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Effect of Two Broad-Spectrum Antibiotics on Activity and Stability of Continuous Nitrifying System

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Abstract

The effects of two broad-spectrum antibiotics, chloramphenicol and oxytetracycline hydrochloride, on the microbial activity and biofilm stability of a mixed nitrifying culture were studied. These antibiotics are present in some wastewaters generated in cattle farms or pharmaceutical industries. A 1-L fermentor, in which nitrifiers grew both in suspension and in a biofilm, was used during the experiments. Chloramphenicol (10–250 mg/L) barely had any effect on biofilm stability and nitrification. Ammonia was fully oxidized to nitrate. However, oxytetracycline caused biofilm sloughing at concentrations of 10 mg/L, but nitrification was not inhibited at antibiotic concentrations up to 100 mg/L. When the concentration of oxytetracycline chlorohydrate was increased stepwise from 100 to 250 mg/L, nitrification was inhibited by 50%. The dissolved organic carbon measurements in both the influent and effluent showed that the antibiotics were neither mineralized by the mixed nitrifying culture nor accumulated in the system. Furthermore, the microbial tests did not reveal the presence of active antibiotics in the effluent. This fact indicates that both cloramphenicol and oxytetracycline were degraded by the nitrifying sludge but not mineralized.

Index Entries: Antibiotic; chloramphenicol; oxytetracycline hydrochloride; nitrification; biofilm.

Introduction

Wastewaters generated in cattle farms or pharmaceutical industries contain important amounts of nitrogen and organic matter, and often

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antibiotics. Biologic treatment systems are widely used to remove both carbon and nitrogen compounds from these kinds of wastewaters. In the treatment systems, removal of organic matter is accomplished by using methanogenic anaerobic reactors as well as aerobic or anoxic biologic systems, while the removal of nitrogen compounds is carried out using nitrification-denitrification processes. During nitrification, ammonia from the hydrolysis of organic matter is aerobically oxidized to nitrite and nitrate by autotrophic nitrifying microorganisms. These nitrogen anions are reduced, in a following stage, to nitrogen gas in the absence of oxygen, by denitrifying heterotrophs, using the organic matter from wastewater as electron donor. In nitrogen removal systems, nitrification is often the limiting step because of the relatively low growth rate of nitrifiers. Furthermore, organic matter, high ammonia concentrations, pH, and toxic compounds may inhibit the nitrifying microorganisms *(1)*.

The presence of antibiotics in wastewaters from cattle farms is a result of their addition to the animal feed. In this case, antibiotics are only partially metabolized by the cattle, and, as a result, it is possible for them or their metabolites to still be bioactive in the wastewater. The effects of antibiotics and their degradation products in surface water and groundwater have scarcely been investigated, and their environmental effects, such as the development of resistant pathogenic microorganisms, are poorly understood *(2)*.

Antibiotics are referred as toxic compounds for microorganisms in wastewater treatment systems. The references in literature are usually related to the effects of these compounds on anaerobic digestion *(3–7)*, their use as inhibitors *(8)*, or their effects on nitrification batch processes *(9,10)*. Since the majority of the results show the toxic effects of the antibiotics, some researchers propose a chemical/physical pretreatment to avoid their presence in biologic treatment systems *(11,12)*. However, the utilization of physical-chemical systems might not be possible, as a result of the high operating costs associated with this kind of treatment. A viable alternative might be the utilization of advanced bioreactors, in which microorganisms are maintained in conditions that could improve their resistance against these toxic compounds.

The utilization of advanced biofilm technologies, for biologic wastewater treatment, has become more and more popular. In these systems, the microorganisms in biofilms tend to be more resistant against toxic or inhibitory compounds than freely suspended cells, which are used, e.g., in the traditional activated sludge systems. The biofilm and associated extracellular polymers provide a resistance to penetration by toxic compounds. Unless the toxic or inhibitory compounds can be diffused to the lower layers of the biofilm, only the microorganisms near the surface are affected *(13–15)*. Nevertheless, a weak point of the utilization of biofilm reactors is the possibility of massive sloughing, which could lead to loss of the biomass and total failure of the process. Sloughing is the incidental loss of large patches of biofilm and may be caused by the presence of certain chemical

compounds in the wastewater *(16)*. For these reasons, it is necessary to evaluate the toxic or inhibitory effects of these compounds on both nitrifying biofilm activity and stability. The effects of two broad-spectrum antibiotics, chloramphenicol and oxytetracycline hydrochloride, widely used in dairy industries on both the nitrification process and the stability of nitrifying biofilms were examined in the present study.

Materials and Methods

Analytical Methods

Ammonia was determined by using a selective electrode as proposed in ref. *17*. Nitrite and nitrate were determined by capillary electrophoresis using a Waters Quanta 4000 system *(18)* and dissolved organic carbon (DOC) using a Shimadzu DOC-500. To identify the structure of the degradation products, a mass spectrometer HP 5988A, an NMR Bruker AmX300 (^{13}C), and a high-performance liquid chromatograph HP Series II 1090 were employed. The high-performance liquid chromatograph was equipped with a diode array detector, and the compounds were separated using a Supelcosil LC-8 column (Supelco-Teknokroma, Barcelona, Spain); the eluent was an acetonitrile:methanol:H_3PO_4 (20 m*M*) (6:20:74) mixture. The antibiotic activity in the effluent was tested by microbial assays using *Escherichia coli* 10536 ATCC and *Staphylococus aureus* 29737 ATCC genera for chloramphenicol and oxytetracycline, respectively, as described in Biological Tests, USP (see ref. *19*).

Experimental Setup

A Biolab Braun fermentor (1 L) with an external settler (Fig. 1) was used. The reactor was inoculated with a mixed nitrifying culture. The biomass growth took place both in suspension and on the inner glass wall of the reactor. Suspended biomass concentration, separated in the external settler, was recycled to the bioreactor using a peristaltic pump.

Temperature was maintained at 28°C, while pH was kept at 7.5 using a solution with 40 g/L of $NaHCO_3$ and 0.4 g/L of $CaCl_2 \cdot 2H_2O$. O_2 concentration was maintained at about 2 mg/L. The composition of the influent (500 mg of N-NH_4^+/L) was an autotrophic solution of 1.18 g/L of $(NH_4)_2SO_4$, 0.96 g/L of NH_4Cl, 1.40 g/L and KH_2PO_4, 0.6 g/L NaCl, 0.5 mL/L of traces solution *(20)*, and a variable antibiotic concentration as described subsequently. Hydraulic retention time was maintained at 3.3 d, and nitrogen loading rate was 0.15 g of N-NH_4^+/(L·d).

The experiment was divided into the following three periods:

1. Days 0–53: Chloramphenicol was added to the influent, and its concentration in the influent was increased stepwise 10, 25, 50, 100, and 250 mg/L, as shown in Fig. 2.
2. Days 53–60: This was a transitory period in which the influent did not contain any antibiotics (data not shown).

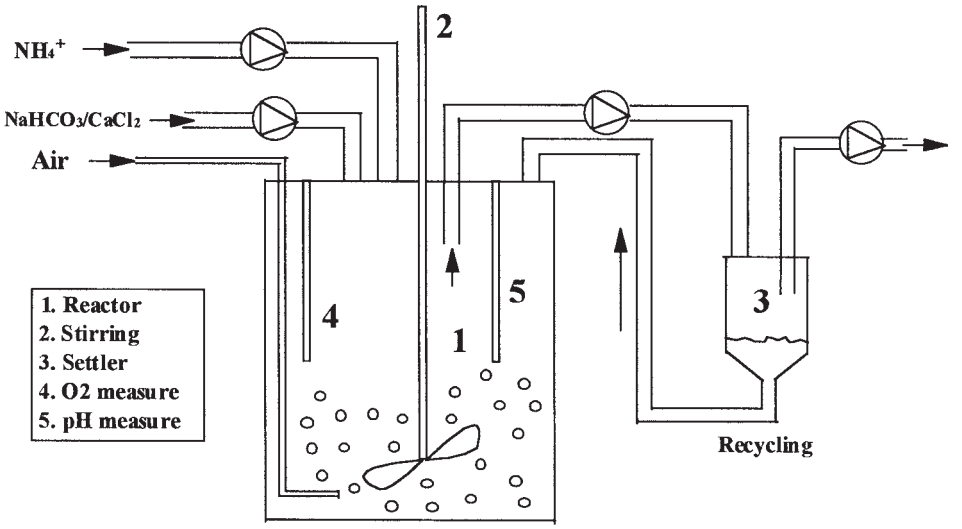

Fig. 1. Schematic representation of the nitrifying unit.

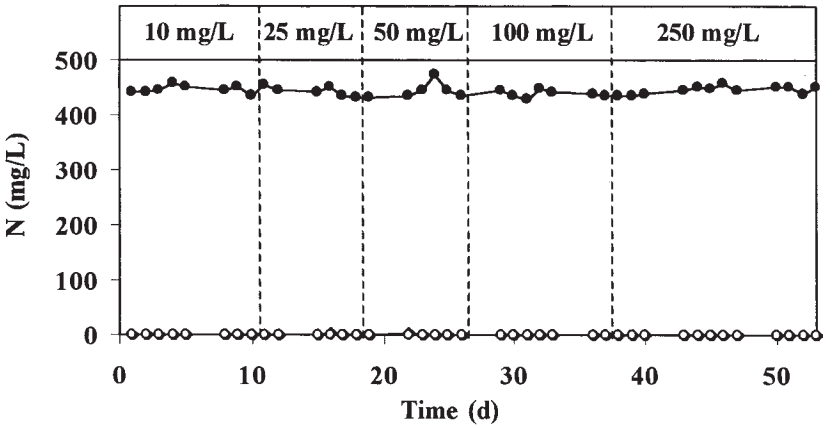

Fig. 2. Evolution of N-NO_3^- (●) and N-NH_4^+ (○) in the effluent during the experiment with chloramphenicol. The concentrations of the antibiotic supplied are given at the top.

3. Days 60–104: Oxytetracycline hydrochloride was added to the influent and its concentration increased stepwise (10, 50, 100, 250, 0 mg/L), as shown in Fig. 3.

The range of antibiotic concentrations was chosen to cover the different amounts of these compounds that may be found in the effluents of farms. The concentration of antibiotics added to the influent, during periods 1 and 3, was modified once the concentrations of nitrogen compounds in the reactor had reached a stationary state.

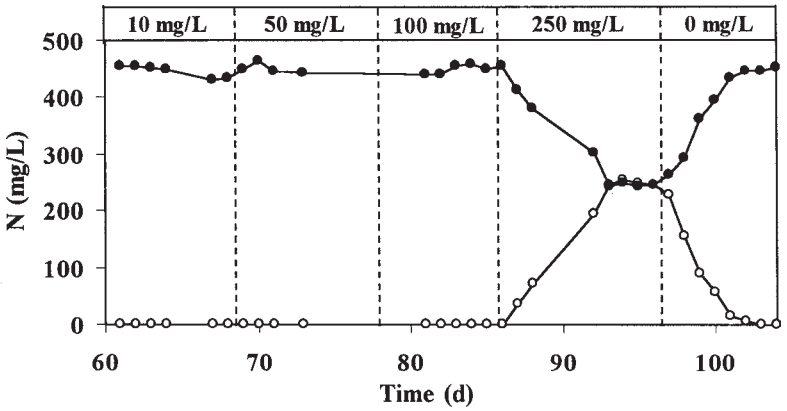

Fig. 3. Evolution of N-NO_3^- (●) and N-NH_4^+ (○) during the experiment with oxytetracycline. The concentrations of the antibiotic supplied are given at the top.

Results and Discussion

Effects of Chloramphenicol (d 0–53)

Before operating d 0, the reactor had been started up and fed with the autotrophic solution, which also contained a mixture of volatile fatty acids (0.4 g/L of acetic acid, 0.1 g/L of butyric acid and 0.1 g/L of propionic acid). This enhanced the formation of a biofilm on the inner glass walls of the reactor, because these organic compounds stimulate the formation of bacterial films. The addition of volatile fatty acids was discontinued once the inner glass wall was fully covered with a biofilm. Day 0 is taken as the day this research started with the addition of chloramphenicol to the influent.

During this stage, we used an antibiotic concentration of 10 mg/L. Afterward, the concentration was increased stepwise to 250 mg/L (Fig. 2). The evolution of the concentrations of nitrogen compounds during the operational period is shown in Fig. 2. Neither conversion of ammonia to nitrite nor nitrite to nitrate was affected by the presence of chloramphenicol. In addition, no change on the surface area covered by the nitrifying biofilm was observed during this stage. There is limited bibliographic information about the effects of antibiotics on mixed nitrifying culture systems *(9,10)* and other mixed microbiologic systems *(3,5,8,21,22)*. Results obtained during the present study are similar to those of Gómez et al. *(9)* using batch assays. They observed that a suspended nitrifying sludge was not inhibited by a lower chloramphenicol concentration of 50 mg/L. Nevertheless, Okpokwasili et al. *(10)* reported the inhibition of pure cultures of *Nitrosomonas* and *Nitrobacter* by chloramphenicol at concentrations of 13.3 mg/L in batch assays. For the anaerobic sludge from a methanogenic reactor, Poels et al. *(3)* observed no effect on organic matter conversion with concentrations up to 166 mg/L of this antibiotic. However, for other anaerobic sludge, Sanz et al. *(5)* observed that this antibiotic strongly inhibited methanogenic activity during the batch assays; a concentration of 25 mg/L

caused an inhibition of 90%. They also noticed that a continuous addition of 40 mg/L of chloramphenicol to an anaerobic reactor totally inhibited methanogenesis activity. Furthermore, acclimation was not observed because the system could not recover its efficiency even when the addition of the antibiotic in the feed was stopped. This last result is quite different from the effects observed by Poels et al. *(3)*, but differences could be related to environmental conditions, adaptation of microorganisms, or differences in hydrodynamic conditions between both experiments. Lai et al. *(22)* found that the effects of this antibiotic depended strongly on the capacity of the microorganisms for acclimation. Other studies concerning the effects of this antibiotic on mixed cultures noted an inhibition of 68% of the Anammox activity of an anammox sludge at 200 mg/L *(8)* and the total inhibition of a denitrifying sludge at a concentration of 300 mg/L *(21)*.

Results of antibiotic activity tests showed no presence of activity in the effluent. The DOC concentration was measured in both the influent and the effluent to determine whether some transformation or accumulation of antibiotic had taken place in the reactor. The DOC concentration in the influent and the effluent were similar during the entire experimental period, chloramphenicol being the only organic carbon source in the influent. This indicated that neither accumulation nor mineralization of chloramphenicol or degradation products to CO_2 occurred in the reactor. An experimental control without biomass was carried out in the system in order to discard some environmental effects on the stability of the antibiotic. Results of these experiments showed that degradation of antibiotic did not take place; therefore, it may be concluded that this compound is biologically inactivated. Since nitrifiers caused the loss of the antibiotic activity, it might be possible to treat a wastewater containing this antibiotic using a postdenitrification treatment system.

DOC analysis and activity tests in the effluent showed that the antibiotic had undergone some changes in the reactor. High-performance liquid chromatography (HPLC) analysis of the effluent revealed differences in the chromatograms of the influent and the effluent. Analytical attempts were made to determine the structure of the degradation products by mass spectroscopy and nuclear magnetic resonance (NMR). Results obtained by mass spectroscopy (MS) analysis indicated that the antibiotic (Fig. 4) had lost the chloride atoms, and that there was an odd number of nitrogen atoms, a possible reduction in the nitro to an amino group, and a minimum of two carbonyl groups. NMR analysis was not carried out because samples might not be dissolved in the different deuterated solvents tested (chlorophorm, deuterated water, and methyl sulfoxide). Bacteria may develop several protecting mechanisms such as the reduction of the permeability *(23)*, the activated flux, or the degradation of the compound *(24)* to avoid the action of this antibiotic. The results obtained seemed to indicate that nitrifying bacteria, in our culture, possibly use the last mechanism.

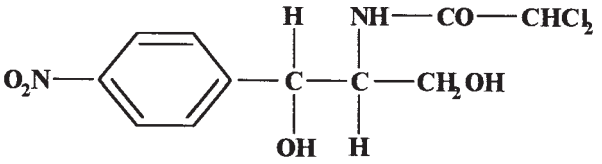

Fig. 4. Structure of the chloramphenicol molecule.

Effects of Oxytetracycline Chlorohydrate (d 60–104)

The research during this stage began with an antibiotic concentration of 10 mg/L in the influent, with the concentration increased stepwise to 250 mg/L (Fig. 3). The evolution of the concentrations of nitrogen compounds during this stage is shown in Fig. 3.

Massive biofilm sloughing was observed 3 d after the beginning of the addition of oxytetracycline, when the concentration was 10 mg/L. The biofilm was completely detached from the wall, indicating that biofilm reactors are not be suitable for treating wastewaters with this antibiotic. However, at 10 mg/L antibiotic conversion was not affected, because an external settler was used, which avoided the washout of the nitrifying biomass. Ammonia was fully oxidized up to an oxytetracycline concentration of 100 mg/L. Nevertheless, a decrease in the efficiency, to 50%, was detected after the concentration was increased from 100 to 250 mg/L (Fig. 3). The addition of oxytetracycline to the influent was discontinued on d 96, in order to check whether the decrease in the activity was persistent or not. Nitrification activity was progressively recovered in 5 d, but formation of a new biofilm was not observed. Nitrite did not accumulate during the period in which ammonia was present in the effluent. There was a partial inhibition of the ammonia-oxidizing microorganisms, but apparently not of nitrite oxidizers. However, Gómez et al. *(9)*, in batch assays with mixed nitrifying sludge, reported that oxytetracycline up to 250 mg/L had no effect on either biomass production or ammonia oxidation. Sanz et al. *(5)* observed that this antibiotic was a powerful inhibitor of anaerobic digestion causing an inhibition of 80% at a concentration of 152 mg/L.

Results of the activity tests showed that no active antibiotic was present in the effluent. Yamaguchi et al. *(25)* observed accumulation of a similar antibiotic, tetracycline, in an *E. coli* pure culture. However, analysis of DOC in the influent and effluent has shown that oxytetracycline hydrochloride, as happened with chloramphenicol, was neither mineralized to CO_2 nor accumulated in the reactor. In addition, a change in the color of the solution from bright yellow in the influent to brown in the effluent indicated possible degradation processes of this compound. These results indicated that degradation products of the antibiotic might be responsible for the inhibition of nitrification. In this case, neither HPLC nor NMR was able to identify these products. These results may suggest that oxytetracycline was degraded by the bacteria, as happened in the case of chloramphenicol.

Park and Levy *(26)* also found that oxytetracycline had been degraded by a mutated strain of *E. coli*.

Data obtained between d 85 and 104 may be used to relate the ammonia inhibition percentage with oxytetracycline in the reactor. If the fermentor is considered a completely stirred tank reactor and the mass balance of the antibiotic in the system is solved, it is possible to obtain an equation (Eq. 1) that describes the evolution of the antibiotic concentration in the system over time, after a change in the oxytetracycline concentration in the feed (Eq. 1):

$$C = C_i - (C_i - C_{i,o}) \cdot e^{-t/HRT} \quad (1)$$

in which C is the concentration of antibiotic in the reactor, C_i is the concentration actually applied to the influent, $C_{i,o}$ is the concentration applied previously to the influent, t is time, and HRT is the hydraulic retention time.

Obviously, the antibiotic in the reactor underwent transformations to intermediate products, but it is possible to solve the mass balance since these intermediate products were not mineralized. In addition, a linear relationship between the antibiotic and its degradation products is assumed. The evolution of the antibiotic concentration in the reactor, after d 85, when it was raised from 100 to 250 mg/L, may be represented according to Eq. 1, with a C_i of 250 mg/L, $C_{i,o}$ of 100 mg/L, and HRT of 3.3 d (Eq. 2):

$$C = 250 - 150 \cdot e^{-t/3.3} \quad (2)$$

The evolution of the antibiotic after d 96, when oxytetracycline concentration was modified from 250 to 0 mg/L, maintaining an HRT of 3.3 d is calculated as follows (Eq. 3):

$$C = 250 \cdot e^{-t/3.3} \quad (3)$$

When Eqs. 2 and 3 and the ammonia concentration in the effluent are taken into account, the nitrification inhibition percentage may be plotted vs the concentration of antibiotic in the reactor (Fig. 5). The graphic representations of both equations do not coincide (hysteresis effect). For the increase from 100 to 250 mg/L, there was a significant effect on nitrification at about 150 mg/L. Fifty percent inhibition was obtained when the concentration in the reactor was about 210 mg/L and remained constant at antibiotic concentrations between 210 and 250 mg/L. Results from Eq. 3, when addition of antibiotic to the influent ceased, showed that inhibition remained at 50% until the antibiotic concentration decreased to 100 mg/L. Ammonia was almost completely oxidized when the concentrations were <50 mg/L.

Conclusion

The effects of two broad-spectrum antibiotics, chloramphenicol and oxytetracycline chlorohydrate, on the biofilm stability or nitrification of a mixed nitrifying culture were different. Additions of up to 250 mg/L of chloramphenicol had barely any effect on both biofilm stability and nitrification, with ammonia being fully oxidized to nitrate. However, oxytet-

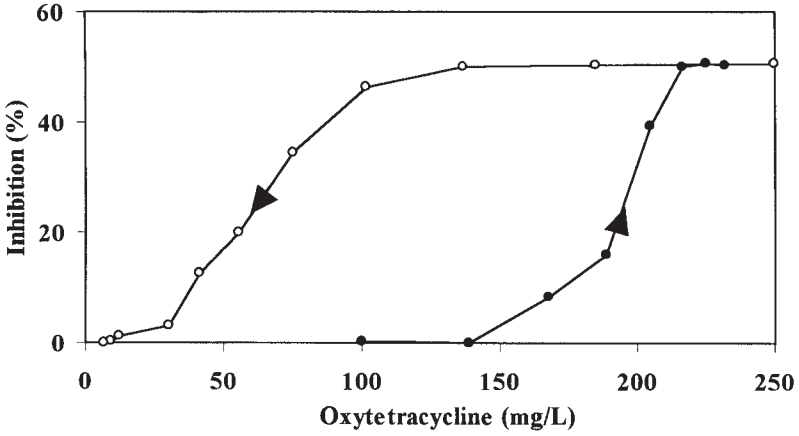

Fig. 5. Percentages of inhibition related to the oxytetracycline concentration supplied during the increase from 100 to 250 mg/L (●) and during the decrease from 250 to 0 mg/L (○).

racycline caused permanent biofilm sloughing at a concentration of 10 mg/L, but nitrification was not inhibited when the antibiotic concentration in the feeding medium was <100 mg/L. Up to 50% inhibition of nitrification was found when the concentration of oxytetracycline chlorohydrate was increased stepwise from 100 to 250 mg/L.

DOC measurements in both the influent and effluent showed that both antibiotics were neither mineralized by the mixed nitrifying culture nor accumulated in the system. Furthermore, microbiologic tests did not reveal the presence of active antibiotics in the effluent. This indicates that both cloramphenicol and oxytetracycline were degraded by the microorganisms but not mineralized. Neither NMR nor HPLC analysis was able to identify the degradation products of these antibiotics. MS analysis of the degradation products of the chloramphenicol molecule showed that it was dechlorinated and possibly reduced one of the nitro group, and the final products had an odd number of nitrogen atoms and a minimum of two carbonyl groups.

Inhibition of denitrification by chloramphenicol has been reported by other investigators *(21)*, but because nitrifiers might cause a decrease in the antibiotic activity, it would be interesting to use a postdenitrification treatment system to treat wastewater containing nitrogen compounds and this antibiotic. Experiments with oxytetracycline indicated that the utilization of biofilm systems to treat wastewater containing this antibiotic might be problematic, because the biofilm could be lost because of sloughing, which causes biomass washout.

Acknowledgments

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References

1. Wiesmann, U. (1994), in *Advances in Biochemical Engineering, vol. 51*, Fletcher A., ed., Springer Verlag, Berlin, pp. 99–108.
2. Kari, F. G., Eckhardt, A., and Holl, U. (1999), *Gas, Wasser, Abwasser* **79(6),** 443–453.
3. Poels, J., van Assche, P., and Verstraete, W. (1984), *Agric. Wastes* **9,** 239–247.
4. Campubrí, M., París, J. M., and Casas, C. (1988), in *Anaerobic Digestion*, Hall, E. R. and Hobson, P. N., eds., Pergamon, Oxford, UK, pp. 329–348.
5. Sanz, J. L., Rodríguez, N., and Amils, R. (1993), *Appl. Microbiol. Biotechnol.* **46,** 587–592.
6. Zabranska, X., Jencek, P., and Dohanyos, M. (1994), *Water Sci. Technol.* **30(3),** 103–107.
7. Hilpert, R., Winter, J., and Kandler, O. (1984), *Agric. Wastes* **10,** 103–116.
8. van de Graaf, A. A., Mulder, A., Jetten, M. S. M., Robertson, L. A., and Kuenen, J. G. (1995), *Appl. Environ. Microbiol.* **61,** 1246–1251.
9. Gómez, J., Méndez, R., and Lema, J. M. (1996), *Appl. Biochem. Biotechnol.* **57–58,** 869–876.
10. Okpokwasili, G. C. and Eleke, F. N. (1997), *J. Natl. Sci. Counc. Sri Lanka* **25(4),** 231–240.
11. Deng, L., Peng, Z., Tang, Y., and Huang, Z. (1998), *Huanjing Kexue* **19(6),** 66–69.
12. Jiang, Y. and Hu, G. (1999), *Taiyangneng Xuebao* **20(2),** 183–186.
13. Sharma, S., Ramakrishna, C., Desai, J. D., and Bhatt, N. M. (1994), *Appl. Microbiol. Biotechnol.* **40(5),** 768–771.
14. Vredenbregt, L. H. J, Nielsen, K., Potma, A. A., Kristensen, G. H., and Sund, C. (1997), *Water Sci. Technol.* **36(1),** 93–100.
15. Boot, T. R. (1992), in *Biofilms—Science and Technology*, Melo, L. F., Bott, T. R., Fletcher, M., et al., eds., Kluwer Academic, Dordrecht, The Netherlands, pp. 567–581.
16. Wobus, A. and Röske, I. (2000), *Water Res.* **34(1),** 279–287.
17. APHA-AWWA-WPCF, eds. (1985), *Standard Methods for Examination of Water and Wastewater*, 16th ed. Washington, DC.
18. Vilas-Cruz, M., Gómez, G., Méndez, R., and Lema, J. M. (1994), in *Proceedings of the III International Symposium of Analytical Methodology for the Environment*, vol. 2, Barcelona, Spain, p. 50.
19. The United States Pharmacopeia (1990), *The National Formulary: NF XVII*, 22nd ed., Board of Trustees, Rockville, MD.
20. Vishniac, W. and Santer, M. (1957), *Bacteriol. Rev.* **21,** 199.
21. Brooks, M. H., Smith, R. L., and Macalady, D. L. (1992), *Appl. Environ. Microbiol.* **58,** 1746–1753.
22. Lai, H., Liu, S. M., and Chien, Y. H. (1994), Abstr. Gen. Meet. Am. Soc. Microbiol., *American Society for Microbiology*, eds., Washington, DC, p. 464.
23. Toro, C., Lobos, S., Calderón, Y., Rodríguez, M., and Mora, G. (1990), *Antimicrob. Agents Chemother.* **34(9),** 1715–1719.
24. Davies, J. and Mazel, D. (1997), *Biofutur* **170,** 14–17.
25. Yamaguchi, A., Ohmori, H., Nomura, T., and Sawai, T. (1991), *Agents Chemother.* **35(1),** 53–56.
26. Park, B. H. and Levy, S. B. (1988), *Antimicrob. Agents Chemother.* **32(12),** 1797–1800.