ROLE OF BIOTRANSFORMATION, SORPTION AND MINERALIZATION OF $^{14}\text{C}$-LABELLED SULFAMETHOXAZOLE UNDER DIFFERENT REDOX CONDITIONS

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ABSTRACT

Sulfamethoxazole biotransformation, sorption and mineralization was studied with heterotrophic and autotrophic biomass under aerobic and anoxic conditions respectively, as well as with anaerobic biomass. The $^{14}\text{C}$-radiolabelled residues distribution in the solid, liquid and gas phases were closely monitored along a total incubation time of 190 hours. The influence of the type of primary substrate and the redox potential was observed in all cases on the biotransformation and mineralization rates, since an enhancement of the removal rate was observed when a carbon source was used as a primary substrate under aerobic conditions, while a negligible effect was observed under nitrifying conditions. Biotransformation was the main removal mechanism, being mineralization and sorption rates remained below 5% in all the cases, although the presence of a carbon source exerted a positive effect on the mineralization rate by the aerobic heterotrophic bacteria. In the supernatant samples collected from all assays, up to three additional peaks corresponding to $^{14}\text{C}$-radiolabelled residues were detected. The highest concentration was observed under anaerobic conditions, where two metabolites were detected representing each around 15% of the total applied radioactivity after 180 h incubation.

Keywords: sulfamethoxazole, biotransformation, mineralization, redox potential

1. Introduction

Sulfonamides antibiotics are frequently used in humans to treat bacterial infection, as well as for their diuretic or anticonvulsant effect. A significant amount of these antibiotics are excreted in slightly changed or unchanged forms due to partial metabolism in human bodies. Therefore, these antibiotics are detected in the different aquatic environments, such as rivers, groundwater or wastewaters. Sulfamethoxazole (SMX), which is used to treat respiratory, gestointestinal and urinary tract and skin infections, is one of the most widely use sulphonamides. There are many studies focusing on the removal of SMX in wastewater treatment plants (WWTP) (Gobel et al., 2007; Xu et al., 2007) although most of them only studied the elimination of the parent compound. As the disappearance of the parent compound does not mean biodegradation of this compound, there is scarce information about the biotransformation rate, metabolites and mineralization rate of SMX. Usually during biological wastewater treatment, low mineralization rates of SMX are expected, for example mineralization rates below 5% within a week were observed in an enrichment culture originating from an aerobic MBR (Bouju et al., 2012).

Most of the previous studies were focused on its biotransformation in aerobic activated sludge, hence information about its fate through other biological wastewater treatment processes is scarce. For instance, previous studies carried out under anaerobic conditions were focused on the sludge line or in the treatment of slurry showed very wide removal efficiencies (2-99 %) in function of the sludge retention time (SRT), temperature or solids concentration (Carballa et al, 2005).
The present study aimed at determining the relevant removal mechanisms of SMX (biotransformation, sorption and mineralization) in the presence of different microorganisms as those developed in: a) heterotrophic; b) nitrifying; c) denitrifying; d) anammox and e) anaerobic sludges. It focuses especially on the effect of the primary metabolism on the biotransformation and mineralization of this compound. $^{14}$C-radiolabelled SMX was used in order to circumvent analytical difficulties in the complex wastewater matrix and to perform a complete mass balance.

2. Calculations performed
2.1. $^{14}$C-SMX fed batch assays
Fed-batch assays were designed in order to follow SMX biotransformation, sorption and mineralization under aerobic, anoxic and anaerobic conditions. The assays were carried out in 150 mL glass bottles, hermetically sealed-off at 25 ºC and constantly stirred. Aerobic conditions were maintained by continuously bubbling wet air. For anaerobic and anoxic incubations, nitrogen gas was bubbled in the culture. In order to trap the $^{14}$C-CO$_2$ resulting from the possible mineralization of $^{14}$C-SMX, the off-gas stream was bubbled through 50 mL of a 5 M NaOH solution. Under anaerobic conditions, a second trap, constituted of a granular activated carbon column was used to retain the $^{14}$C-CH$_4$ produced. 50 mL of each biomass was centrifuged for 15 minutes at 5 000 g and washed twice in the culture medium. Two fractions of 20 mL were placed in glass bottles, constituting the two duplicates. The final suspended solids concentration was 1 g/L. Different primary substrates were used to assess the possible effect of cometabolism: ammonium for the nitrification experiments (6 and 14 mg N/Ld), acetic acid for the aerobic heterotrophic tests(5.3 and 48 mg COD/Ld), nitrate and nitrite for the anammox activity tests (150 mg N/Ld), acetic acid and nitrate for the anoxic heterotrophic tests (5 and 50 mg N/Ld) and diluted skimmed milk and bicarbonate in order to obtain a complex substrate in the anaerobic tests (480 and 640 mg COD/Ld). An abiotic control was prepared. Both abiotic and biotic experiments were spiked with 272 µg/L of $^{14}$C-SMX. Once the primary was exhausted, it was again fed into the culture medium using freshly prepared stock solutions.

2.2. Sample preparation
All along the 190 hours incubation time, 2 mL of each culture were regularly collected and extracted in order to determine the biotransformation rate. The total radioactivity was measured in the samples, which were then centrifuged at 21500 g for 10 minutes. The supernatant was collected and freeze-dried. After lyophilisation, the sample was resuspended in 1 mL of methanol, filtered and analysed with HPLC-DAD-LSC (Agilent Tech. HPLC 1200 Series coupled to a radioisotope detector 'Ramona Star', Raytest, Germany). The pellet (solid fraction) was resuspended in 1.5 mL of distilled water, centrifuged for 10 minutes at 21500 g and the total radioactivity was assessed in the supernatant. The sum of radioactivity allocated to $^{14}$C-residues measured in both MLSS and aqueous samples represent the bioavailable fraction. Each pellet was then rinsed and subsequently centrifuged for 10 minutes at 21500 g three times with 1.5 mL of ethylacetate. The radioactivity was measured in each supernatant sample, the sum of the three constituted the extractable fraction. Finally the remaining pellet was freeze-dried, weighed and combusted; the radioactivity measured is the non-extractable fraction.

10 µL aliquot of each liquid sample was taken in order to measure the total radioactivity amount in the samples with Liquid scintillation Counting (LSC) (Perkin Elmer 2800TR Tri Carb). Additionally, 2 ml of each CO$_2$ trap were collected and the radioactivity was measured in the LSC analyser. Finally, pellets recovered after the extraction steps were combusted in a 307 PerkinElmer Sample Oxidizer.

3. Results
3.1. Sulfamethoxazole biotransformation and effect of the primary substrate
With all biomass types, no effect on their metabolism was observed after the spike of SMX. Under aerobic conditions, the effect of the nitrifying bacteria and the heterotrophic bacteria
responsible of the degradation of organic matter was followed. The bioavailable SMX fraction decreased from 98 to 94 % of the total radioactivity measured in the samples of the incubations using acetic acid as primary substrate, while it remained constant (99%) in the experiment under nitrifying conditions. Moreover, a higher removal rate was observed in the presence of an external carbon source (i.e. 27 µg SMX/Ld were biotransformed when the heterotrophic removal rate was 48 mg COD/Ld). With the anaerobic biomass, a similar trend was observed and the percentage of radioactivity allocated to the bioavailable fraction decreased with time (from 97.5% to 94% over 159 hours). Therefore, the external carbon source played an important role in the biotransformation of SMX (Gauthier et al., 2010) and the biotransformation of SMX was strongly dependent on the heterotrophic activity under aerobic and anaerobic conditions.

Under anoxic conditions, heterotrophic and autotrophic bacteria can be responsible of the denitrification. The percentage of total radioactivity allocated to the bioavailable fraction decreased with time in the same range in both experiments under anoxic conditions, although the biotransformation rate of SMX was higher in the presence of heterotrophic biomass. As observed under aerobic and anaerobic conditions, biotransformation was dependent on the primary substrate degradation. In fact, a correlation between SMX biotransformation and the denitrification rate was evidenced.

![Figure 1](image)

**Figure 1:** Biotransformation of sulfamethoxazole under aerobic (a), anoxic (b) and anaerobic (c) conditions in function of the primary substrate removal rate

Supernatant samples were analysed with HPLC-DAD-LSC, to detect the different metabolites produced. Under nitrifying conditions, no metabolites could be detected, while an additional $^{14}$C-labelled metabolite was detected when sodium acetate was used as primary substrate. After 187 hours of incubation, this metabolite represented 7% of the total radioactivity measured in the bioavailable fraction. Under anoxic conditions, up to three additional peaks corresponding to $^{14}$C-residues were detected after 190 hours of incubation in the experiments with autotrophic and heterotrophic bacteria. In the supernatant from the anammox culture (autotrophic denitrifying bacteria), two new peaks were detected, representing together 5.9 % of the total radioactivity, while in the supernatant from the heterotrophic denitrifying bacteria cultures a third additional peak was detected and these three $^{14}$C-labelled metabolites represented respectively 7%, 8.5% and 8% of the total radioactivity. Under anaerobic conditions, two metabolites were
detected after 190 hours of incubation. Each metabolite represented respectively 10.6% and 10.3% of the total radioactivity of the supernatant. The first peak was detected at the same retention time than the first metabolites under anoxic conditions.

### 3.2. Mineralization

The maximum mineralization rate (3 %) was achieved under aerobic conditions when sodium acetate was used as primary substrate. As observed with biotransformation, SMX mineralization rate was dependent on the primary substrate, as well as on the redox potential. Additionally, the biomass activity exerted a strong influence on the percentage of mineralization, even stronger than its effect on biotransformation, i.e. the mineralization rate was 22 times higher when the aerobic heterotrophic rate changed from 5.3 to 48 mg COD/Ld, while biotransformation rate was only 4 times higher.

### 3.3. Distribution of 14C-residues between bioavailable, extractable and non-extractable fractions

The total radioactivity measured in the MLSS was divided into three fractions, bioavailable, extractable and non-extractable (Fig.2). As SMX is a hydrophilic compound, its elimination in the biological treatment is expected to be mainly due to biotransformation. This tendency was confirmed by demonstrating its poor tendency to sorb on the biomass (<6%).

![Figure 2](image_url)

**Figure 2:** 14C-labelled residues distribution as a function of time for the following incubation conditions: a) heterotrophic degradation under aerobic conditions, b) heterotrophic degradation under anoxic conditions, c) autotrophic degradation under aerobic conditions, d) autotrophic degradation under anoxic conditions and e) heterotrophic degradation under anaerobic.

In the case of the secondary sludge, the amount of radioactivity associated to the sludge was different in the three experiments performed and increased with the time: from 2% to 5 % when organic matter was used as substrate and from 0.6 to 2 % when ammonium was used as primary substrate under aerobic conditions, while the total radioactivity associated to solids under anoxic conditions increased from 1.7% to 5.5 %. The non-extractable fraction increased with time in all the experiments. In the case of the anammox biomass, the increase of the non-extractable fraction was more marked (from 0.9-5.3% of the total radioactivity after 190 hours). This fate might be due to the sorption mechanism occurring in granular biomass, which considered two stages: firstly, a quick sorption onto the specific surface followed by an intramolecular diffusion inside the granules (Shi et al., 2011). Under anaerobic conditions, a slightly higher extractable fraction was observed compared to the experiments carried out under aerobic and anoxic conditions. This could be the consequence of the disaggregation of the granules, i.e. the extractable fraction corresponded to 3% in anaerobic vs. 0.6% in heterotrophic conditions.
anoxic experiments of the total radioactivity on MLSS over 30 d, when the primary substrate removal rate was 640 mg COD/Ld and 50 mg N/Ld, respectively.

4. Conclusions
A new procedure to determine the fate (biotransformation, mineralization and sorption) of SMX under different redox potentials using SMX radiolabelled was explained. Additionally, the influence of primary substrate removal rate on the SMX removal mechanisms was followed. The main results achieved have been summarised:

- SMX mineralization was lower than 5% in all tested cases
- The $^{14}$C residues radioactivity (corresponding to the extractable and no-extractable fraction) was lower than 7% of the total radioactivity. The sorbed residues increased with the time.
- Mineralization and biotransformation were dependent on the primary biomass activity, mainly when organic matter was used as primary substrate. The maximum mineralization (2.9%) and biotransformation (48.5%) were observed with a heterotrophic activity of 48 mg COD/Ld under aerobic conditions.
- The metabolites generated during the biotransformation of SMX depended on the type of biomass and the redox potential.

REFERENCES