0273-2289/96/5758—0869$7.00

The Effect of Antibiotics on Nitrification Processes

Batch Assays

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ABSTRACT

The effect of different antibiotics at several concentrations of ampicillin (0–250 mg/L), benzylpenicillin (0–250 mg/L), novobiocine (0–150 mg/L), oxytetracycline (0–250 mg/L), and chloramphenicol (0–50 mg/L) on a stabilized nitrifying sludge was evaluated under aerated and lithoautotrophic conditions. No effect resulting from the presence of antibiotics on the biomass and nitrate production was noticed. The specific growth rate and volumetric nitrification rate average values for the controls were $8.28 \times 10^{-3}/h^{-1}$ and 2.74×10^{-3} g/L·h, respectively. Similar rate values were found when different kinds of antibiotic and concentrations were tested. These results may be explained by the nature of the floc or the instability of the antibiotics.

Index Entries: Antibiotics; nitrification; waste water; sludge.

INTRODUCTION

Organic carbon and nitrogen are the most important pollutants in both municipal and industrial waste water. Efficient carbon removal is usually carried out by anaerobic or aerobic processes. However, nitrogen removal is a slow process with poor efficiency especially in waste water with high ammonia content *(1)*. Biological nitrogen removal is a two-step reaction: (1) An aerobic biological oxidation, in which nitrogen is oxidized to NO_3^- (nitrification process); and (2) a nitrate reduction to N_2 in the denitrification process *(2,3)*.

The rate-limiting step of nitrogen removal through nitrification is the sensitivity of the nitrification process to many factors, such as the substrate (ammonia-nitrogen) and the product concentrations (nitrite, nitrate), pH, and others *(4)*. Since nitrification is an obligate aerobic-lithoautotrophic process, the presence of organic compounds even at low concentrations may cause a decrease in the nitrification rate *(5)*.

Waste waters from the food and fertilizer industries or piggery manure have high nitrogen concentrations and very often contain substances, such as antibiotics, at high concentrations *(6)*. For instance, monensin is routinely used as animal food

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to increase the productivity; chlortetracycline is added to fodder to prevent liver infection in animals *(7)*. On many occasions, antibiotics are only partially metabolized by animals, and therefore, it is possible to find them in waste waters.

The effect of antibiotics on microorganisms is observed at different levels. Accordingly, it is possible to find antimicrobials that can inhibit cell-wall, protein, or nucleic acid synthesis *(8)*. Thus, the biological action of antibiotics on a particular sludge may present different effects *(9)*. Nitrifying sludge may be affected either in its microbial growth rate or in its nitrification rate. Nitrifying microorganisms are gram-negative *(10)*, although the nitrifying sludge involves a consortium of many microorganisms that may be affected in different ways.

It was reported early that methanogenesis may be affected by the presence of antibiotics, and the level of inhibition depended on the type of antibiotic and its concentration *(9,11,12)*. Information available regarding the effect of antimicrobials on nitrification is very scanty. The object of this work was to examine the effect of five antibiotics at different concentrations on the nitrification process using a stabilized nitrifying sludge under lithoautotrophic and aerated culture conditions.

MATERIAL AND METHODS

Analytical Methods

Growth of the biomass in the sludge cultures was followed by measuring protein *(13)*: 1 mL of suspension was digested at 90°C for 15 min using 0.1 mL of 10*N* NaOH, and the blue color was measured at 750 nm in UV-visible spectrophotometer (Shimatzu UV-160A). The protein of the culture was correlated to a standard curve that was prepared with a pure bovine serum albumin (Sigma Co., St. Louis, MO). The variation coefficient of the method was of 4%. Nitrite and nitrate were measured by capillary electrophoresis (Waters Quanta 4000) using a microcapillary tube (fused silica, 50 cm × 75 μ). Samples were filtered through a 0.45-μ cellulose membrane. Electrostatic application, at 20 kV, 38 μA, and UV zinc lamp of fixed wavelength of 214 nm were the analytical conditions employed. Sodium sulfate solution 10 m*M* was employed as the electrolyte. Reproducibility of the results was >97% throughout the experimental work. Initial and final concentration of NH_4^+-N consumption was measured by specific ammonia electrode *(14)* and compared to a standard curve prepared from ammonium chloride from 10 to 1000 ppm of NH_4^+-N. The response of the electrode followed a linear relationships with the range of concentration used, and the variation coefficient of the slope was of 5%. Each sample of culture was diluted with deionized water up to 50 mL and then 0.5 mL of 10*M* NaOH was added.

Inoculum

The nitrifying inoculum was taken from a 2-L continuous aerated reactor (type Hussman, with recycled biomass) operating in steady-state conditions at a hydraulic retention time (HRT) of 3.3 d, at pH 7.7, and stirred in a shaker at 290 rpm. The temperature was kept constant at 26°C, and the O_2 concentration was maintained at 70% of the saturation value. In order to decrease precipitation and light oxidation, the culture medium for the continuous nitrification was separated in two opaque plastic flasks. One of them had (g/L): $(NH_4)_2SO_4$, 1.18; NH_4Cl, 0.956; NaCl, 0.2; K_2HPO_4, 1.4; $MgSO_4$, 0.6; and $FeSO_4$ 0·15; and the other had (g/L) $CaCl_2 \cdot 2H_2O$, 0.4

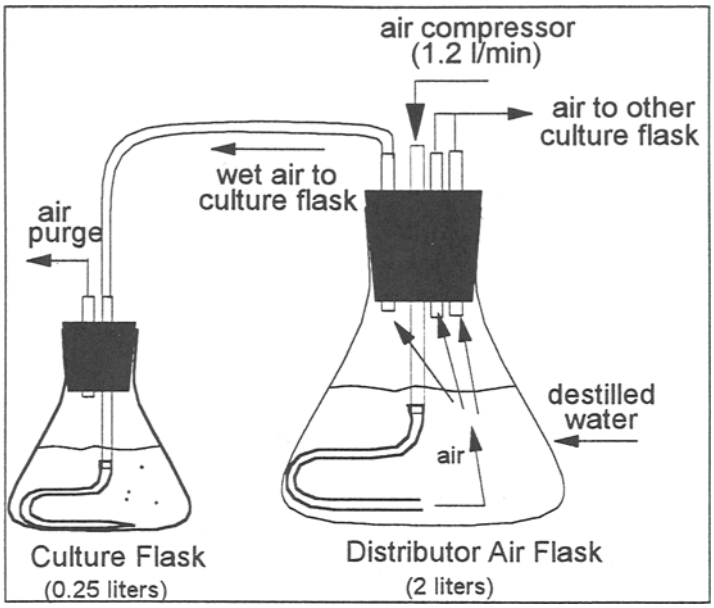

Fig. 1. System employed for batch culture nitrification in aerated conditions. The work volume was 110 mL. Flasks were agitated at 290 rpm and incubated at 26°C.

and $NaHCO_3$, 40. The same stationary conditions were maintained during 6 mo in order to reach a complete stabilization of the nitrifying microflora. The concentrations of nitrate and microbial protein inside the reactor were 1.80 ± 13 g/L and 0.375 ± 0.025 g/L, respectively. The nitrification rate under this condition was 2.91 g NO_3-N/g N_{prot}·d. NH^+_4-N removal was above 95% with an inlet concentration of 0.5 g NH_4^+-N/L. Nitrite was not observed when operating under steady-state conditions.

Batch Assays

The experiments were carried out in 250-mL flasks, as shown in Fig. 1, which were kept on a shaker (190 rpm at 26°C). Each flask was inoculated with 22 mL of nitrifying sludge, accounting for a 20% of the total working volume and 88 mL of lithoautotrophic basal medium. In order to decrease error in the inoculation level, the source of inoculum was stirred for 10 min at 500 rpm. The variation in the inoculated biomass into various flasks was <5%. Air was sparged continuously into the flasks by means of a compressor at a constant flow rate, in order to assure an efficient oxygenation. The initial pH was adjusted to 7.7 with 10*M* NaOH. During the nitrification process, the initial pH increased to a more alkaline value, but was again adjusted to 7.7 using 5*M* HCl. All the experiments were carried out in the absence of light.

The chemical composition of the basal medium was (g/L): $(NH_4)_2SO_4$, 0.59; NH_4Cl, 0.47; NaCl, 0.1; $FeSO_4·H_2O$, 0.15; $CaCl_2·2H_2O$, 0.1; K_2HPO_4, 0.7; $MgSO_4$, 0.3; $NaHCO_3$, 5. $FeSO_4$ was added at the time of inoculation to decrease loss of Fe^{2+} owing to its rapid oxidation to avoid culture medium turning black.

Table 1
Specific Growth and Volumetric Nitrification Rates of the Nitrifying Sludge in the Presence of Different Kinds of Antibiotics at Different Concentrations, Under Alkaline pH and Aerated Culture Conditions

Antibiotic	Concentration, mg/L	μ, h^{-1}	Q, $gNO^-_3/L \cdot h$
Benzylpenicillin	0–250	$8.2 \times 10^{-3} \pm 8.9 \times 10^{-5}$	$2.95 \times 10^{-3} \pm 5.5 \times 10^{-5}$
Chloramphenicol	0–50	$8.3 \times 10^{-3} \pm 9.8 \times 10^{-5}$	$2.57 \times 10^{-3} \pm 5.2 \times 10^{-5}$
Ampicillin	0–250	$8.3 \times 10^{-3} \pm 7.5 \times 10^{-5}$	$2.93 \times 10^{-3} \pm 1.0 \times 10^{-4}$
Oxtetracycline	0–250	$8.2 \times 10^{-3} \pm 5.5 \times 10^{-5}$	$3.15 \times 10^{-3} \pm 8.4 \times 10^{-5}$
Novobiocine	0–150	$8.2 \times 10^{-3} \pm 9.8 \times 10^{-5}$	$2.41 \times 10^{-3} \pm 9.8 \times 10^{-5}$

The antibiotics tested were: chloramphenicol (Analema, 99.85%); oxytetracycline-HCl (Analema, SA of 0.879 g antibiotic/g protein); novobiocine (Fluka, 95%); benzylpenicillin sodium salt (Fluka, 99%); and trihydrated ampicillin (Analema, 99%). The concentration range chosen (Table 1) for each antibiotic supplied to culture medium was established from the literature *(15)*.

The maximum specific growth rate μ (h^{-1}) of the microorganisms was calculated in the exponential growth phase by means of the equation: $\mu = (1/t)\ \mathrm{Ln}\ (x/x_0)$, where t is the time (h), x_0 the initial microbial concentration of inoculum (measured as g/L of protein), and the x is the protein concentration at any time (g/L). The volumetric nitrification rate, Q (g/L·h) of the process was determined after the end of the lag phase as $Q = \Delta NO_3^-/t$, where ΔNO_3^- is the increase of nitrate concentration (g/L), in the elapsed time (t).

RESULTS AND DISCUSSION

Different kinds of antibiotics may be found in waste waters, and consequently, different physiological effects on the nitrifying sludge may be produced. Because the nitrifying sludge is a complex microbial consortium, it was important to study antibiotics with different biochemical actions on the microbial flora. In all cases, the measurement of the NH_4^+-N concentration at the end of the experiment was approx 6% of the initial value.

The microbial growth in cultures containing different concentrations of chloramphenicol is shown in Fig. 2. It can be seen that within the range studied, the microbial growth pattern was practically the same in all flasks. It was evident that no effect owing to the presence of antibiotic was noticed. The nitrification profiles in this same experiment are shown in Fig. 3. The nitrate produced was similar in the control and with 50 mg/L of antibiotic. Chloramphenicol is reported to exert a drastic inhibitory effect on the anaerobic digestion process *(11,16)*, however, this effect was not observed in our studies.

Nitrification under several concentrations of ampicillin is shown in Fig. 4. As in the previous case, nitrate content was similar to that in the control. When benzylpenicillin, novobiocine, and oxytetracycline were tested at the same culture conditions, similar nitrification profiles were observed (Fig. 4).

Microbial growth profiles in the presence of various concentrations of ampicillin give the impression of increased protein synthesis, as can be seen in Fig. 5. However, it should be remembered that the level of inoculation was the same in all cases (20%

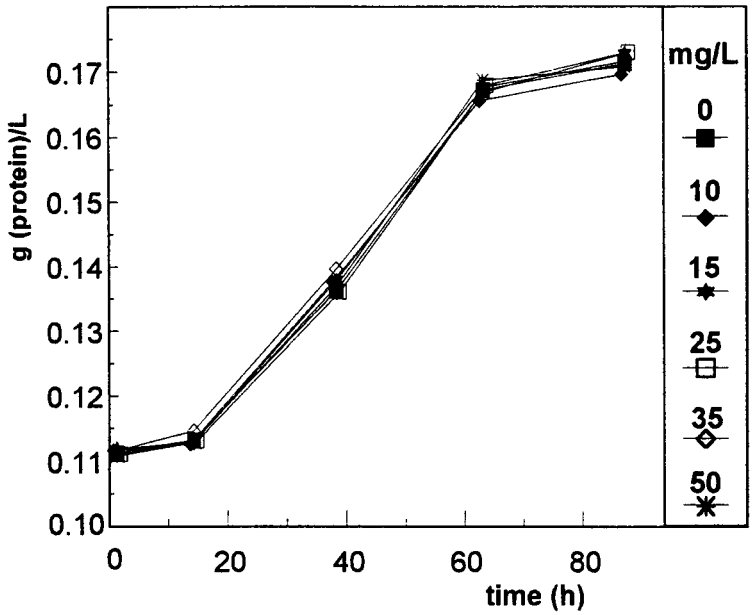

Fig. 2. Growth of the microflora measured as protein of the nitrifying sludge in the presence of different concentrations of chloramphenicol. Symbols at the right margin represent the antibiotic concentration.

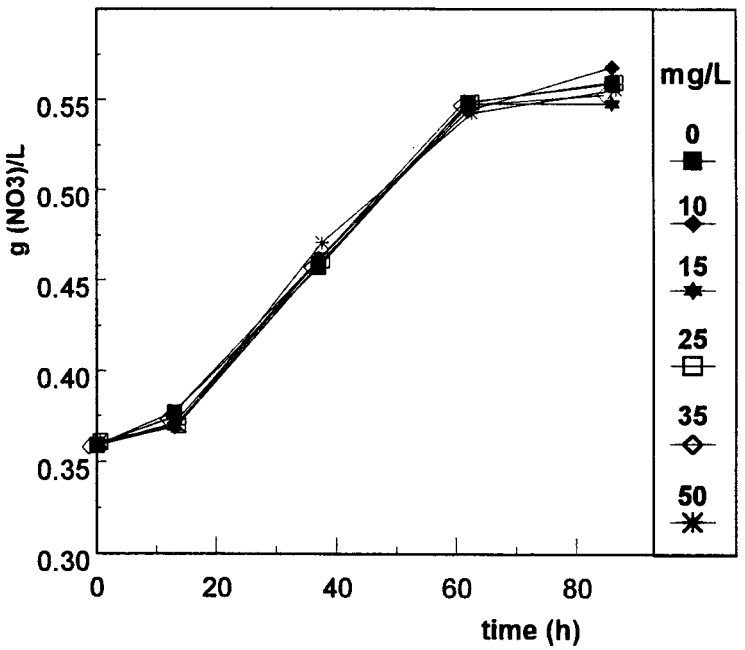

Fig. 3. Nitrate formation at different concentrations of chloramphenicol. Symbols at the right margin indicate the antibiotic concentration.

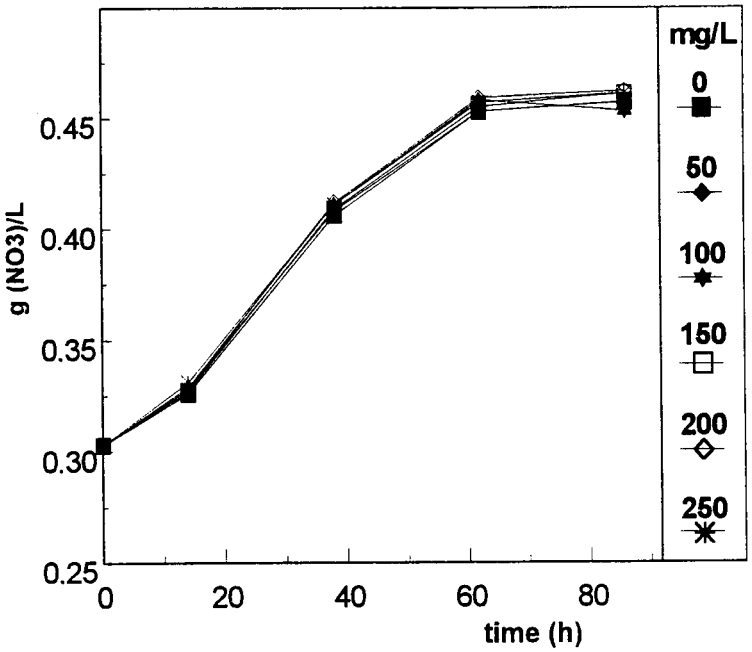

Fig. 4. Nitrate production with a nitrifying sludge at different concentrations of ampicillin. Symbols at the right represent the antibiotic concentration.

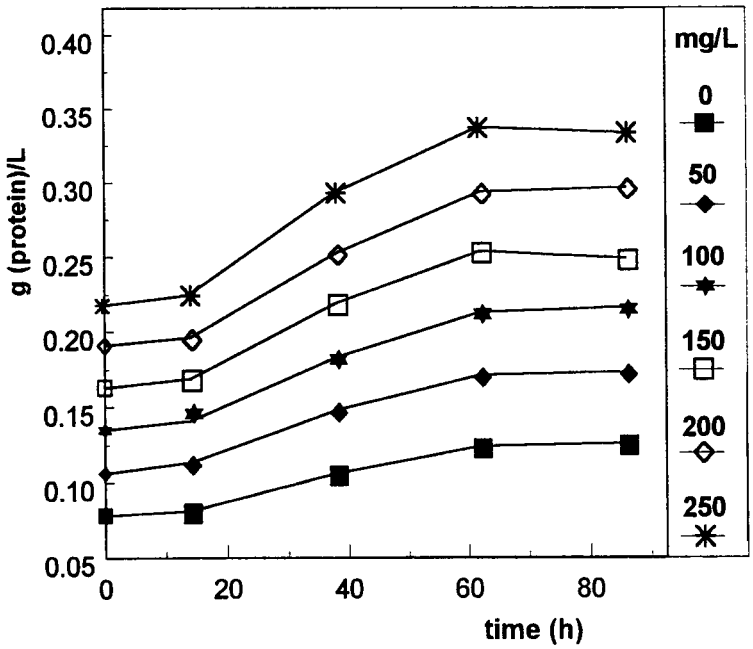

Fig. 5. Microbial growth patterns measured as protein of the nitrifying sludge at different concentrations of ampicillin. Symbols at the right show the concentration of the antibiotic.

of total volume), and one would not expect any difference in protein levels. These increases were probably the result of the presence of antimicrobial drugs at different concentrations. However it did not affect the determination of the specific rate values of the microbial growth in any case, as can be corroborated by the data of growth rate presented in Table 1. The average of specific growth rate, μ, and volumetric nitrification rate, Q, for all antibiotics and control were $8.28 \times 10^{-3} \pm 8.4 \times 10^{-5}$ h^{-1} and $2.74 \times 10^{-3} \pm 2.8 \times 10^{-4}$ g/L·h, respectively. Comparing this value with those of Table 1, it may also be stated that no significant effect was produced on the nitrification rate process.

The β-lactamic antibiotics are known to inhibit cell-wall development in gram-positive microorganisms, and obviously no effect on the nitrifier microorganisms was expected. However, the nitrifying sludge is a mixed culture the microorganisms that are sensitive to cell-wall development and should have been inhibited changing the physiological behavior of the nitrifying sludge. However, on the contrary, the nitrification profiles were very similar in all cases, including control. These results might be related to the microbial interactions within the consortium, as well as the culture conditions, or to the chemical structure of the floc.

Nitrification takes place at alkaline pH, but β-lactamic antibiotics are unstable under these conditions. Furthermore, the stability of β-lactamic drugs decreases in aqueous solution. A similar situation is also seen with novobiocine. Since nitrification was carried out under high aeration, which might also have contributed to the increased instability of the antibiotics. Work is being carried out in our laboratory to elucidate the fate of the antibiotics during the nitrification process.

The nitrifying microorganisms are considered floc forming, similar to the methanogenic flora. The main difference between these two sludges is in the operating redox potential, where the former is positive and the latter is negative. It has been generally considered that the floc is a protective and selective barrier *(17)* and, hence, might explain why the nitrifying sludge was not sensitive to the antibiotics studied here.

Antibiotic accumulation can affect the global nitrification and denitrification processess. It has been mentioned that denitrification is strongly inhibited in presence of chloramphenicol *(18)*, so that nitrogen removal from waste waters might be scarcely performed if the antimicrobials are accumulated in the floc. Preliminary studies in our laboratory indicate that in the nitrification process, chloramphenicol is modified in some way that its antimicrobial activity is lost. Further work is being done to know which are the new compounds produced through nitrification. It will also be important to know if any changes on the nitrifying population owing to the presence of the antibiotic were effected.

It can be concluded that none of the antibiotics tested here, whether protein inhibitor (chloramphenicol and oxytetracycline) β-lactamic cell-wall inhibitors (benzylpenicillin and ampicillin), or DNA inhibitor (novobiocine), had any effect on either biomass production or nitrite formation. Likewise, the specific growth rate and the volumetric nitrification rate of the nitrifying sludge with different antibiotics at various concentrations were similar to those of control, indicating no change.

ACKNOWLEDGMENT

Funding for this work was provided by the European Union (grant No. CI1-CT93-0040). The National Council of Science and Technology of Mexico supported the stay of Jorge Gomez in the Santiago de Compostela University. Oscar Monroy is thanked for helpful discussions.

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