린산 처리 기간 중에 출현 빈도가 증가하였다. 해산어 양식 환경에서 세균의 ETS 활성은 옥소린산 처리 기간 중 42-67 % 로 줄어들었지만, 옥소린산의 처리가 종료된 후에 세균은 다시 회복 되었다. 해산어 양식장에서 옥소린산의 빈번한 사용은 옥소린산에 대한 세균의 내성을 증가시키는 것으로 관찰되었다

핵심용어 : 양식, 내성, microcosm, 옥소린산, 항생제

1. INTRODUCTION

The microbial community in the aquatic environment plays an important role in the nutrition cycle as a part of biogeochemical processes and in the degradation of some pollutant compounds in the system. Releases of large amounts of nitrogen, phosphate and organic materials as uneaten feed from fish farms have been reported in aquaculture area (Enell, 1995; Shahidul, 2005; Antonio et al., 2000; John, 1998).

Employment of antibacterials for the purpose of disease treatment in the fish farm has been common practice. However, the effect of antibacterial treatment on the bacterial community or biogeochemical processes, in particular, has been rarely studied. Possible impacts of antibacterials on sediment ecosystems are inducing the changes in bacterial composition, suppressed rate of organic degradation (Kupka-Hansen et al., 1993) and the development of bacterial resistance to antibacterials, which may result in problems in treating infections (Nygaard et al., 1992; Sandaaand Enger, 1996).When antibiotics are used for treating bacterial disease, the bacteria most sensitive to the drug are likely killed or inhibited. However, those bacteria that possess, or acquire the ability to resist the drugs may persist and

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replace the sensitive bacteria. In fact, following the wide use of these agents in aquaculture, the multiple drug resistant strains among fish pathogens have increased drastically(Barnes et al., 1990; Inglis et al., 1991; O'Grady and Smith, 1992; Karunasagar et al., 1994). The places where drugs were frequently used show higher rate of emerging resistance than places with no drug history. This pattern could be spread along with expansion of aquaculture industry unless there is any other replacement of alternative therapy.

Little is known about functional disruption of microbial community by antibacterials introduced into the aquatic system. As heterotrophic bacteria usually prevent an accumulation of organic matter, studies on reduction of bacterial activity by antibacterial treatments are required to understand the consequences for the interaction between use of antibiotics and organic decomposition in marine ecosystem.

In this study, the resistance patterns of bacterial community against oxolinic acid treatment were investigated under a simulated and controlled environment. An artificial bacterial community of unknown composition was created by introducing waste from a fish farm into a simulated environment. These methods can make it possible to assess the environmental implications of new antibiotics under various conditions of simulated environment and to see the response of bacterial community to antibacterial treatment. Therefore a microcosm, as the simulated fish farm environment, was employed and applied to investigate the reduction of metabolic activities against oxolinic acid treatment compared to untreated stages. Also recovery of bacterial community in the microcosm with time was monitored after ceasing treatment. Overall feasibility of applying microcosms to microbial ecology was assessed by the results obtained (Melanie and Andrea, 1997; Awong et al., 1990)

2. MATERIALS AND METHODS

2.1 Composition of the microcosm

Each microcosm consisted of 100 g of artificial sediment which was overlaid with 350 ml of artificial seawater. Artificial sediment was composed of sand, clay, peat moss (dry weight) in a 75 %, 20 % and 3 % mixture, respectively. Calcium carbonate (1−2 g) was mixed with formulated sediment as a pH buffer and 1−2 g of commercial salmon feed (Hyper 50, BOCM & Pauls Ltd.) was added as the source of organic materials from the fish cages

Artificial seawater was made from distilled water (Aquatron, Model A8S, U.K.) mixed with artificial seasalts (Instant Ocean, Aquarium Systems, Inc. Sarrebough, France). Artificial seawater was kept at room temperature (26±2.2℃) for at least 3 days before use to ensure that the salts had been dissolved completely. Salinity was measured using a refractometer (S/Mill, Atago Co., Tokyo, Japan).

2.2 Establishment of the bacterial community in the microcosm

In order to establish the microbial community in simulated environment system, bacteria were collected from a fish farm. The sample was cultured for 3 days at 10℃. After amplifying the bacterial community associated with waste in the rearing tank or with free living in the water column, 5 ml or 10 ml of well mixed suspension was introduced into the system. Initial bacterial community was confirmed by measuring the proportion of bacterial species in a microcosm.

2.3 Introducing oxolinic acid into microcosm

50 g/ml of oxolinic acid standard was prepared and kept in dark condition. 1 ml of standard oxolinic acid was mixed with 1−2 g of pellet powder in a bijou just before introduction into the microcosm. The concentration prepared was adjusted within a range of 20−30 mg/kg of biomass which is commonly applied in the field. Pellet powder associated with oxolinic acid was poured into the microcosm and the bijou was washed with 3−5 ml of overlaying water three times to ensure that there was no residue left. Introducing 1 ml of oxolinic acid made the final concentration in the microcosm 0.14 g/ml. Major environmental parameters; pH, salinity and DO, were measured during experiment in both simulated environments.

2.4 Bacteria identification and community analysis

Based on the visual observation and routine method of bacterial identification, generic level of bacterial species was identified.

Bacterial activity was measured by the amount of tetrazolium dye reduced by bacterial electron transport system (ETS) of bacterial cells. Samples (5 ml) were filtered using 0.22 ㎛ cellulose acetate membrane filters. The membrane filter was put into a glass universal and suspended in 1 ml of 50 mM-phosphate buffer (pH 7.8) containing 75 M-MgSO4, 80 mM-sodium succinate, 0.768 mM NADH and 0.22 mM NADPH. Toluene (10 ℓ) and 0.1 % (v/v) sodium deoxycholate solution (20 ℓ) were added to make the bacterial cells permeable. The mixture was incubated by shaking for 20-30 minutes at room temperature. Next, 200 ℓ of 40 mM INT (2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyl tetrazolium chloride) was added and incubated at 20℃ for 60 minutes in the dark. The formazan formed from tetrazolium during respiration was assayed as follows. 3 ml of acetone (100 %, v/v) was added to the sample and shaking continued overnight at 20℃ in capped glass tubes. The resultant suspension was centrifuged at 3,500 rpm for 20 minutes at 20℃ and the violet color of the supernatant was measured by spectrophotometer at 460 nm wavelength .

2.5 HPLC analysis

The overlaying water sample was taken by 1 ml pipette and mixed with the same volume of 0.5 N NaOH using a vortex mixer. After centrifugation at 3,000 rpm for 10 minutes, 1 ml of supernatant was collected and mixed with the same volume of mobile phase before injection into the HPLC system. The sediment slurry sample was taken by pushing a glass tube into the sediment and suspending in 4 ml of 0.5 N NaOH. The mixture was homogenized on vortex mixer for at least one minute and then centrifuged at 3500 rpm for 25 minutes. The precipitant was washed twice with 2 ml of 0.5 N NaOH. 1 ml of supernatant from suspended sample was taken and the extracts were combined. After centrifuging at 3500 rpm for 15 minutes, 1 ml of supernatant was taken and injected into the HPLC system. Concentration was calculated by standard curve with peak height obtained.

2.6 Bioassay

Iso-sensi test agar (IST, Oxoid Ltd., U.K.) in 100 ml was prepared. IST agar was supplemented by addition of 1 ml of overnight culture of *Yersinia ruckeri* Italy. The media were thoroughly mixed and poured into a bioassay plate (Nalgen Nunc Int., Naperville, U.S.A.) on a level platform. The mixture was spreaded evenly for avoiding air bubbles and the agar was allowed to solidify. The plate was closed in a plastic bag and placed in the refrigerator (4℃) for 1-2 hours. The plate was placed on the bench and 36 wells (6 x 6) were punched using a flamed 8 mm diameter core cutter. The agar plugs were removed with a flamed wire loop or fine forceps.

The bioassay plates were always prepared on the day of use, and HPLC analysis was conducted at the same time as the bioassay test was used on the day of preparation. The mobile phase was tested in a bioassay plate as a positive control was compared with the blank as a negative control.

3. RESULTS

3.1 Proportion of initial bacteria community

Changes of environmental factors in control microcosm and the oxolinic acid treated microcosm during experiment are given in Table 1. Community structure for fish farm environment is shown in Fig. 1. From marine fish farm sample, *Vibrio* sp. was the dominant species with 68 % of the total bacteria. Other species *Pseudomonas* sp., *Alcaligenes* sp., *Altermonas* sp., *Micrococcus* sp., *Flavobacterium* sp. and *Bacillus* sp. were detected at the level of 1 % of the total bacterial number. Out of 57 colonies isolated, the unidentified bacterial portion was 11 %.

3.2 Changes in bacterial community structure

Community structure shown by the original bacterial population was likely sustained during the experiment in simulated environment.

Structural changes of bacterial community in control microcosm are shown in Fig. 2 and Fig. 3. Vibrionaceae was isolated as the dominant bacteria in microcosm consisting of the 65-75 % of the total bacteria. *Pseudomonas* sp. and unidentified bacteria followed as the second and the third dominant proportion, respectively. Although there were fluctuations of bacterial composition, the overall bacterial community showed a similar pattern to the initial bacterial community structure

Table 1. Changes of environmental factors through time in microcosm control and the oxolinic acid treated microcosm during the experiment

MFFM* control					MFFM treated with oxolinic acid				
Day	DO (㎎/ℓ)	Salinity (‰)	pH	Temp. (℃)	Day	DO (㎎/ℓ)	Salinity (‰)	pH	Temp. (℃)
0	10.3	34	8.3	10.4	0	10.6+0.6	33.5+0.2	8.1+0.2	10.4
4th	10.7	34	8.1	10.6	4th	11.2+0.3	34+0.4	8.2+0.2	10.6
6th	11.3	33.5	7.9	9.8	6th	10.36+0.4	34+0.2	7.9+0.3	9.8
9th	10.2	34	7.9	10.4	9th	10.21+1.5	33.5+0.3	7.8+0.2	10.4
13th	9.7	34.2	8	10.6	13th	10.4+0.6	34+0.3	7.9+0.4	10.6
16th	10.7	33.5	7.8	10.7	16th	11.1+0.3	33.5+0.2	8+0.2	10.7
25th	10.5	34	8.1	11.1	25th	10.2+0.9	33.5+0.2	8.1+0.1	11.1
28th	11	33.5	8	10.5	28th	10.3+0.7	34.5+0.3	8+0.4	10.5

* MFFM : Marine Fish Farm Microcosm

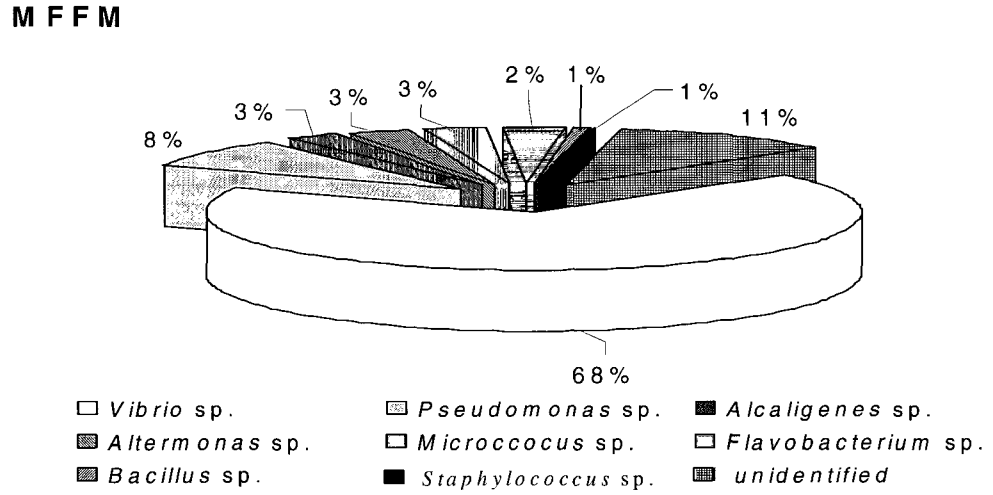

Fig. 1. Proportion of initial bacterial community introduced into a simulated environment. The Proportion of bacterial community was obtained from 57 isolates for a microcosm.

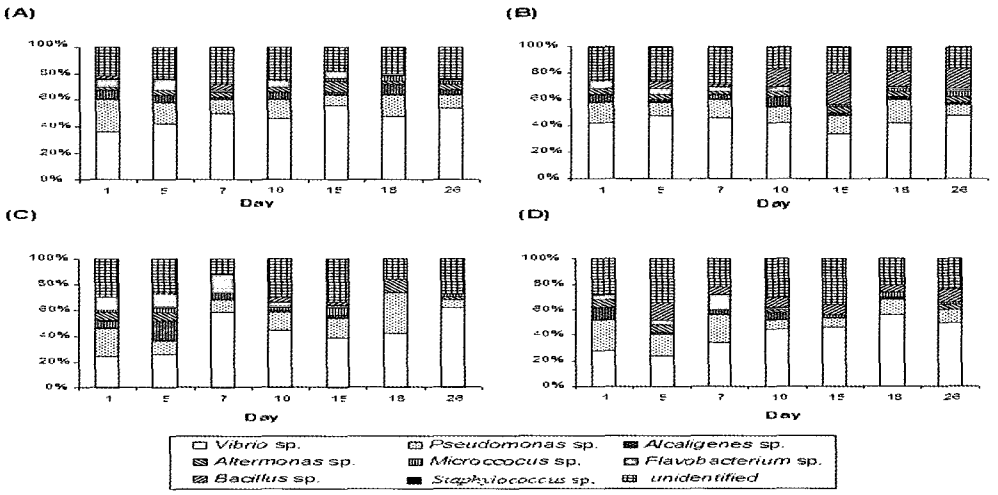

Fig. 2. Changes of bacterial community in marine fish farm microcosm (MFFM) treated with oxolinic acid. Sample was taken from overlaying water and sediment slurry before replacing 30 % of overlaying water.
(A): Overlaying water in MFFM control, (B): Sediment slurry in MFFM contro.
(C): Overlaying water in MFFM treated, (D): Sediment slurry in MFFM treated

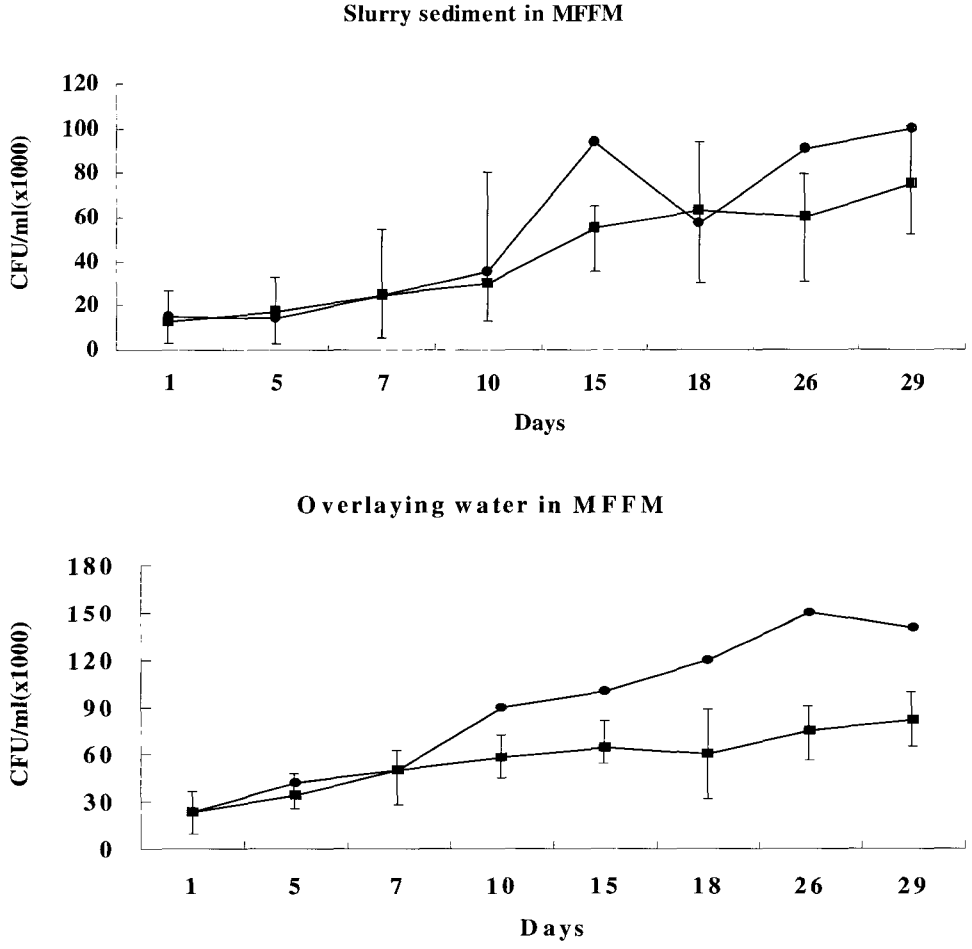

Fig. 3. Variable bacteria number by colony forming unit (cfu) from overlaying water (upper) and sediment slurries (below) at marine fish farm microcosm (MFFM) (● control, ■ treated)

3.3 Changes of bacterial numbers in microcosms

Initial viable bacterial number (as cfu) in overlaying water ranged from 1.1−2.3 x 10^4 cfu/ml from treated and control microcosm and 0.2−1.4 x 10^4 cfu/ml in sediment slurry (Fig. 3). Until 5th day of experiment, bacterial growth both in overlaying water and slurry sediment was sustained at 2.1−3.9 x 10^4 cfu/ml and 1.5−1.7 x 10^4 cfu/ml, respectively. In order to investigate the effect of oxolinic acid treatment on the bacterial community, bacterial number in samples was compared with that in the control group using reduction rate.

3.4 Changes in bacterial activity

Bacterial respiration measured by the ETS method is given in Fig. 4. Reduction of ETS activity during oxolinic acid treatment measured 42−67 %. However, bacterial activity in simulated environment in overall period of experiment showed continuous increase.

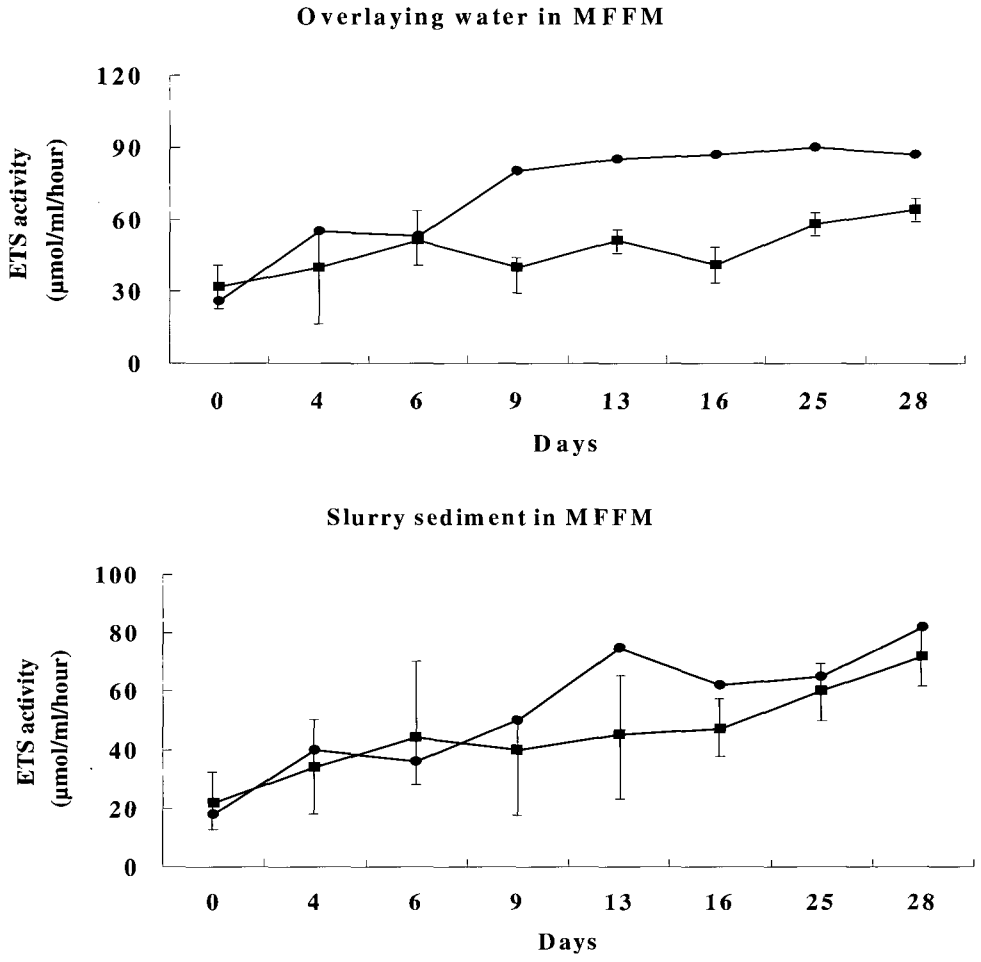

Fig. 4. Bacterial activity by measuring activity of bacterial respiration. Electron trasport system (ETS) activity was measured by the formazan produced from overlaying water (upper) and sediment slurries (below) at marinefish farm microcosm (MFFM) (● control, ■ treated).

3.5 Residue of oxolinic acid measured by HPLC

By HPLC measurement, shown in Fig. 5, oxolinic acid was measured from overlaying water as 0.14 g/ml after the 3rd day of treatment and increased sharply to 0.23 g/ml after 9 days of treatment. The concentration of oxolinic acid in overlaying water in simulated environment increased twice during treatment and showed the same residue level even after cessation of the treatment. However, at the end of experiment oxolinic acid was reduced in overlaying water, whereas the waste composite was still at high concentration in the sediment sample. The oxolinic acid in the waste composite increased to 0.25 g/ml after ceasing treatment but was slightly reduced at the end of experiment. The concentration of oxolinic acid at the end of experiment was 0.2 g/ml.

3.6 Bioassay of oxolinic acid from waste composite during experiment

Susceptibility of overlaying water and sediment containing the oxolinic acid was monitored daily as shown in Fig. 6. In general, bioassay result in the overlaying water sample was higher than that in sediments. The susceptibility in overlaying water increased with time over the treatment and then sharply decreased after stopping treatment, whereas maintained its bio-sensitivities in the sediment until day-18 and dropped at the end of experiment. The accumulated waste from a microcosm showed same level of susceptibility with overlaying water.

Day

Fig. 5. Oxolinic acid detected from water column, sediment and from waste water after replacement of overlaying water. Initial concnetration of oxolinic acid in microcosm was 0.14 g/ml.

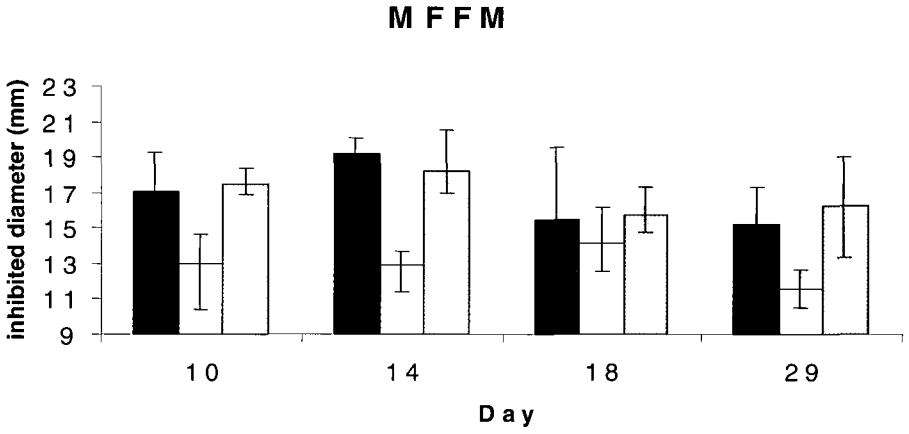

Fig. 6. Bioactivity of oxolinic acid taken from MFFM during and after treatment.

4. DISCUSION

The bacterial community for the marine microcosm contains *Vibrio* sp., *Pseudomonas* sp., *Alcaligenes* sp., *Altermonas* sp., and *Micrococcus* sp., *Flavobacterium* sp., *Bacillus* sp. which are the most commonly isolated bacteria in coastal environments. It is reported that bacterial populations at fish farm outlets are more diverse than the bacterial species collected further down stream (Spanggaard et al., 1992; Carr and Goulder, 1993; Bedwell and Goulder, 1996). This may be explained by the presence of food borne bacterial contamination from the frozen fish and pellet supplied to the fish on a daily basis contributing to bacterial diversity (Kerry et al., 1995). Even though there was no microbiological data available at the sampling sites, introducing the bacterial community sampled from target area into the artificial environment was acceptable for conducting a partial microbial ecology study.

Bacterial settlement in a microcosm was compared by water/sediment proportion in bacterial number. Bacteria settlement was faster in sediment than water column. This assumption was based on the fact that bacterial number in sediment was always more than in the water column (Atlas and Bartha, 1998). The microbial community introduced into the microcosm in this study also required 3 days to be settled in the marine fish farm environment. However, the bacterial population in sediment reaches the same numbers as in water column a few days later. Bacterial growth performance depends on the optimal temperatures for each individual bacterial species, further study would be needed to understand this process.

The reduction in bacterial numbers during treatment was to ensure the drug effects on bacterial community. The results showed certain level of reduction in bacterial number with 42-69 % in overlaying water and 17-46 % in sediment slurry. This result showed the bacterial communities in the overlaying water were more susceptible to antibacterial treatment than those in sediment communities. Although more detailed work is needed to interpret these results, number of particles or electric charge of particles would be an obvious factor to the bacterial response under stress (Kirchman et al., 1982; Holm et al., 1992; Tranvik, 1994; Waiser and Robarts, 1997).

Bacterial community in natural habitat may change its number or species structure depending upon the energy source in the system or direct effects of stress. It was suggested by Weston(1996) even if antibiotics were used in aquaculture environment, bacterial function was still not changed. This suggests that the bacterial community was more affected by water temperature. It is well known that temperature influences the metabolic activities of microorganism. In general, higher temperatures result in higher metabolic activities affecting the stability of bacterial diversity. Thus, although temporal succession is occurring due to drug treatment, the ecological stability in the system may recover faster in a higher temperature environment.

Measuring the bacterial respiration is an indication for the activities of metabolism, though ETS activity relatively determines metabolic activity of microbial community (Peroni and Rossi, 1986; Burton and Lanza, 1986; Garcia-Lara et al., 1991; Vosjan and Olanczuk-Neyman, 1991). Bacterial activity, in particular with organic materials, was studied by Sugita et al(1988). and shown that the growth of heterotroph was enhanced by the substrate in culture pond. A few dominant species normally account for most of the energy flow within a community since these can persist against antibacterial treatments. Similar changes were reported by the study of transformation patterns of fatty acids in sediment from a marine fish farm site (Johnsen, 1994)

Oxolinic acid was measured by HPLC from the microcosm and from waste composite. As expected, residue of oxolinic acid increased during treatment in water column, suggesting a large portion of oxolinic acid still remained or was bound with suspended solids in water column. On the other hand, comparing the water samples with sediment samples, relatively low levels of oxolinic acid were detected in sediment. Oxolinic acid residues in waste were not as high as expected which could be due to the dilution effect of replaced water.

A similar pattern was obtained by bioactivity test although reading of the inhibition zone was hindered by many factors such as bacterial contamination, thickness of media, and susceptibility of standard strain, so on. Increasing pattern of oxolinic acid residue level during the treatment was detected. Eventually bioactivities in all the tested groups were reduced, implying that most of oxolinic acid remained was likely to bind with particles and so lose their activity.

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