On the effect of pharmaceuticals on bacterial nitrite oxidation

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Abstract
Pharmaceuticals or their metabolites are partially excreted with urine or faeces ending up in raw sewage. Many of these substances are not biodegradable and their presence in influents of municipal wastewater treatment plants may cause adverse effects to sensitive biological processes such as nitrification, while on the other hand, they may go through the activated sludge process unreacted. The second step of nitrification, i.e. oxidation of nitrite to nitrate is particularly sensitive. Inhibition of this step under uncontrolled conditions may lead to accumulation of nitrite nitrogen in the plant effluent, a form of nitrogen which is particularly toxic. The effects caused by the presence of seven different pharmaceuticals to a culture of nitrite-oxidizing bacteria isolated from activated sludge are presented. These pharmaceuticals were ofloxacin, propranolol, clofibrate, triclosan, carbamazepine, diclofenac and sulfamethoxazole. Different effects were observed for each of the pharmaceuticals tested in this study. In the cases of ofloxacin and sulfamethoxazole significant inhibition was observed. Triclosan presented a substantial inhibitory effect on the substrate (nitrite) reduction rate. The long-term effect of triclosan on nitrite oxidizers was also examined in a CSTR reactor and conclusions were drawn regarding the reversibility of the inhibition caused by this compound.

Keywords
Inhibition; nitrification; nitrite oxidizers; pharmaceuticals

Introduction
Pharmaceutical compounds, due to their growing and uncontrolled use, have recently become a new environmental concern (Dietrich et al., 2002). Many of the pharmaceuticals applied in human medical care are not completely eliminated in the human body. Often they are excreted with urine or faeces only slightly transformed or even unchanged mostly conjugated to polar molecules (e.g. glucuronides) (Heberer, 2002), thus finding their way to raw sewage (Hirsch et al., 1999). A large variety of pharmaceutical residues have been detected in STP effluents and surface waters including antiphlogistics, lipid regulators and beta-blockers. Globally, concentrations measured in STP effluents and in aquatic environment ranged up to several micrograms per litre. A comparable data base is given by Richardson and Bowron (1985).

Several investigations have shown some evidence that pharmaceutical substances are often not eliminated during waste water treatment and also not affected by natural physicochemical degradation processes (Ternes, 1998; Zwiener et al., 2000). Also, they may escape through the STP effluent into the aquatic environment affecting marine life and resulting in bioaccumulation. Most recently, the aquatic environmental impact of three common pharmaceuticals (carbamazepine, clofibrate and diclofenac) towards bacteria, algae, invertebrates and fish has been evaluated in standard conditions and the results have shown that carbamazepine seems to be the most dangerous tested compound for aquatic environments (Ferrari et al., 2003). Of particular concern is also the speculation that the presence of pharmaceuticals in the environment may be leading to subtle but hitherto unrecognized or undetected effects causing irreversible damage of the ecosystem (Daughton, 2001).
Given the fact that many pharmaceuticals are often compounds very persistent to biodegradation their presence in the raw sewage may influence the performance of sensitive sewage treatment plant (STP) processes, such as nitrification. Nitrification is the initial step in the removal of nitrogen from wastewater. It involves a two step conversion of ammonia to nitrite (ammonia oxidation) and nitrite to nitrate (nitrite oxidation) (Halling-Sørensen, 1993). The second step of nitrification is particularly sensitive. Inhibition of this step under uncontrolled conditions may lead to accumulation of nitrite nitrogen in the STP effluent, a form of nitrogen which is particularly toxic, significantly more than nitrate.

The goal of this study was to investigate the effects caused by the presence of seven different pharmaceuticals on a mixed culture of nitrite oxidizing bacteria isolated from activated sludge. These pharmaceuticals were ofloxacin, propranolol, clofibrate, triclosan, carbamazepine, diclofenac and sulfamethoxazole.

Materials and methods

Isolation of nitrite oxidizing bacteria

Mixed liquor from the aerobic stage of the Wastewater Treatment Plant of the University of Patras (Greece) was collected and used as inoculum for the isolation of nitrite oxidizing bacteria. The isolation of these bacteria was based on feeding the inoculum with an autotrophic bacteria selective feed consisting of 0.30 g/l NaNO₂, 3.52 g/l NaHCO₃, 10.52 g/l K₂HPO₄ and 4.72 KH₂PO₄ g/l in a draw-and-fill reactor.

Test substances

Sulfamethoxazole is an antibiotic with CAS no. 723-46-6, Diclofenac is a nonsteroidal anti-inflammatory drug with CAS no. 15307-79-6, Triclosan is an antibacterial agent with CAS no. 3380-34-5, Carbamazepine is an antiepileptic with CAS no. 298-46-4, Ofloxacin is an antibiotic with CAS no. 82419-36-1, Propranolol is an antihypertensive (non selective b-blocker) with CAS no. 3506-36-1 and Clofibrate is a lipid regulator with CAS no. 882-09-7. All the substances were purchased by Sigma-Aldrich Chemical Co.

Batch experiments with pharmaceuticals

Four batch experiments were conducted in parallel for each of the pharmaceuticals. Three different initial concentrations of 2, 6 and 10 mg/l of each pharmaceutical were tested. Appropriate amounts of the tested compound were added to the same concentration of active nitrite oxidizers and the behavior of these cultures was monitored and compared with the performance of a control culture that contained no pharmaceutical. Batch reactors with a volume of 250 ml were used to grow nitrifying bacteria with the solutions containing pharmaceuticals and NaNO₂ as a substrate for nitrite oxidation. The nitrification rate was measured for 5 hours and compared with the rate calculated from reference samples obtained from the control culture. The temperature was kept constant at 25°C throughout the experiment using a temperature controller. The pH of the culture medium was maintained in the range of 7.40 to 8.40 by keeping the medium buffered with KH₂PO₄ and K₂HPO₄.

Long-term effect of the most inhibiting pharmaceutical with the help of a CSTR reactor

The long-term effect of triclosan (most inhibiting pharmaceutical) on nitrite oxidizers was also examined in a CSTR reactor for several days of operation and conclusions were drawn regarding the reversibility of the inhibition caused by this compound. For this case two different CSTR reactors were used with 550 ml working volume operating at 12 days retention time and with 60 mg/l of N-NO₂ in the feed. The mixed culture used to inoculate these two CSTRs came from an other CSTR with 1,254 ml working volume operated at 12
days retention time and fed with 60 mg/l of N-NO$_2$. The first CSTR was used as a control without any addition of triclosan in the feed. The other one was used to monitor the behavior of this culture with the feed containing triclosan. The initial concentration of triclosan in the feed was 1 mg/l. After the tenth day of operation, the concentration of triclosan in the feed was raised to 5 mg/L and finally to 10 mg/L during the last 5 days of experimentation. The performance of the two CSTRs was monitored and compared.

**Chemical analysis**

Nitrite-N (NO$_2^-$-N) was determined spectrophotometrically according to the colorimetric method 4500-B (A.P.H.A, 1995). The pH was measured with a Hanna electrode (HI 8224).

**Results and discussion**

The nitrite concentration profiles for each batch containing a different pharmaceutical at different concentrations is shown in Figures 1(a)–(d) and 2(a)–(c). Triclosan (Figure 2(c)) presented a substantial inhibitory effect on the substrate (nitrite) reduction rate. In the case of ofloxacin (Figure 1(a)) a significant level of inhibition was also observed, while sulfamethoxazole (Figure 1(b)) had a moderate inhibitory effect on this process. On the other hand, propranolol (Figure 2(a)) and diclofenac (Figure 1(d)) caused minor, if any, inhibition on nitrite consumption rate, while carbamazepine (Figure 2(b)) and clofibrate (Figure 1(c)) did not affect the performance of nitrite oxidizing bacteria.

In order to assess the long-term effect of triclosan (most inhibiting pharmaceutical) on nitrite oxidizers two CSTR reactors were operated for several days and conclusions were drawn regarding the reversibility of the inhibition caused by this compound. The behavior of the two CSTRs running in parallel is shown in Figure 3.

Triclosan caused an inhibition in nitrite oxidizers during the first days of operation in agreement with the behavior observed in the batch experiments. However, no inhibition was observed for longer periods of operation and for higher concentrations of triclosan. Therefore, nitrite oxidizers proved to be able to adapt to the presence of triclosan in the culture medium even at concentrations as high as 10 mg/l.

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**Figure 1** Effect of pharmaceuticals at different concentrations on nitrite consumption a) ofloxacin, b) sulfamethoxazole, c) clofibrate, d) diclofenac

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The inhibition of ofloxacin, sulfamethoxazole and triclosan to nitrite consumption was modelled as non-competitive using the following kinetic equation for the specific nitrite consumption rate:

\[
    r_{\text{NO}_2} = r_{\text{NO}_2}^{\text{max}} \frac{C_{\text{NO}_2}}{K_{\text{NO}_2} + C_{\text{NO}_2}} \frac{K_I}{K_I + I} 
\]

where \( r_{\text{NO}_2}^{\text{max}} \) is the maximum specific nitrite consumption rate in the case of zero inhibitor concentration, \( K_I \) is the inhibition constant and \( I \) is the concentration of each inhibitor (ofloxacin, sulfamethoxazole, triclosan). For nitrite concentrations well above \( K_{\text{NO}_2} = 0.94 \) mg \( \text{NO}_2\text{-N/l} \) (our data) the term \( C_{\text{NO}_2}/(C_{\text{NO}_2} + K_{\text{NO}_2}) \) in Eq. (1) is close to unity. Thus, rewriting the above equation in the form:

\[
    \frac{1}{r_{\text{NO}_2}} = \frac{1}{r_{\text{NO}_2}^{\text{max}}} + \frac{1}{r_{\text{NO}_2}^{\text{max}} \cdot K_I} \frac{I}{K_I} 
\]

the values of \( r_{\text{NO}_2}^{\text{max}} \) and \( K_I \) can be determined from a plot of \( 1/r_{\text{NO}_2} \) vs \( I \) (Figure 4). The estimated values for \( r_{\text{NO}_2}^{\text{max}} \) and \( K_I \) were 0.014 mg \( \text{NO}_2\text{-N/mg biomass} \cdot \text{h} \) and 12 mg/l, 13.8 mg/l and 0.045 mg/l for ofloxacin, sulfamethoxazole, triclosan respectively.
The effect of the presence of seven common pharmaceuticals on a mixed culture of nitrite oxidizing bacteria isolated from activated sludge was investigated. The pharmaceutical compounds used in this study were ofloxacin, propranolol, clofibrate, triclosan, carbamazepine, diclofenac and sulfamethoxazole.

Triclosan exhibited the most inhibitory effect on nitrite consumption rate at a short-time basis. In fact the estimated value of $K_I$ (inhibition constant) for triclosan was found to be very low as a result of the almost complete retardation of nitrite reduction observed in our batch experiments. This type of behaviour was anticipated since triclosan is a known antibacterial agent with a bacteriostatic efficacy against a broad spectrum of microorganisms (Council Directive 76/768/EEC). However, nitrite oxidizers operating in a continuous system presented the ability to overcome the inhibitory effect from triclosan in just a few days.

In the presence of ofloxacin and sulfamethoxazole a significant level of inhibition was also observed during the experiments carried out in batch reactors. The values of $K_I$ estimated for these two compounds were found to be very close indicating the same level of nitrite reduction inhibition.

On the other hand, propranolol, diclofenac, carbamazepine and clofibrate caused minor, if any, inhibition on the performance of nitrite oxidizing bacteria.

Figure 4  Estimation of kinetic parameters $r_{\text{NO}_2}^{\text{max}}$ and $K_I$ of Eq. (1) for ofloxacin and sulfamethoxazole

Conclusions
The effect of the presence of seven common pharmaceuticals on a mixed culture of nitrite oxidizing bacteria isolated from activated sludge was investigated. The pharmaceutical compounds used in this study were ofloxacin, propranolol, clofibrate, triclosan, carbamazepine, diclofenac and sulfamethoxazole.

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References


