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Acute sensitivity of activated sludge bacteria to erythromycin

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ABSTRACT

The presence of antibiotics in water resources has been disturbing news for the stakeholders who are responsible for public health and the drinking water supply. In many cases, biological wastewater treatment plants are the final opportunity in the water cycle to trap these substances. The sensitivity of activated sludge bacteria to erythromycin, a macrolide widely used in human medicine was investigated in batch toxicity tests using a concentration range of 1–300 mg L^{-1}. Erythromycin, a protein synthesis inhibitor, has been found to significantly inhibit ammonification, nitritation and nitratation at concentrations higher than 20 mg L^{-1}. The degree of inhibition increased with greater concentrations of the antibiotic. Exposure to erythromycin also clearly affected heterotrophs, particularly filamentous bacteria, causing floc disintegration and breakage of filaments. Cell lysis was observed with the concomitant release of organic nitrogen (intracellular proteins) and soluble COD. Although erythromycin exhibits properties of a surfactant, this characteristic alone cannot explain the damage to heterotrophs: the effects from erythromycin were greater than those of Tween 80, a commonly used surfactant. Floc disruption can lead to the release of isolated bacteria, and possibly antibiotic resistance genes, into the environment.

1. Introduction

The occurrence of pharmaceutical substances (primary compounds or human/animal metabolites) in the environment has become a subject of general concern in recent years. Improved analytical techniques have permitted the detection of pharmaceuticals in water resources in various countries [1,2]. These substances are by nature biologically active, hydrophilic and persistent. Human pharmaceuticals are discharged mainly through domestic wastewater (a mixture of urine, feces and water) in the sewer network. Manufacturing facilities can also be an important source if their wastewater treatment is insufficient [3]. Runoff from agricultural facilities is another source of antibiotic contamination [4,5]. One way to avoid the release of pharmaceuticals into the environment [6] and their potential presence in drinking [7] and reclaimed [8] water is to improve their degradation in wastewater treatment systems. There are many discussions, contradictions, and data variability in the literature regarding the fate of these substances in wastewater treatment systems [9], and drawing any general conclusions is difficult due to their diversity. Many of these molecules do not have direct effects on the bacteria involved in wastewater treatment. This is not the case with antibiotics [10], which can also induce damage in aquatic ecosystems [11].

Antibiotics are designed to inactivate bacteria, which are the chief actors in wastewater treatment systems that employ activated sludge. Bacteria exposed to antibiotics over long periods of time can develop resistance, which can then be transferred to pathogenic bacteria [12]. Incomplete degradation or confinement [13] leads to discharge of these antibiotics into water bodies [14,15] where other organisms can acquire resistance [16,17]. The release of antibiotics and their metabolites into the environment increases the risk of developing bacterial resistance. Resistance to these antibiotics has been reported [18], and resistance genes themselves are considered emerging contaminants [19].

Macrolide antibiotics are effective against Gram-positive bacteria as well as some Gram-negatives. They constitute an important treatment alternative in patients allergic to penicillin. Used in both human and veterinary medicine, the macrolides are based on a 14- to 16-member lactone ring with sugars linked via glycosidic bounds. Erythromycin is one of the most commonly used macrolides in human medicine. This compound and its derivatives have been detected in different aquatic compartments (up to 6 μg L^{-1} in wastewater treatment plant effluents [20] and 1 μg L^{-1} in river water [21]), and they affect microbial populations in aquaculture [22]. Erythromycin acts primarily via protein synthesis inhibition. By binding to the 23S rRNA molecule in the 50S ribosomal subunit, the antibiotic inhibits the translocation of peptidyl-tRNA. However, it was observed that Gram-negative *Legionella pneumophila* cells treated with erythromycin had irregular membranes and could be

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partially or fully lysed [23]. At subminimal growth inhibitory doses (0.05 μg L^{-1}), erythromycin has also been reported to repress the production of lectin in *Pseudomonas aeruginosa* [24] and to modify the cell surface structure [25].

Reports on the exact effects of antibiotics on activated sludge are scarce. Halling-Sørensen [26] investigated the effects of some bactericides such as tylosin, a macrolide for veterinary use which is similar to erythromycin, and a half-maximal effective concentration (EC_{50}) of 54.7 mg L^{-1} was determined for this antibiotic. Christensen et al. [27] studied the ecotoxicity of various antibiotics used in aquaculture and an EC50 of 41 mg L^{-1} was found for erythromycin. However only the global effect on activated sludge bacteria was discussed, i.e. without discrimination between heterotrophs and nitrifiers. According to Campos et al. [10], chlorampicol does not hinder nitrification, which is reduced by oxytetracycline. Lincomycin, an antibiotic with an action mode similar to macrolides, inhibited nitrification in the experiments described by Carucci et al. [28]: approximately 80% inhibition was observed in the presence of 10 mg L^{-1}. However, there is no clear position regarding the sensitivity of nitrifiers to toxic substances of any kind. Henze et al. [29] stated that nitrifiers are not more sensitive to toxics than other bacteria, while Principi et al. [30] indicated a higher sensitivity of nitrifiers with respect to metals (copper, zinc and nickel) and Stasinakis et al. [31] showed similar results with triclosan (a broad spectrum antibacterial agent).

There are two ways to investigate the effect of an antibiotic on a bacterial consortium. Long-term laboratory-scale continuous cultures can be performed using a well-defined synthetic substrate that includes antibiotics. This method can assess the effect of very low antibiotic concentrations, similar to those found in real wastewater. The disadvantage of this method is that the consortium becomes well adapted to a synthetic, easily biodegradable substrate, which is a significantly different scenario from biomass in a full-scale plant. In contrast, short-term batch experiments performed with biomass and wastewater directly sampled from a plant avoid this problem. The disadvantage of batch experiments is that the concentrations of the antibiotics must be much larger than those found in real wastewater, as the substrate (i.e. the carbon and nitrogen based pollution contained in wastewater) is quickly depleted. Using the latter method, the present work focused on assessing the effects of erythromycin on activated sludge through batchwise short-term toxicity tests. This method is often used in acute toxicity testing. The fate of erythromycin itself in the reactor was not investigated, although it has been previously reported that this antibiotic is relatively stable in wastewater treatment processes, with limited biodegradability [32,33].

2. Materials and methods

2.1. Experimental setup

Four identical batch reactors (3 L working volume) were run in parallel. Experiments were carried out at 20 ± 2 °C, and the reactors were aerated by an air diffuser placed at the bottom. At the beginning of each experiment, each reactor was filled with 1 L of fresh activated sludge. Wastewater and fresh activated sludge were collected (after grid removal and in the recycle line, respectively) from an urban wastewater treatment plant (Nancy-Maxéville, France) for each series of experiments. In this plant, the daily average characteristics of the wastewater are as follows: Chemical Oxygen Demand (COD): 260 mg L^{-1}; Biological Oxygen Demand $(BOD)_5$: 120 mg L^{-1}; Kjeldahl nitrogen (NTK): 30 mg L^{-1}; $N–NH_4$: 18 mg L^{-1}. One reactor was systematically used as the control, and 2 L of wastewater was added to the sludge. Wastewater (2 L) spiked with erythromycin (CAS 114-07-8 from Sigma–Aldrich) was added to the other reactors. The final erythromycin concentration varied between 1 and 267 mg L^{-1}. Different amounts of erythromycin were used in each reactor of a series. Altogether, fifteen 48-h experiments (each including one control and three spiked reactors) were run over a period of 18 months. Replicates were performed at different dates to take into account the variability of the biomass and the wastewater. An experiment was also conducted in the presence of 4×10^{-3} mg L^{-1} of Tween 80 (CAS 9005-65-6 from Sigma–Aldrich).

2.2. Analytical methods

Samples were taken from the reactors every two hours, except at night, for at least 48 h. After filtration (paper filter of pore size ≈1.5 μm), a portion of each sample was used to determine ammonia concentrations using a mini-Nessler method (based on Hach method 8038) on a Hach DR 2400 (Hach, Loveland, Colorado) (error ± 0.5 mg $N–NH_4$ L^{-1}). The remaining part of the sample was used for UV–visible spectroscopic analysis, conducted between 200 and 600 nm (Anthelie Light, Secomam, Domont, France) with 1 cm-path quartz cuvettes and synchronous fluorescence (Hitachi F2500). Absorbance at 254 nm (A_{254}) was found to be a suitable surrogate parameter to monitor dissolved chemical oxygen demand on small samples [34]. The tryptophan-like fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 330 nm was used to monitor the fate of soluble organic nitrogen, which is linked to human urine in domestic wastewater [35]. Nitrate concentrations were obtained either by application of the second-derivative technique on the UV–vis spectra [36], the cadmium reduction method (Hach method 8039), or by ion chromatography (Dionex) (error ± 1 mg $N–NO_3$ L^{-1}). Nitrites were measured by the cadmium reduction method (Hach method 8192) after 10- or 20-fold dilution with a Hamilton automated diluter (error ± 0.02 or 0.04 mg L^{-1}, depending upon dilution), or by ion chromatography. Total solids were quantified by drying 25 mL of mixed liquor at 105 °C for 24 h. Total volatile solids were measured by calcination at 550 °C of the residue for 2 h. Their amount is a rough approximation of the organic matter present in the sample.

2.3. Biomass characterization

For activated sludge morphology assessment, a smear of mixed liquor (100 μL) was carefully spread on a clean glass slide with a wide-bore pipette and thoroughly air dried. The slide was examined at 100× magnification with direct illumination on a Dialux 20 (Leitz, Solms, Germany) microscope equipped with a Sony 3CCD EXWAVE HAD color camera (Aires, Châtillon, France) connected to a PC via a Meteor (Matrox, Dorval, Quebec, Canada) grabbing board. Fifty images (768 × 576 pixels on 24 bits; 1 pixel = 0.63 μm^2) were obtained from each slide by a systematic examination (no overlap of fields). Only the green plane of the color images was considered for morphology assessment. The procedure developed by da Motta et al. [37] was adapted to evaluate filament abundance and floc size. The average values of the total biomass (flocs + filaments), biomass as flocs and as cell debris, and number of filaments detected per analyzed field (≈0.28 mm^2) were determined. Cell debris were defined as objects whose projected surface was smaller than 10 μm^2. The Gram Hucker staining protocol (RAL, Martillac, France) was used to assess the Gram type of filamentous bacteria in some of the runs. A glass slide was examined under oil immersion at 250× magnification as above. The images obtained from each slide were analyzed using the procedure described by Pandolfi and Pons [38], using features embedded within the Visilog 5 software (Noésis, Les Ulis, France).

Table 1
Loss of total solids and total volatile solids during toxicity tests (nd = not determined) after 48 h.

Erythromycin (mg L^{-1})	0 (control reactor)	7	20	30
Total suspended solids lost (%)	3.7	32.6	38	43
Total volatile suspended solids lost (%)	7.4	nd	22.6	nd

2.4. Surface tension

The surface tension of erythromycin and Tween 80 (UltraSigma) solutions (prepared with deionized water) were measured on a Tracker apparatus (Teclis, Longessaigne, France) using the rising bubble method.

3. Results

Foaming was observed immediately at the start of the experiment in reactors containing erythromycin for all tested concentrations. The biomass transferred slowly from the liquid phase to the foam layer, and then to the reactor wall above the waterline. The transfer was more pronounced at higher antibiotic concentrations. For the largest tested concentrations (above 30 mg L^{-1}), the biomass completely transferred to the foam after 48 h. The concentrations of total solids and total volatile solids confirmed that the overall biomass loss via transfer to the foam layer or by death was larger in presence of erythromycin (38% and 22.6%, respectively, for 20 mg L^{-1}) than in the control reactor (3.7% and 7.4%, respectively; Table 1). Slight foaming was observed in the control reactor, probably due to the detergents contained in the raw wastewater used in the experiments.

The deterioration of the activated sludge was monitored by image analysis. Fig. 1 compares the variation over time of the total biomass (Fig. 1a), the biomass as flocs (Fig. 1b), the number of flocs (Fig. 1c), and the total length of filaments per image between the control reactor and a reactor spiked with 20 mg L^{-1} of erythromycin. In the presence of erythromycin, biomass decreased and the number of flocs increased drastically. This indicates a breakdown of the large flocs, which are initially present in the inoculum, with a concomitant release of fragments of filamentous bacteria. The total length of detected filaments (longer than 60 μm) in each image initially increased at the beginning of the experiment, followed by a decrease. Approximately 80% of the filamentous bacteria initially protruding from the flocs were Gram-positive; this percentage remained constant throughout the run for the control reactors as well as in the reactors spiked with 10 and 20 mg L^{-1} of erythromycin.

As shown in Fig. 2, erythromycin inhibited ammonia removal. The degree of inhibition depended upon the antibiotic concentration. There was no noticeable inhibition at 1.25 mg L^{-1} erythromycin. In contrast, inhibition was significant at 2.5 mg L^{-1} erythromycin: after 24 h, the ammonia concentration in the spiked reactor was 6 mg L^{-1} and only 1 mg L^{-1} in the control reactor. However, the ammonia pool was consumed after 48 h. Almost no ammonia consumption was observed at 20 mg L^{-1} of erythromycin, and an apparent increase in ammonia concentration occurred at 267 mg L^{-1} of erythromycin. Nitrification is the result of two sequential steps: ammonia → nitrites (nitritation) followed by nitrites → nitrates (nitratation). In the control reactors, the typical behavior of an A → B → C reaction, where the first step is initially faster than the second, was observed (Fig. 3). In the reactor spiked with 20 mg L^{-1} of erythromycin, the nitrite build-up was very slow. The nitrate production rate decreased as the erythromycin concentration increased (Fig. 4). In the control reactors, the final nitrate concentration was always higher than the predicted concentration calculated from the initial ammonia content, because organic nitrogen contained in the wastewater was ammonified and transformed ultimately into nitrates. In the reactors spiked with erythromycin, the nitrate concentration obtained after 48 h depended upon the balance between nitrification inhibition (which reduces the final concentration) and cell lysis (which increases the nitrogen pool in terms of ammonia and organic nitrogen, thus increasing the final concentration).

The fluorescence intensity of the band at λ_{exc} = 280 nm/λ_{em} = 330 nm corresponds to tryptophan-like proteins contained in human urine, and is a good tracer of organic nitrogen present in domestic sewage. In the control reactors, this fluorescence intensity decreased. In the reactors spiked with erythromycin, the fluorescence intensity tended to decrease during the first hours of the test, but the final level remained higher than in the reference reactors

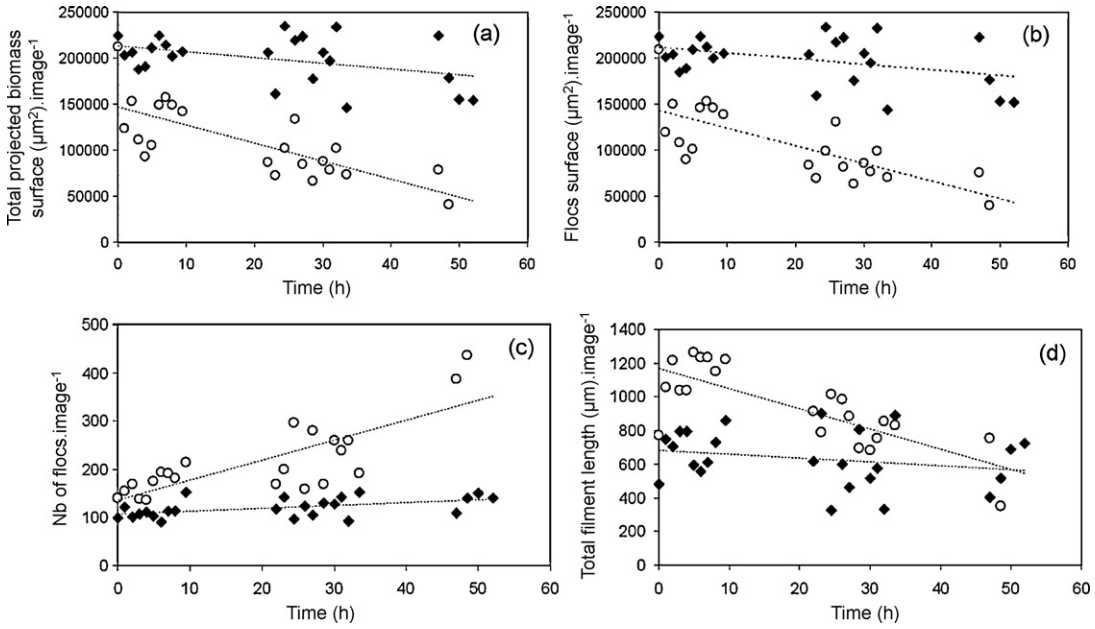

Fig. 1. Variation with respect to time of the total bacterial surface (a), the bacterial surface occupied by flocs (b), the number of flocs (c) and the total length of filaments (d) per image: (♦) control reactor without erythromycin, (○) reactor spiked with erythromycin (20 mg L^{-1}).

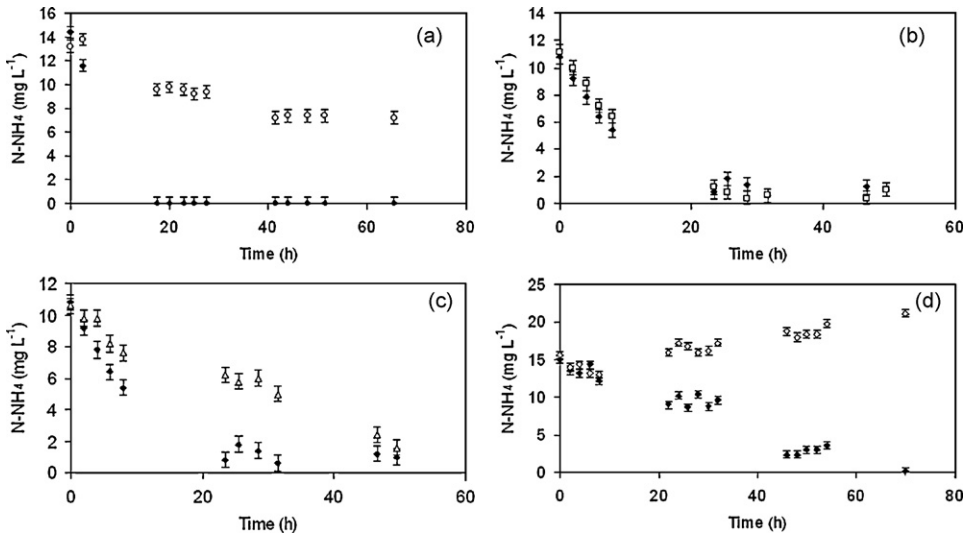

Fig. 2. Ammonia removal under different erythromycin concentrations: (♦) 0 mg L^{-1} (reference reactors, a–d); (□) 1.25 mg L^{-1} (a); (Δ) 2.5 mg L^{-1} (b); (○) 20 mg L^{-1} (c); and (◊) 267 mg L^{-1} (d).

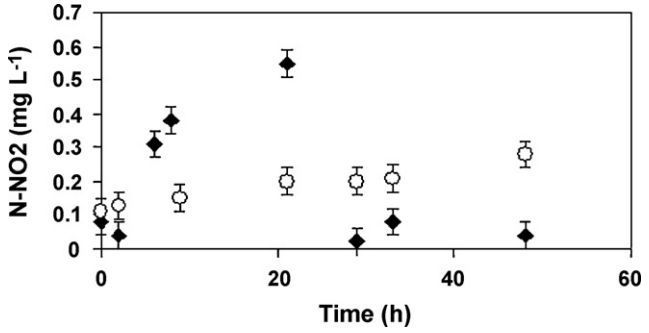

Fig. 3. Concentration of nitrites versus time in the control reactor (♦) and in a reactor spiked with 20 mg L^{-1} of erythromycin (○).

(Fig. 5). In the reactor spiked with 100 mg L^{-1} of erythromycin, an increase of the fluorescence intensity occurred, which can be interpreted as a release of protein-like substances into the reactor broth. Absorbance at 254 nm, which is an indicator of soluble COD, exhibited similar trends (Fig. 6), indicating a rapid consumption of soluble biodegradable pollution by heterotrophs and an accumulation of microbial by-products in the liquid phase in the presence of high concentrations of erythromycin. An exact soluble COD balance is difficult to establish due to the competition between consumption and release.

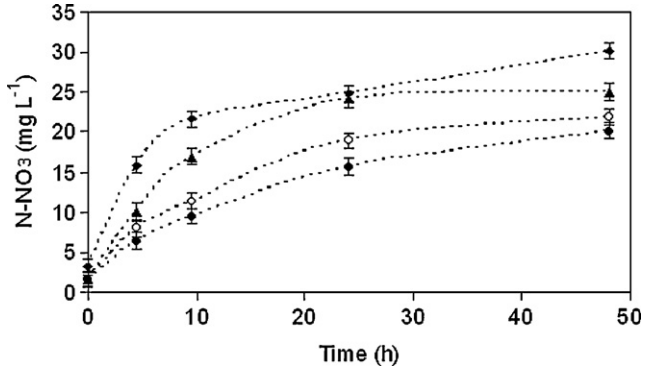

Fig. 4. Concentration of nitrates versus time as a function of the erythromycin concentration: (♦) 0 mg L^{-1} (control reactor), (▲) 7 mg L^{-1}, (○) 20 mg L^{-1}, and (●) 30 mg L^{-1}.

In order to assess whether the effects observed on the biomass were due to inhibition or transfer to the foam layer, an experiment was run in presence of Tween 80. For that purpose, the surface tension of erythromycin and Tween 80 solutions were measured at 20 °C (data not shown). A concentration of 4×10^{-3} mg L^{-1} of Tween 80 was used to match the effect of 20 mg L^{-1} of erythromycin. The same foam layer thickness was observed in both spiked reactors, with no foam present in the control reactor. After 24 h, the total suspended solid loss was 8.8% in the Tween 80 reactor and 19% in the erythromycin reactor, with no loss in the control reactor. The kinetics for ammonia, nitrites, nitrates and A_{254} as well as cell debris abundance (the ratio between the amount of cell debris and total biomass) are shown in Fig. 7. During the first 3 h, the behavior in the three reactors was similar. After 3 h, nitrite accumulation stopped in the erythromycin reactor, which was accompanied simultaneously with an increase in A_{254}, indicating a release of soluble organic substances. After 6 h the debris abundance started to increase in the erythromycin-spiked reactor. The mean floc size at the end of the experiment in the control reactor was 74% of the initial size, compared to 55% in the Tween 80-spiked reactor and only 12% in the erythromycin-spiked reactor.

4. Discussion

The procedure used in the present study was designed to keep the activated sludge under conditions as close as possible to the environment in the wastewater treatment plant. Wastewater was repeatedly sampled from the same plant to avoid adaptation to a synthetic substrate, which can modify the bacteria consortium. The main limitation is that it is difficult to run experiments for extended time periods, as the rapidly biodegradable substrates and nutrients (such as ammonia) are quickly consumed. The antibiotic concentrations used in these experiments (between 1 and 300 mg L^{-1}) might seem very large with respect to the levels reported in wastewater by various authors (6 ng L^{-1} in sewage water, Kümmerer [39]; 2.5–6 μg L^{-1} in wastewater treatment plant effluents, Ternes [40]). This range, which is the same order of magnitude as the tylosin EC_{50} for nitrifiers [21], has been previously used for similar tests [10] with other antibiotics. In addition, concentrations up to 31 mg L^{-1} of nalidixic acid (an antibiotic of the quinolone group) have been reported in the effluent of a large industrial treatment plant in India [3], and 45 mg L^{-1} of nalidixic acid was measured in pharmaceutical wastewater by Sirtori et al. [41]. Furthermore, previous authors

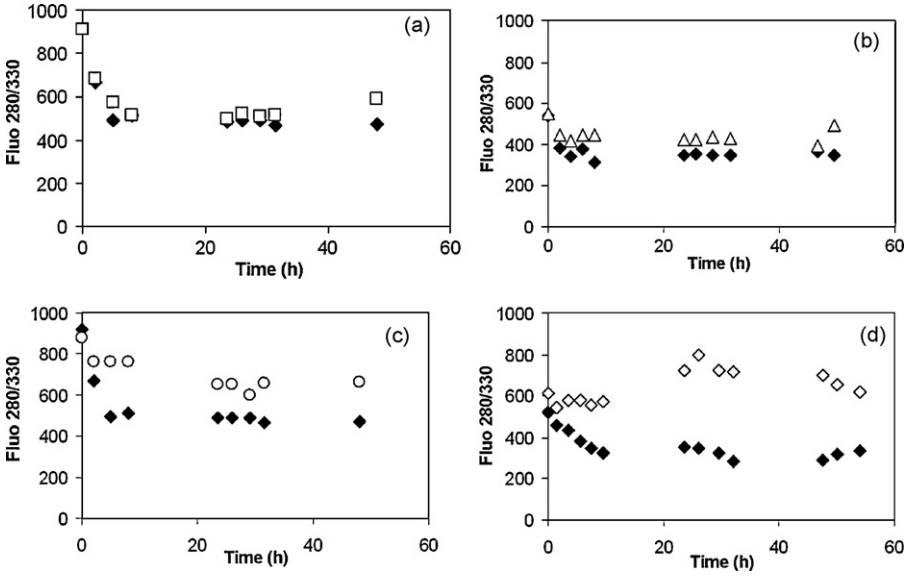

Fig. 5. Fluorescence intensity at λ_{exc} = 280 nm/λ_{em} = 330 nm with respect to time: (♦) 0 mg L^{-1} (control reactors, a–d); (□) 1.25 mg L^{-1} (a); (Δ) 2.5 mg L^{-1} (b); (○) 20 mg L^{-1} (c); and (◊) 100 mg L^{-1} (d).

have used similar concentrations of erythromycin: Amin et al. [42] exposed their anaerobic biomass to 200 mg L^{-1}, and concentrations between 20 and 383 mg L^{-1} were used by Drillia et al. [43].

Part of the erythromycin added to the spiked reactors is adsorbed onto the biomass. A fast sorption rate is usually assumed [14]. Based on a sorption coefficient for activated sludge equal to 0.165 L g_{SS}^{-1} [44] and a suspended solids concentration of 2 g L^{-1} (used in the present experiments), the ratio of total to soluble erythromycin would be 1.33. Under aerobic conditions, the biodegradability of erythromycin is very low [14,45,46]. The results are discussed as a function of the total erythromycin present in the reactor.

As expected from previous experimental results obtained by various authors on the sensitivity of nitrifiers to toxics, these bacteria were sensitive to erythromycin: ammonification, nitritation and nitratation rates decreased in the presence of this macrolide. Fig. 8 summarizes the results obtained from 50 toxicity tests performed in 15 series over a period of 18 months. Ammonia accumulation was observed at the highest erythromycin concentration (Fig. 8a). The initial specific ammonia uptake rate decreased when the initial erythromycin concentration increased (Fig. 8b). An erythromycin concentration of 20 mg L^{-1} had a statistically significant effect (Student test at a significance level of 95%), but this was not the case for 5 mg L^{-1}. As only a limited number of experiments were run with 10 mg L^{-1} of erythromycin, no definite conclusion can be drawn for this concentration. However, sub-inhibitory levels of an antibiotic can induce a notably higher metabolic turnover of the cells [30]. Overall, it appears that nitritation and nitratation rates are both reduced: nitrite accumulation as well as nitrate production were slower in the presence of erythromycin. The nitrate concentration after 48 h was lower at high erythromycin concentrations, even if potentially more ammonia was available due to cell lysis. Average inhibition rates were calculated for 10 and 20 mg L^{-1}. Their order of magnitude (46% and 72% respectively) are similar to the val-

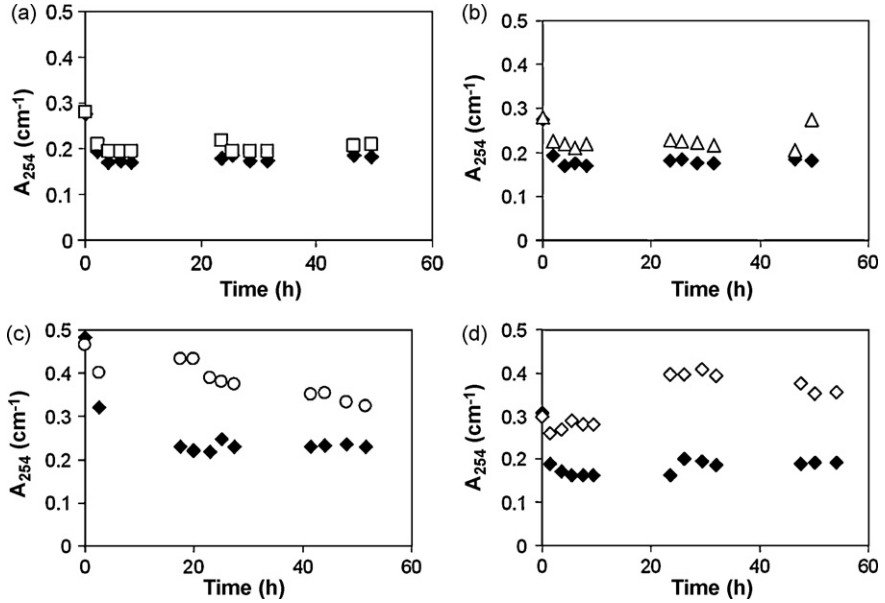

Fig. 6. Absorbance at 254 nm with respect to time: (♦) 0 mg L^{-1} (control reactors, a–d); (□) 1.25 mg L^{-1} (a); (Δ) 2.5 mg L^{-1} (b); (○) 10 mg L^{-1} (c); and (◊) 100 mg L^{-1} (d).

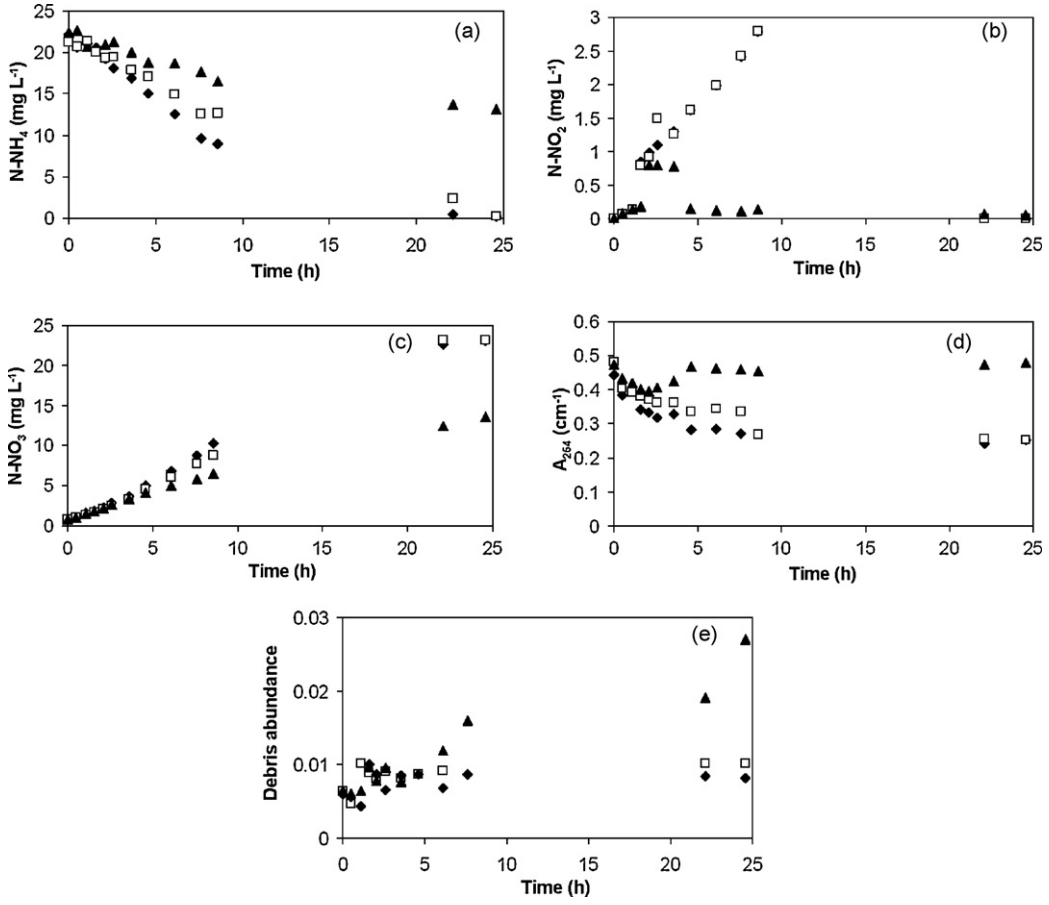

Fig. 7. Variations over time of N–NH_4 (a), N–NO_2 (b), N–NO_3 (c), A_{254} (d) and cell debris abundance (e) in the presence of 20 mg L^{-1} of erythromycin (▲), 4.10^{-3} mg L^{-1} of Tween 80 (□), and in the control reactor (♦).

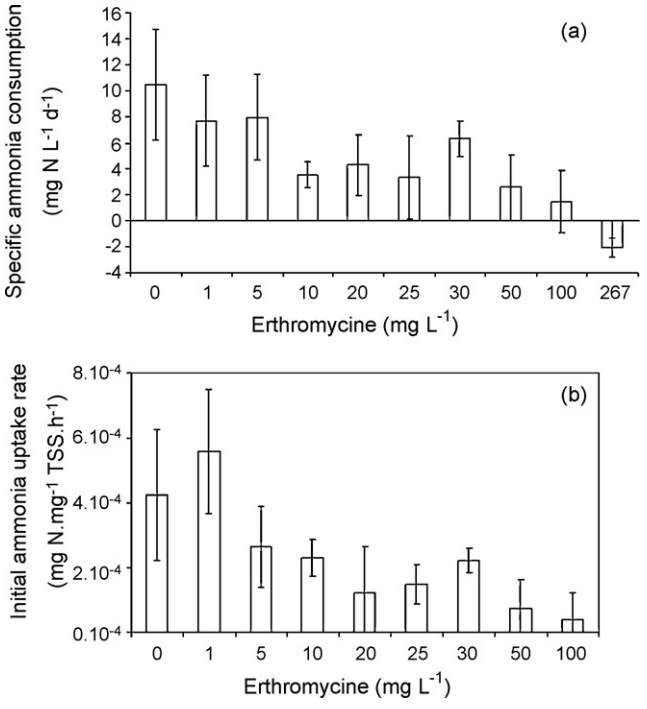

Fig. 8. Volume-specific ammonia consumption over the first 24 h of the tests (a) and initial specific ammonia uptake rate (b) (bar = ± standard deviation).

ues obtained by Carucci et al. [28] for lincomycin (78% and 36% for 11 mg L^{-1} lincomycin for sludge with low and high nitrification activity.

The variability observed in the control reactors is related to the variability of the inoculum sludge sampled at the wastewater treatment plant. Lower rates were generally observed in winter, when cold temperatures are not favorable to the development of nitrifiers in a full-scale plant. The nature of extracellular polymeric substances, which can act as a barrier against transfer of toxics [47], may change over time as a result of seasonal composition variations in the wastewater.

What has apparently not been reported so far in the literature is the sensitivity of activated sludge heterotrophs to erythromycin, which acts primarily on the bacterial ribosome. In our experiments, global floc disintegration was observed in the presence of this macrolide, with partial transfer of biomass to the foam layer at the surface of the liquid phase. Under high concentrations, cell lysis is suspected: the increases in ammonia, protein-like substances and soluble COD release can be linked to the dispersion of intracellular material in the liquid phase. Part of this effect could be due to the surfactant potency of erythromycin, which has been previously reported in literature and has been related to its cytotoxicity [48]. Even at low concentrations, surfactants can cause cell death [49]. Therefore, it could be hypothesized that the filamentous bacteria, which are mainly Gram-positive and form the floc backbone, are at least partly sensitive to erythromycin due to the detergent properties of the molecule. Once the floc skeleton is destroyed, the floc falls apart into smaller pieces. However, the surfactant properties of the antibiotic are probably not the sole cause of floc disintegration. The experiment with Tween 80 showed that at similar surface tensions,

less biomass was lost with the surfactant than with erythromycin. Furthermore, nitrification was not inhibited in the Tween 80 experiment. Most nitrifiers are Gram-negative, and thus theoretically rather insensitive to erythromycin. This is due to the relative impermeability of the Gram-negative membrane and the hydrophobicity of the antibiotic [50]. However, some Gram-positive nitrifying bacteria have been detected in soils [51] and in reactors [52], and the behavior of Gram-positive bacteria is certainly altered by this macrolide. The Gram type assessment performed in the present work was aimed at the filamentous bacteria (which were mostly Gram-positive), and not at the nitrifiers, whose colonies are generally embedded in the floc exopolymeric matrix and cannot be well visualized by optical microscopy. The Gram type assessment performed on the filamentous bacteria did not reveal a shift in their distribution, indicating no preferential action of erythromycin on those organisms.

5. Conclusions

Despite the inherent variability of fresh activated sludge sampled from a real wastewater treatment plant, the inhibition of activated sludge nitrifiers by erythromycin was confirmed in batchwise tests at concentration higher than 5 mg L^{-1}. At the concentrations used (between 1 and 267 mg L^{-1}), which are in the range of what has been found in pharmaceutical wastewater, the antibiotic also had a detrimental effect on the heterotrophic bacteria. This effect was more pronounced as the antibiotic concentration was higher. Although erythromycin is known to inhibit protein synthesis, its surfactant potency is an additional property which favors floc disintegration and bacterial lysis. This effect increases the risk of dissemination of bacteria and possibly of antibiotic resistance genes in the environment by the release of fine particulate pollution that is not retained in secondary clarifiers. Although the concentrations used in the study are larger than those commonly encountered in urban wastewater treatment plants, the observed phenomena could be indicative of dysfunctions that could result from a sudden increase in antibiotic concentrations due to unexpected discharge of hospital effluent, or to increased antibiotic use in response to an epidemic.

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