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Full Length Research Paper

Degradation of antibiotics by bacteria and fungi from the aquatic environment

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The potential of using bacteria and fungi from aquatic environment to degrade a mixture of two antibiotics, ciprofloxacin and erythromycin, was investigated. The antibiotics were added to river water at a concentration of 4 and 120 mg.L^{-1} for ciprofloxacin and erythromycin respectively. Three variations of the study unit were setup. The first having increased phosphate concentration; the second having slight increase in pH; and the last having increased phosphate concentration and slight increase in pH. The pH of all the study units was reduced on day 15. Preliminary biodegradation tests showed that though biodegradation of the antibiotics were achieved, phosphate augmentation and variation in pH had no significant effect on the biodegradability of the antibiotics. The results indicate that some bacteria and fungi from aquatic environment exposed to antibiotics will develop the ability to degrade the antibiotics, and develop resistance to the antibiotics and some other antibiotics. The degrading ability of these organisms does not depend on phosphate augmentation and variation in pH of the external environment.

Key words: Aquatic environment, antibiotics, phosphate augmentation, pH variation, biodegradation.

INTRODUCTION

Antibiotics have wide spread use outside their conventional use in medicine. Antibiotics are used in genetic engineering, research experiments, animal breeding, crop production, fish farming and aquaculture (Dietze et al., 2005; Prescott et al., 1999; Yanong, 2006). Because of the wide spread use of antibiotics, containment of waste accumulating from their use poses the challenge of the organisms developing resistance to the antibiotics. This is due to the exposure of these organisms to the antibiotics in the environment, at inappropriate concentrations, leading to the metabolism of the antibiotics by the organisms and subsequent resistance to the antibiotics. Pharmaceutical industries involved in the production of antibiotics discharge their waste openly which contains some quantity of the active compounds. Antibiotics administered to humans and animals are not 100% metabolized by the body (Rang et al., 2003). Some active quantity is excreted after body metabolism and may find their way to municipal sewage treatment plants from the excretions. Leaking city sewer systems can thus provide avenues for the migration of effluents containing antibiotics to underlying aquifers. Pharmaceutical drugs detected in a high school septic tank effluent were also detected in the underlying sand and gravel aquifer (Godfrey et al., 2006). The presence of these drugs in the aquifers was reported to correlate with local usage. Antibiotics are also likely to be released into the aquatic environment via wastewater effluent as a result of ineffective treatment, or improper disposal. Sulfamethazine, sulfamethoxazole, tetracycline, ciprofloxacin, erythromycin and trimethoprim have been detected in several wastewater treatment facilities discharging their treated effluents to both surface and ground waters (Karthikeyan and Meyer, 2006).

A reduction in the general productivity of the ecosystem and a disruption in the food chain may occur as a result of impairment in the normal physiological and biochemical functions of some major primary and secondary producers affected by antibiotics (Halling-Sorensen et al., 1997, 1999, 2000; Wollenberger et al.,

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Table 1. Preparation of the mixture for MIC determination of ciprofloxacin.

100 µg.ml^{-1} CPX solution (ml)	Distilled water (ml)	Distilled culture (ml)	Total volume (ml)	CPX concentration (µg.ml^{-1})
1	0	9	10	10
0.9	0.1	9	10	9
0.8	0.2	9	10	8
0.7	0.3	9	10	7
0.6	0.4	9	10	6
0.5	0.5	9	10	5
0.4	0.6	9	10	4
0.3	0.7	9	10	3
0.2	0.8	9	10	2
0.1	0.9	9	10	1

CPX – Ciprofloxacin.

2000). The community structure will thus be remarkably changed leading to a reduction in biodiversity. Also, increase in the number of antibiotic resistant bacteria strain in the environment may occur and multiple antibiotic resistant bacteria may be generated as a result of selective adaptation and transfer of antibiotic resistance-encoding plasmid (Rhodes et al., 2000; Aminov et al., 2001).

Environmental concentrations of antibiotics may be below acutely toxic levels. However, the chronic and/or synergistic effect of the variety of antibiotics on humans in the environment is of great concern. Some microorganisms in the aquatic environments may have developed the ability to breakdown antibiotics released to the aquatic environments, as they have done with other substances such as petroleum products (Leahy and Colwell, 1990). Oxygen, moisture, the absence of alternative sources of carbon and nitrogen, and the presence of acclimatized consortium of microbes have been shown to be required for the biodegradation of antibiotics (Gartiser et al., 2007; Drillia et al., 2005; Fenton et al., 1973; Wang et al., 2006; Badalucco et al., 1994). Other conditions may also be required for the biodegradation of antibiotics. Phosphate which has been shown to be one of the requirements for enhanced biodegradation of oil/hydrocarbons (Abu and Ogiji, 1996) may also enhance the biodegradation of antibiotics, taking into cognizance that some of the above mentioned conditions required for the biodegradation of antibiotics have been shown to enhance the biodegradation of oil/hydrocarbons (Abu and Atu, 2008; Leahy and Colwell, 1990).

This study was embarked upon to investigate the effect of phosphate augmentation and variation in pH (while providing some of the conditions required for the biodegradation of antibiotics) on the biodegradation of two broad spectrum antibiotics, ciprofloxacin and erythromycin, which are among the antibiotics commonly found in wastewater treatment facilities, by bacteria and fungi from aquatic environment.

METHODS

River water collection and analysis

River water was obtained from the new Calabar River located about 1 km from the University of Port Harcourt Teaching Hospital. Concentration of selected ions in the river water were determined. Bacterial enumeration of the river water was carried out using nutrient agar. Different bacterial isolates were characterized and identified based on colonial morphology as described by Cheesbrough (2006). Pure colonies of these were developed and preserved. Sabouraud's dextrose agar was used in the isolation of fungi. Pure colonies of the different fungal isolates, identified based on macroscopic characteristic were developed.

Determination of the concentration of the antibiotics to be used

The bacterial isolates were subjected to ciprofloxacin and erythromycin sensitivity testing using the disc method. The minimum inhibitory concentration (MIC) of these antibiotics was determined for their most sensitive isolates. A modification of the broth dilution method as described by Andrews (2001) was used for the MIC determination. The broth of each test bacterium was prepared by transferring three colonies of a bacterium to 100 ml of nutrient broth then incubated for six hours. Afterwards, 9 ml of the culture was transferred to ten sterile test tubes. The most sensitive isolate to ciprofloxacin and erythromycin were used as the test bacterium for ciprofloxacin and erythromycin MIC determination respectively.

A ciprofloxacin solution of 1 mg.ml^{-1} was prepared then 10 ml of this solution transferred to 90 ml distilled water to obtain a solution of 100 µg.ml^{-1}. One ml of the 100 µg/ml solution was then transferred to 9 ml culture of the test bacterium to obtain a 10 µg.ml^{-1} ciprofloxacin solution, 0.9 ml solution + 0.1 ml sterile distilled water to 9 ml culture to obtain 9 µg.ml^{-1} solution, 0.8 ml solution + 0.2 ml sterile distilled water to 9 ml culture to obtain 8 µg.ml^{-1} solution, and lower dilutions (Table 1).

An erythromycin solution of 15 µg.ml^{-1}was prepared then 10 ml of this solution transferred to 90 ml distilled water to obtain a solution of 1500 µg.ml^{-1}.

Table 2. Preparation of the mixture for MIC determination of erythromycin.

100 µg.ml^{-1} CPX solution (ml)	Distilled water (ml)	Distilled culture (ml)	Total volume (ml)	CPX concentration (µg.ml^{-1})
1	0	9	10	150
0.9	0.1	9	10	135
0.8	0.2	9	10	120
0.7	0.3	9	10	105
0.6	0.4	9	10	90
0.5	0.5	9	10	75
0.4	0.6	9	10	60
0.3	0.7	9	10	45
0.2	0.8	9	10	30
0.1	0.9	9	10	15

ETM – Erythromycin.

Transfers were made similar to that of ciprofloxacin to obtain the various erythromycin concentrations (Table 2).

A tube of the broth culture of each test bacterium containing no antibiotic was included as growth control. Stock solutions of the antibiotics were prepared to attain the MIC of the antibiotics when added to the biodegradation media. The stock solutions were sterilized with the aid of a membrane filtration unit, and stored at 4°C.

Experimental setup

About 2 L of the river water was filled into 2.5-L Erlenmeyer flasks. Three study units were set up. Study unit SII (SII) was used to assess the effect of pH on the biodegradability of antibiotics. The pH of the river water was adjusted to slight alkalinity with the aid of potassium hydroxide (KOH). Study unit SIII (SIII) was used to assess the effect of phosphate on the biodegradability of antibiotics. Phosphate (in the form of KH_2PO_4) was added at a concentration of 1 g.L^{-1}. Study unit SV (SV) was used to assess the effect of both pH and phosphate on the biodegradability of antibiotics. The pH of the river water was adjusted to slight alkaline and phosphate was added at a concentration of 1 g.L^{-1}.

The antibiotics were added to the study units at concentrations equivalent to their MIC against their most sensitive isolates. Air passed through lime water was bubbled through the study units. This was done as an attempt to reduce the amount of carbon dioxide in the air thereby excluding it as a carbon source for the microbes. The pH of all the study units was reduced on the 15th day.

Negative and positive controls (Ctrl (-) and Ctrl (+)) were also set up. Air did not bubble through them. All the study units were incubated at ambient conditions in a slightly dark environment.

Assessment and monitoring of biodegradation

Bacterial enumeration was carried out for the study units (Ctrl (-), Ctrl (+), SII, SIII, and SV) on day one, three, and at five days interval after day three, to assess the variation in the total bacterial population.

Chemical oxygen demand (COD) measurement was carried out as described by Rastogi (2005) on day 0, 7, 14, 21, and 28. A sample volume of 20 ml of the biodegradation medium from each study unit was collected with the aid of sterile 20 ml syringes and used for the measurement.

The pH of the biodegradation medium from each study unit was measured at seven days interval. The pH measurement was achieved by collecting about 10 ml of the medium using sterile 20 ml syringes and measuring with the aid of a portable pH meter.

At ten days interval, about 5 ml of the biodegradation medium from each study unit was collected, filtered with the aid of a membrane filtration unit, and tested on identified sensitive bacteria (which were obtained from the preserved stock), using the well in agar method. This was done to assess the decrease in potency of the antibiotic mixture.

Isolation of isolates that survived

At the end of the study period (28 days), bacteria and fungi were isolated from the study units. Different isolates were identified based on colonial morphology for bacterial isolates, and macroscopic characteristic for fungal isolates. Pure cultures of the bacterial isolates were developed and subjected to antibiotic sensitivity testing using the following antibiotics: linocin, gentamycin, ampiclox, revamping, floxapen, streptomycin, norfloxacin (norbactin), and chloramphenicol.

RESULTS

The concentration of selected ions in the river water measured in this study is presented in Table 3 and Figure 1. From the figure it can be seen that chloride and sulphate ions are relatively high in concentration compared to the other ions, while phosphate, manganese, zinc, nickel, and copper ions are present in relatively little or no amount.

The result of the antibiotic sensitivity testing on the bacterial isolates is presented in Figure 2. From the figure it can be seen that NA7 and NA9 are the most sensitive isolates to ciprofloxacin and erythromycin respectively. The MIC determination result is presented in Table 4. From the table it can be deduced that the MIC for ciprofloxacin and erythromycin against their most sensitive isolate falls within the range of 3.1 to 4 µg/ml and 106 to 120 µg/ml respectively. The concentration of

Table 3. Concentration of selected ions in the river water.

Ions	Concentration ($mg.L^{-1}$)
Cl^-	145.55
SO_4^{2-}	123.84
PO_4^{3-}	0.25
Na^+	7.35
K^+	6.09
Mg^{2+}	2.90
Ca^{2+}	1.81
Mn^{2+}	0.05
Zn^{2+}	0.02
Ni^{2+}	0.02
Cu^{2+}	0.01

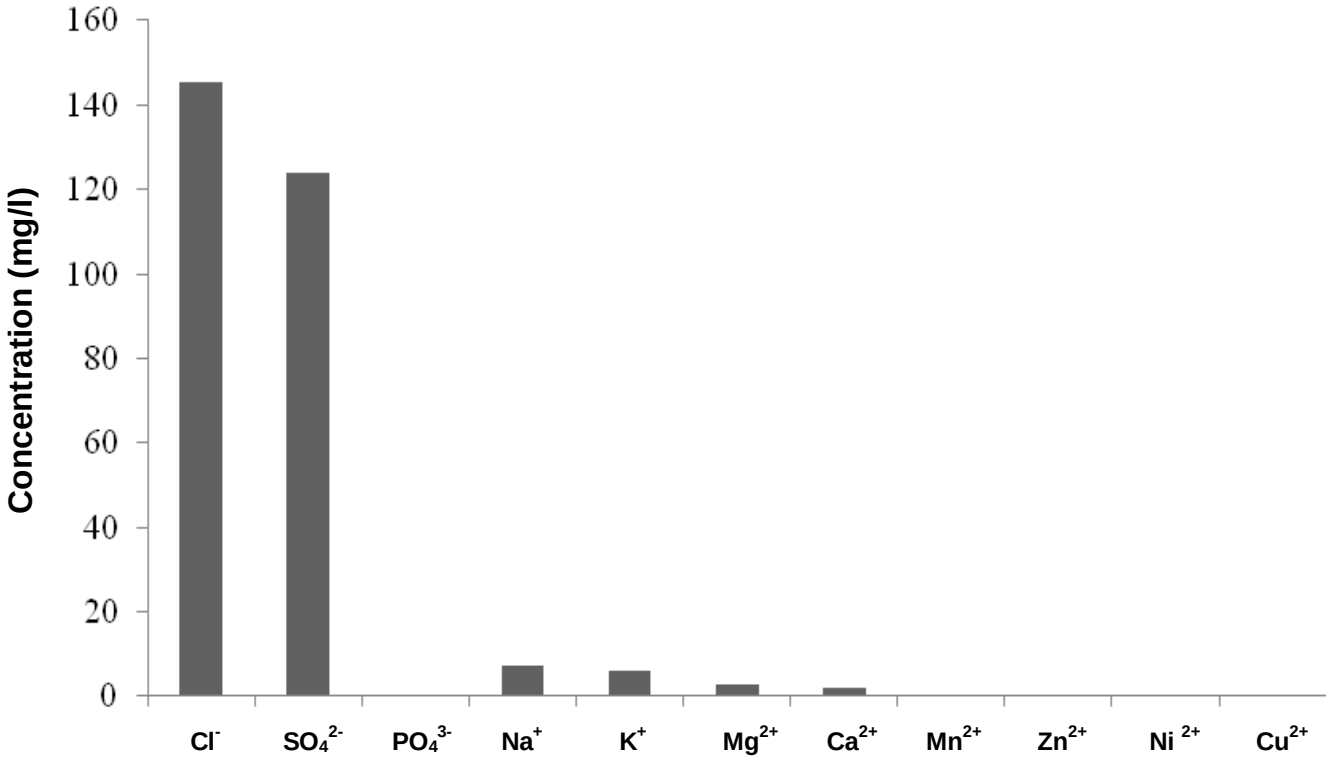

Figure 1. Concentration of selected ions in the river water.

these antibiotics used for this study is 4 and 120 mg/L for ciprofloxacin and erythromycin respectively.

The change in the bacterial numbers of the study units is presented in Figure 3. From the figure, it can be seen that the bacterial growth pattern in the negative control closely follows that of the normal bacterial growth pattern in a closed system. The drop in the growth curve of the other study units on day one shows that the antibiotics reduced the bacterial numbers. After the drop, bacterial numbers increased sharply then steadily and terminated at a peak. After the peak a steep reduction in bacterial population occurred in study units SIII and SV, while the reduction in the positive control and study unit SII were gradual. Also, from the figure it can be seen that the negative control had a higher bacterial population over the various study units for most part of the study period. The change in the COD of the study units is presented in Figure 4. The shows that the COD in the negative control was relatively low and followed a gradual decrease, while the COD in the other study units were high and there was no clear cut difference in their drift.

The change in the pH of the study units is presented in

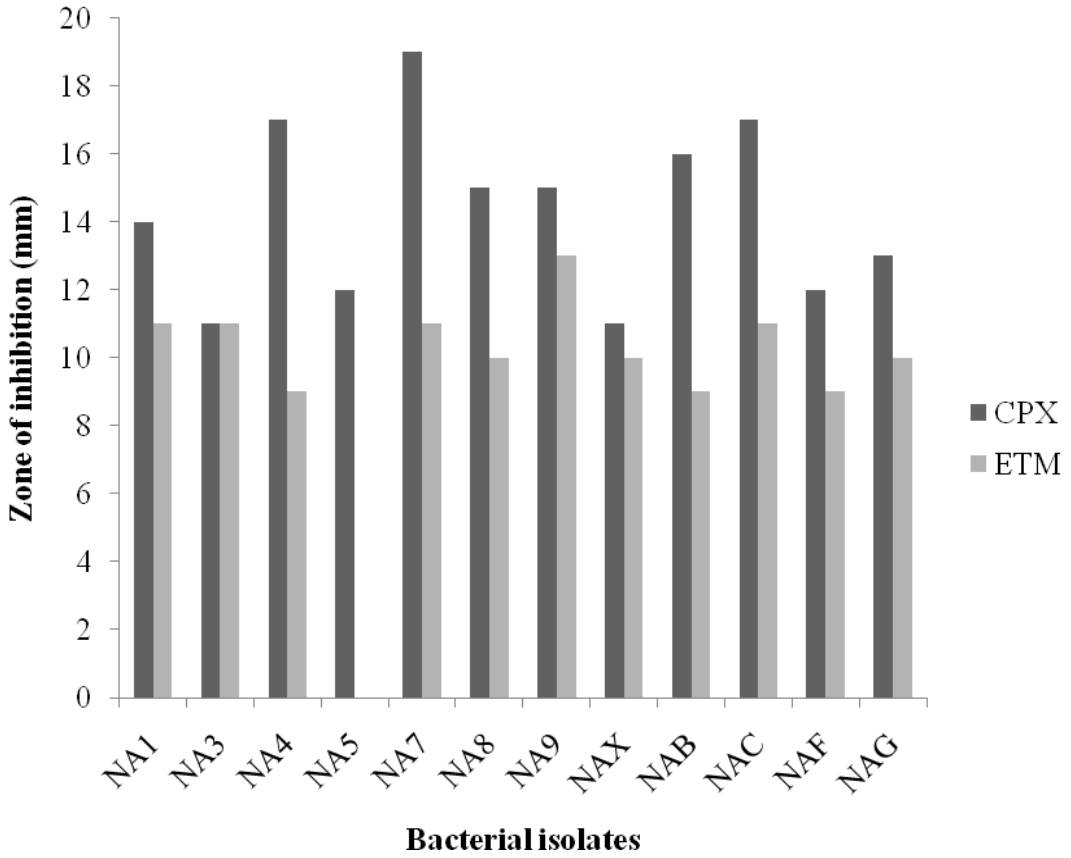

Figure 2. Inhibition of growth of the bacterial colonies by the antibiotics. CPX-ciprofloxacin, ETM-erythromycin.

Table 4. MIC determination of the antibiotics to their most sensitive isolates.

CPX (µg/ml)	Turbidity (NA7 culture)	ETM (µg/ml)	Turbidity (NA9 culture)
10	–	150	–
9	–	135	–
8	–	120	–
7	–	105	+
6	–	90	+
5	–	75	+
4	–	60	+
3	+	45	+
2	+	30	+
1	+	15	+

CPX – ciprofloxacin, ETM – erythromycin.

Figure 5. The sharp decline in pH of study units SII, SIII and SV represents the reduction in pH, effected on day 15, to see if there would be a considerable reduction in their COD. After the reduction, the trend in pH followed the initial trend before pH was reduced. The pH trend in SV and SIII are similar except that the pH in SV remained higher than that of SIII. Also, the pH trend in the negative and positive control is similar.

The loss in antibacterial activity of the study units are presented in Table 5 and Figure 6. The shows that the loss in antibacterial activity of study units SII, SIII and SV on day 10, though not quite different from each other,

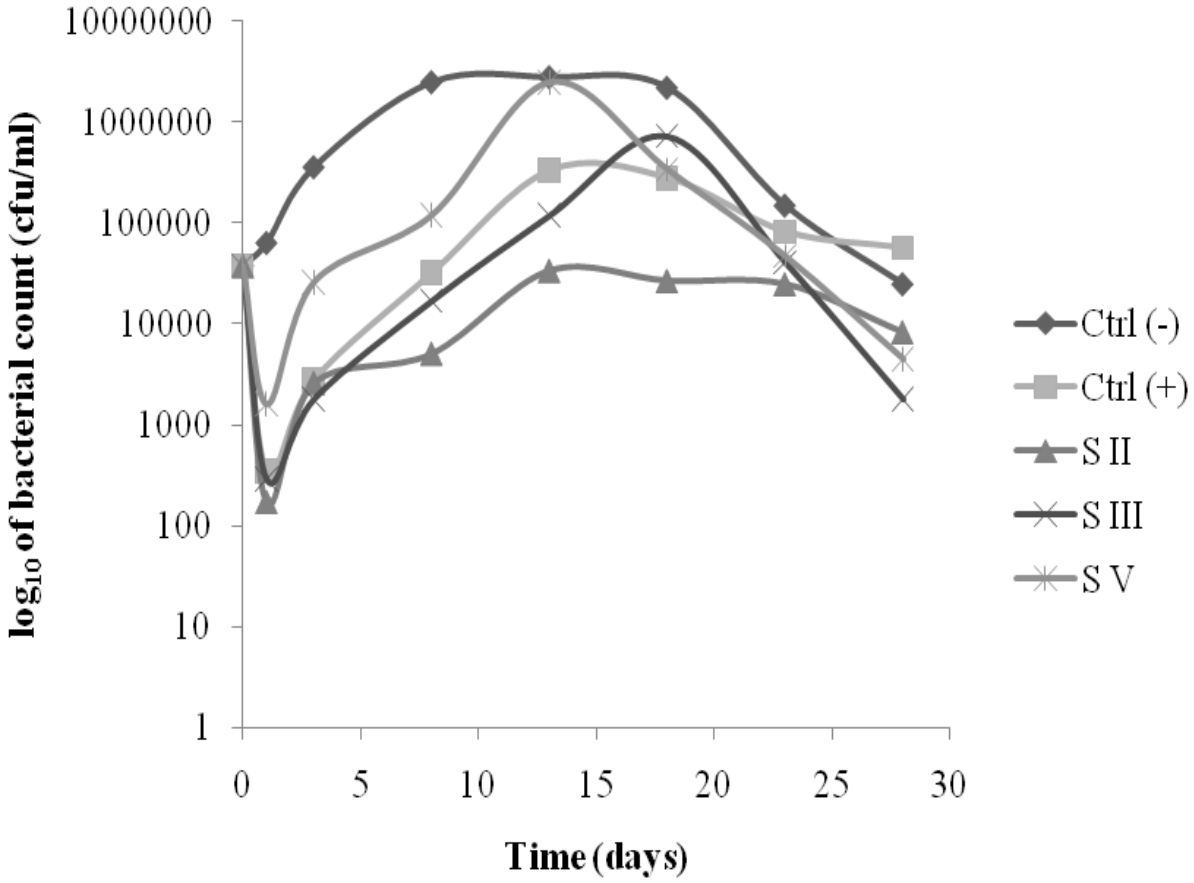

Figure 3. Total heterotrophic bacteria counts of the study units. Ctrl (-) – negative control, Ctrl (+) – positive control, SII – study unit SII, SIII – study unit SIII, SV – study unit SV.

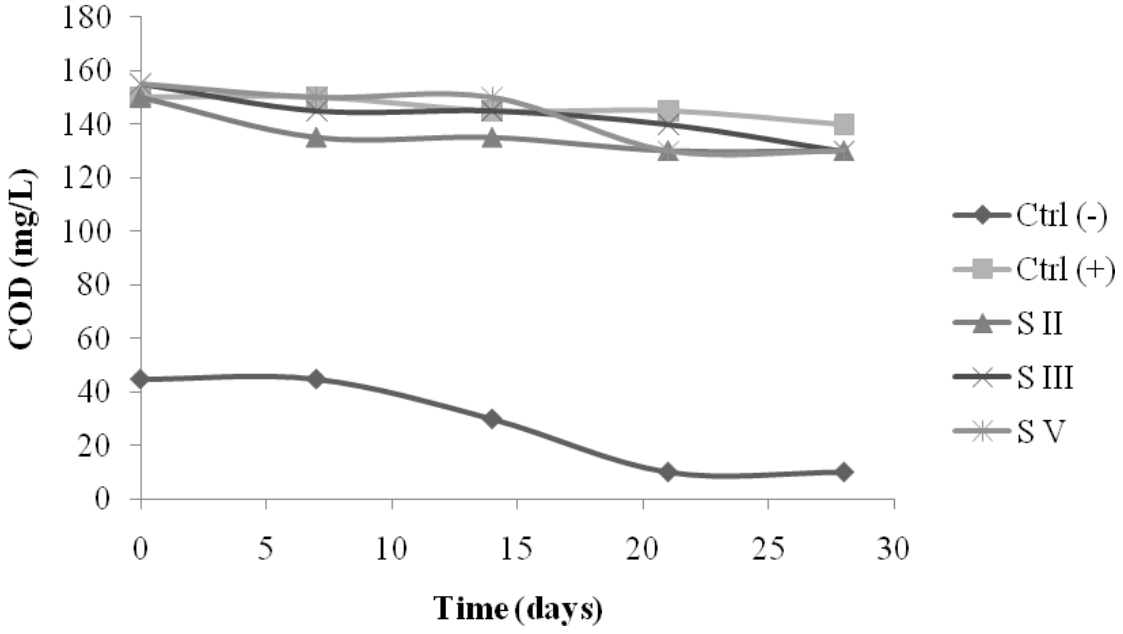

Figure 4. Change in the COD of the study units. COD – chemical oxygen demand, Ctrl (-) – negative control, Ctrl (+) – positive control, SII – study unit SII, SIII – study unit SIII, SV – study unit SV.

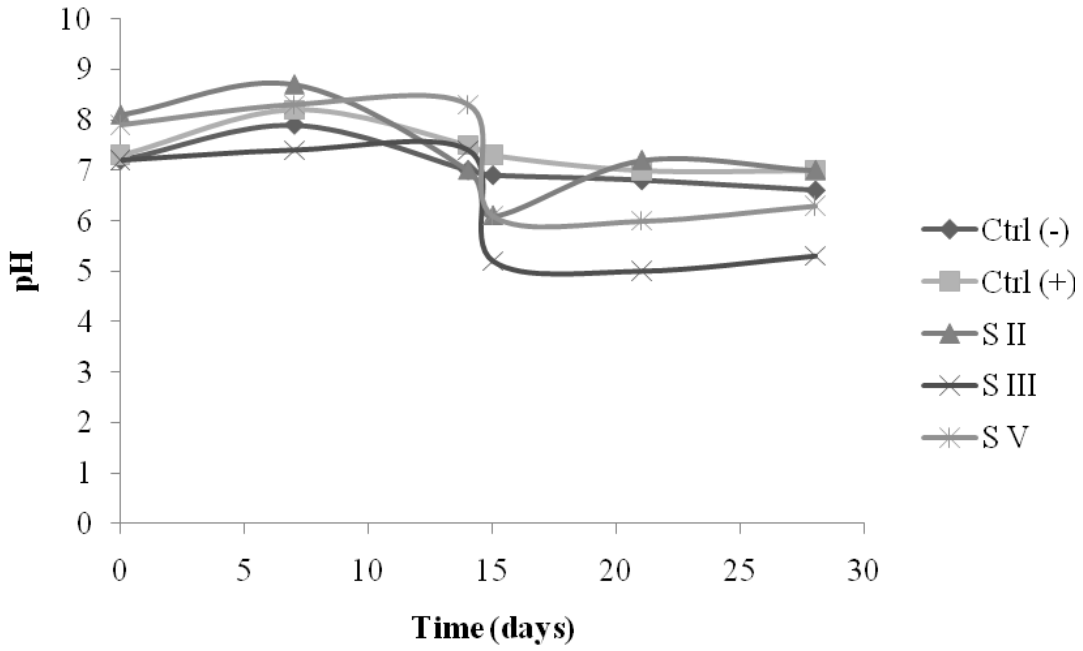

Figure 5. pH changes of the study units**.** Ctrl (-) – negative control, Ctrl (+) – positive control, SII – study unit SII, SIII – study unit SIII, SV – study unit SV.

Table 5. Loss of antibacterial activity.

Day	SU	Zn (cm)	An (cm)	VA (cm^3)	LA (%)
	AB	1.6	0.8	1.6091	_
	+Ctrl	1.5	0.8	1.4143	12.11
10	SII	1.4	0.7	1.0780	33.01
	SIII	1.3	0.8	1.0623	33.98
	SV	1.3	0.8	1.0623	33.98
	AB	3.0	0.7	4.95	_
	+Ctrl	2.0	1.0	3.1429	36.51
20	SII	2.1	0.7	2.4255	51.00
	SIII	2.1	0.9	3.1185	37.00
	SV	1.9	0.9	2.5528	48.43

SU – Study units, AB – referenced antibiotic mixture, Zn – zone of inhibition, An – agar thickness, VA – agar volume.

$$LA = \frac{VA(AB) - VA(i)}{VA(AB)} \times 100$$

having activity; $VA = \pi (Zn/2)^2 X An$, LA – Loss in antibacterial activity; .

were higher than that of the positive control. On day 20 the loss in antibacterial activity of the positive control increased considerably, though not as high as that for study units SII and SV.

The result of antibiotic sensitivity testing on the bacteria isolated from the study units at the end of the study period is presented in Figure 7. From the figure it can be seen that the isolates were sensitive only to rifampin, except isolate SB2 which was still sensitive to ciprofloxacin. The initial fungal isolates from the river water isolated from each study unit at the end of the test period is presented in Table 6. From the table it can be seen that study unit SII contained more of the fungal isolates, while the positive control contained only one of the fungal isolate.

The identity and code of the bacterial and fungal isolates are as follows: NA1 and SB4 – *Micrococcus* sp., NA3 and SB6 – *Bacillus* sp., NA4 – *Citrobacter* sp., NA5

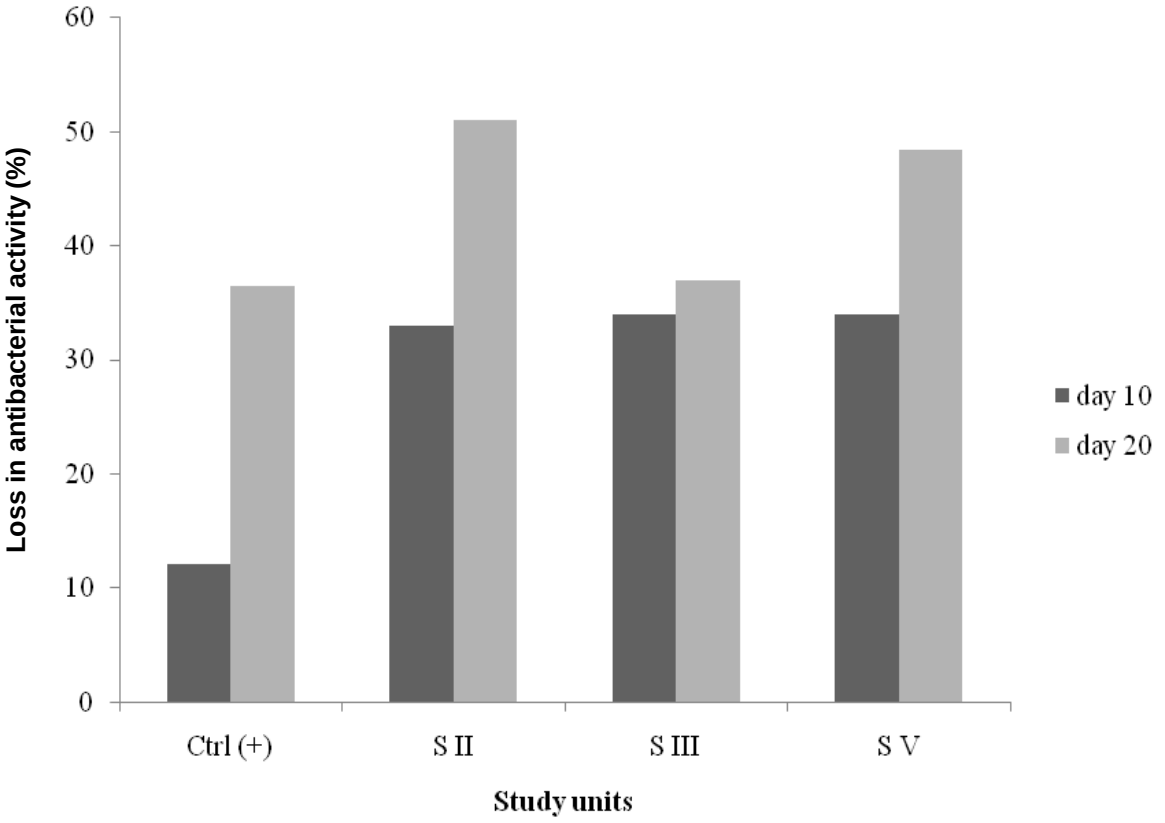

Figure 6. Loss in antibacterial activity of the study units. Ctrl (+) – positive control, SII – study unit SII, SIII – study unit SIII, SV – study unit SV.

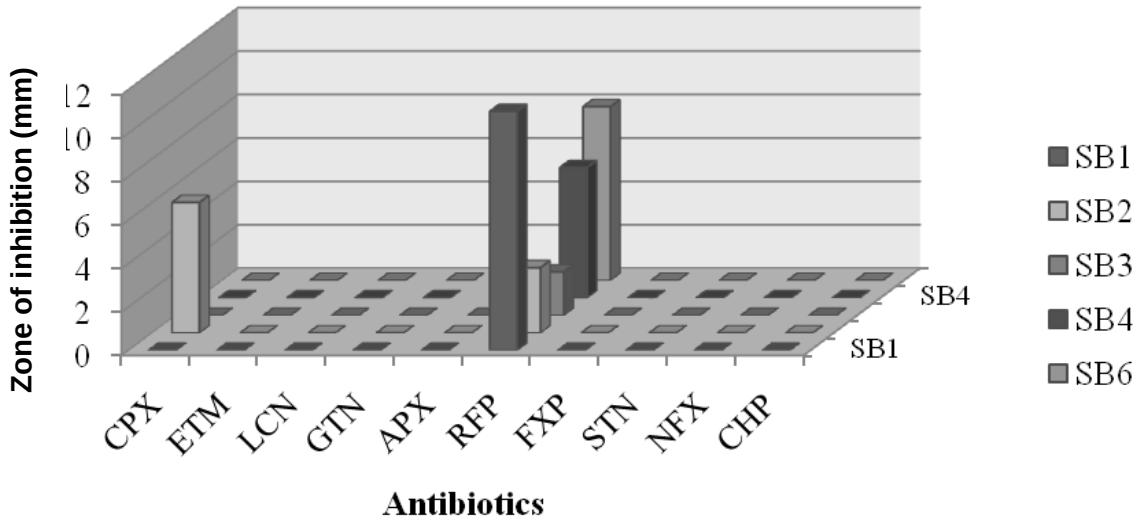

Figure 7. Antibiotic sensitivity of bacteria isolates that survived. CPX – Ciprofloxacin, ETM – Erythromycin, LCN – Lincocin, GTN – Gentamycin, APX – Ampiclox, RFP – Rifampin, FXP – Floxapen, STN – Streptomycin, NFX – Norfloxacin (Norbactin), CHP – Chloramphenicol.

and SB3 – *Pseudomonas* sp., NA7 – *Escherichia* sp., na8 – *Shigella* sp., NA9 and SB2 – *Proteus* sp., NAX and SB1 – *Staphylococcus* sp., NAB – *Vibrio* sp., NAC – *Enterobacter* sp., NAF – *Klebsiella* sp., NAG – *Serratia*

Table 6. Presence/absence data on initial fungal isolates in the study units at the end of the test period.

Study unit	Fungal isolates					
	GA1	GA2	GA4	GA6	YG1	YG2
– Ctrl	+			+		+
+ Ctrl	+					
SII	+		+	+	+	+
SIII	+	+	+			+
SV		+	+		+	+

Ctrl (-) – negative control, Ctrl (+) – positive control, SII – study unit SII, SIII – study unit SIII, SV – study unit SV.

sp., GA1 – *Aspergillus* sp., GA2 – *Penicillium* sp., GA4 – *Mucor* sp., GA6 – *Fusarium* sp., YG1 – *Rhodotorula* sp., YG2 – *Candida* sp.

DISCUSSION

Effluent from pharmaceutical manufacturing plants and wastewater treatment facilities collecting wastewater from sewage, hospitals and veterinary clinics are some of the sources responsible for the release of antibiotics into the environment (Karthikeyan and Meyer, 2006). Antibiotics released into the environment usually end up in surface and ground waters (Holm et al., 1995). Some researchers have shown that antibiotics are biodegradable when certain conditions are amended (Drillia et al., 2005; Gartiser et al., 2007; Wang et al., 2006). This study was carried out in an attempt to enhance the biodegradability of antibiotics.

The antibiotics used for this study are ciprofloxacin, a broad spectrum antibiotic mostly effective against gram negative bacteria, and erythromycin, a relatively broad spectrum antibiotic mostly effective against gram positive bacteria (Prescott et al., 1999). These antibiotics are among the antibiotics commonly found in wastewater treatment facilities (Karthikeyan and Meyer, 2006). The concentration of these antibiotics used for this study is 4 and 120 mg/L for ciprofloxacin and erythromycin respectively. These concentrations were chosen based on the MIC determination of the antibiotics on their most sensitive isolates.

In an attempt to provide some of the conditions necessary for the biodegradation of antibiotic (presence of oxygen and absence of alternative source of carbon and nitrogen (Gartiser et al., 2007b; Drillia et al., 2005) air passed through lime water was bubbled through the study units. The air was passed through lime water as an attempt to reduce the amount of carbon dioxide in the air thereby excluding it as a carbon source for the microbes. Potassium dihydrogen phosphate (KH_2PO_4) was added to the study units marked for phosphate augmentation to provide for phosphorus. From Table 3 it can be seen that phosphorus, a major element, in the form of phosphate ion is present in the river water in micrograms quantity. Since the major elements (carbon, hydrogen, oxygen, nitrogen, sulphur, and phosphorus) are needed in gram quantities in a liter culture medium (Prescott et al., 1999), phosphorus in the form of KH_2PO_4 was added at a concentration of 1.0 g/L to augment for the reduced concentration. Sulphur in the form of sulphate ion is present in the river water in adequate quantity (Figure 1). The other major elements (carbon, hydrogen, and nitrogen) are present in the structure of the antibiotics.

The reduction in COD values and antibacterial activity of the various test systems show that the antibiotic mixture is biodegradable. However, the difference in the reduction of COD values and antibacterial activity of the various test system were not significant ($p < 0.05$). Thus, the variation in pH and the phosphate augmentation did not enhance the biodegradability of the antibiotics. Increase in pH was shown to enhance the degradation of tetracyclines, but increase in phosphate concentration in the form of ionic strength measurement was shown to have no effect on the degradation of the antibiotics (Loftin et al., 2008). The deviation of the observed result concerning pH increase in this study from that of the work of these researchers may be attributed to the unique conditions in a soil environment and an aquatic environment, and the nature of the process applied in the studies. Though the finding of Loftin and his coworkers concerning phosphate was associated with a non biological process, it could possibly occur in a biological process.

In comparing Figures 4 and 5 it can be seen that adjusting the pH of the test systems assisted in reducing the COD which did not change after a period of one week. Though the reduction was not significant, this may be an indication that reduction in pH can play a role in the degradation of antibiotics.

The total heterotrophic bacterial counts (Figure 3) showed that the overall reduction in bacterial population was least in the test system having phosphate and at alkaline pH (SV). The percentage loss in antibacterial activity (Figure 6) supports this pattern. The result could be attributed to the actions of the fungal population on the antibiotics thus giving the bacterial population an advantage over their counterparts in the other test systems.

The test system having only alkaline pH (SII) had more fungal genera than the other test systems. The high percentage loss in antibacterial activity of this test system could thus be attributed to the actions of the fungal population. The percentage loss in antibacterial activity is supported by the COD pattern, but not by the total heterotrophic bacterial counts.

The survival of some of the organisms at the end of the study period may be an indication of their potential to degrade antibiotics. Thus this ability may be undertaken in future studies to determine their potential for use in waste treatment facilities where antibiotics need to be eliminated.

At the end of the study period the isolates became resistant to most antibiotics. Only rifampin was effective on all the isolates even though it has similar mechanism of action as ciprofloxacin; they both inhibit nucleic acid synthesis. The bacterial isolates have become resistant to many antibiotics as a result of exposure to just two antibiotics. The effectiveness of rifampin against these isolates may not be attributed to non prior exposure. Its effectiveness could be attributed to the slight difference in mechanism of action from other antibiotics especially ciprofloxacin. Rifampin blocks ribonucleic acid (RNA) synthesis by binding to and inhibiting the deoxyribonucleic acid (DNA) – dependent RNA polymerase, whereas ciprofloxacin and other quinolones interfere with DNA replication and transcription by inhibiting DNA gyrase.

Conclusion

The biodegradation tests showed that biodegradation of the antibiotics were achieved, however phosphate augmentation and variation in pH had no significant effect on the biodegradability of the antibiotics. Thus phosphate and pH are not factors of importance compared to the other factors (oxygen, moisture, absence of alternative sources of carbon and nitrogen, etc) which have been shown to be necessary for the biodegradation of antibiotics to occur.

Some bacteria and fungi from aquatic environment exposed to antibiotics will develop the ability to degrade the antibiotics. In addition, the bacteria will develop resistance to the antibiotics and some other antibiotics. However, degradation of antibiotics can be attributed mostly to the fungi since, as indicated by the results, the decrease in the number of fungal genera is small compared to the decrease in the number of bacteria genera.

Further studies on the biodegradation of antibiotics should be focused on fungi, and other factors that have been identified to be necessary for the biodegradation of anthropogenic and organic molecules, and on the optimization of the factors already identified as necessary for the biodegradation of antibiotics.

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