Effects of long-term exposure to antimicrobial colistin sulfate (polymyxin E) over mixed cultures of nitrifiers and methanogens

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Abstract
Nitrifying bacteria and methanogens are among the microorganisms most sensitive to inhibitory compounds in the aerobic and anaerobic environments, respectively, and are useful as biosensors to access contaminant toxicity information in the wastewater treatment plants. In order to assess colistin chronic toxicity to microorganisms involved in the biological wastewater treatment, a nitrifier sequential batch reactor (SBR) and a methanogenic upflow anaerobic sludge blanket (UASB) reactor were monitored under progressive increments of antimicrobial concentration at the influent medium. Methanogens showed a high tolerance to colistin, maintaining acetate consumption and biogas production even at high concentration tested (100 mg colistin.L^{-1}). Nitrifiers, on the other hand, showed lower tolerance to colistin; an expressive inhibition can be seen at 50 mg colistin.L^{-1}, and at 100 mg colistin.L^{-1} the reactor performance was strongly affected.

Introduction
Colistin sulfate is widely used in animal farming as a food additive, especially at intensive pig farms, being one of the strongest antibiotics for treating intestinal infections caused by gram-negative bacteria such as *Escherichia coli*, *Salmonella sp.*, and *Pseudomonas sp.*. Besides the therapeutic applications, colistin is commonly used at lower concentrations as a growth promoter (prophylactic use) [1, 2]. Additionally, colistin use is also re-emerging in human medicine as an option for the treatment of multiresistant gram-negative strains such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* [3]. Therefore, the importance of knowing the effects of this antibiotic on wastewater treatment plant microbial communities goes beyond farming. The possibility of these environments acting as a "reservoir" of colistin resistance genes is an issue that has also become important from the human health standpoint [4]. Colistin is an antibiotic that has been used in medicine for a long time, but data concerning about its pharmacology and microbiological properties are very scarce [5].

The term colistin (polymyxin E) actually refers to a group of alkaline cyclic polypeptides having colistins A and B (polymyxins E1 and E2, respectively) as their main components. This group is structurally and pharmacologically related to polymyxin B, acting on microbial cells as cationic detergents. When orally administered, colistin is eliminated in faeces virtually unaltered; thus, potentially reaching wastewater treatment plants and water bodies and in its active form. Apart from the risks associated with development of antimicrobial resistance and environmental toxicity issues, the presence of antimicrobials in wastewater can, additionally, interfere with biological processes commonly used to treat these effluents. Nitrifiers and acetoclastic methanogens are among the most sensitive microorganisms to inhibitory compounds, including pharmaceuticals, and are useful as biosensors to access toxicity data of contaminants in wastewater treatment plants [6, 7].

In this work, chronic exposition of nitrifier and methanogen mixed cultures to colistin sulfate are evaluated using bench scale bioreactors in a long-term experiment.

Materials and Methods
Nitrifier Sequential Batch Reactor (SBR) – inoculum, culture medium and reactor characterization
Mixed nitrifier biomass were obtained from a nitrifier culture maintained in a bench scale SBR originally enrichment from activated sludge of a swine wastewater treatment plant [8]. Inorganic mineral medium modified from Tanaka et al. (1981) and containing only ammoniacal nitrogen as

substrate was used to feed nitrifying reactor. Composition of mineral medium include: NH_4Cl (1.91 g.L^{-1}); $(NH_4)_2SO_4$ (2.36 g.L^{-1}); KH_2PO_4 (0.25 g.L^{-1}); $MgSO_4$ (0.12 g.L^{-1}); $FeSO_4$ (0.008 g.L^{-1}); CaCl (0.008 g.L^{-1}); $NaHCO_3$ (11.96 g.L^{-1}); and micronutrient solution (0.15 mL.L^{-1}), resulting a 1000 mg N-NH_3.L^{-1} medium.

Nitrification inhibition tests were performed using a cylindrical bench scale SBR with 12.5 cm diameter and 19.5 cm total height, operated at 1.8 L reaction volume. Reactor was operated at fill and draw mode under an 8 h operational cycle, that include a 7 h aerated reaction time and 1 h for settling sludge, draw and re-fill steps. Replacement volume adopted is 0.4 L/cycle, resulting in a HRT of 1.5 day. Aeration was provided using an air stone connected to an air pump (BIG AIR model A420). Peristaltic pumps were used to fill (MILAN, model BP-200) and draw effluents (MILAN, model BP-600), and a magnetic stirrer (FISATOM model 752A) was used for mixing. pH was maintained at 7.5 by NaOH addition using a pH controller (DIGIMED, model TH-44). All equipment (fill and draw pumps, stirrer, aerator, and pH controller) was automated through a programmable logic controller (PLC) (Dexter, model μDX series 100).

Methanogenic Upflow Anaerobic Sludge Blanket (UASB) – inoculum, culture medium and reactor characterization
Methanogens were enriched from a UASB sludge used in swine wastewater treatment plant [8] using a bench scale UASB reactor and acetate as the only organic substrate. After start-up reactor (data not showed) DQO medium was fixed at 5.4 g COD.L^{-1}. Mineral medium used was composed of: CH_3COONa (6.92 g.L^{-1}), NH_4Cl (2.27 g.L^{-1}), KH_2PO_4 (0.5 g.L^{-1}), $MgSO_4.7H_2O$ (0.15 g.L^{-1}), $CaCl_2.2H_2O$ (0.1 g.L^{-1}), and micronutrient solution (2 mL.L^{-1}).

To evaluate toxicity of colistin over methanogens a bench scale UASB reactor with 6 L reaction volume (10 cm diameter and 85 cm total height) was used. Biogas production was measured with a floating dome type gasometer connected to a gas collector at the top of the reactor. Continuous feed operation at 5 L.d^{-1} was done using a peristaltic pump (MASTERFLEX, model 7550-62).

Chronic inhibition tests
In both cases, after inoculation with enriched biomass and stabilization of bioreactors at desired substrate loading rate, they were then submitted to progressive increments of colistin concentration in the affluent medium along the course of reactors operation. Substrate consumption and/or products formation was monitored to identify inhibitory effects. Nitrogenous ions involved in nitrification process (NH_4^+, NO_2^- e NO_3^-) were monitored to characterize nitrifying system; and for the methanogen UASB reactor, COD consumption and biogas production was used to monitor process.
Since dose–response models for antibiotics commonly shows an inhibitory concentration range where the inhibition is nearly linearly correlated to the logarithm of the antimicrobial concentration, it was used in the assays colistin concentration scale representing a logarithmic progression, ranging from 0.32 to 100 mg.L^{-1} (a high concentration which is very unlikely to be found in swine farming). Hence, the colistin concentrations used in the chronic toxicity tests were 0.32 ($10^{-0.5}$), 1 (10^0), 3.16 ($10^{0.5}$), 10 (10^1), 31.6 ($10^{1.5}$), and 100 (10^2) mg.L^{-1}.

Analytical determinations
The nitrogenous forms were determined through colorimetric methods with the use of a HACH spectrophotometer (model DR 5000 UV–vis). The Nessler method was used to establish total ammoniacal nitrogen (TAN) concentration [10], while the analytic kit Nitriver 2 (HACH) and the salicylic acid method [11] were used to determine nitrite and nitrate concentrations, respectively. COD determinations and suspended solids analysis were carried out in accordance with the Standard Methods for the Examination of Water and Wastewater [12].

Results and Discussion

Chronic toxicity of over nitrification
Expressive effects over reactor performance were observed at concentrations over 50 mg colistin.L^{-1}, and at 100 mg colistin.L^{-1} nitrification was severely affected resulting in failure of nitrification process

(Figure 1). Chronic inhibitory concentrations are higher than values derived from acute tests over similar biomass, and nitrite accumulation was not observed at any moment, corroborating data from acute toxicity tests that point to a higher tolerance of nitrite-oxidizing bacteria (NOB) in relation to ammonia-oxidizing bacteria (AOB). [13]. Usual colistin concentration in swine wastewater, at farms that use it, still remains unknown at this time. Other antimicrobials reported in literature generally occur at lower concentrations in swine wastewater than inhibitory concentrations observed in this paper, but it was shown that in some situations the concentration range of some antimicrobials can be higher, reaching the same order of magnitude as those observed in this work [14, 15].

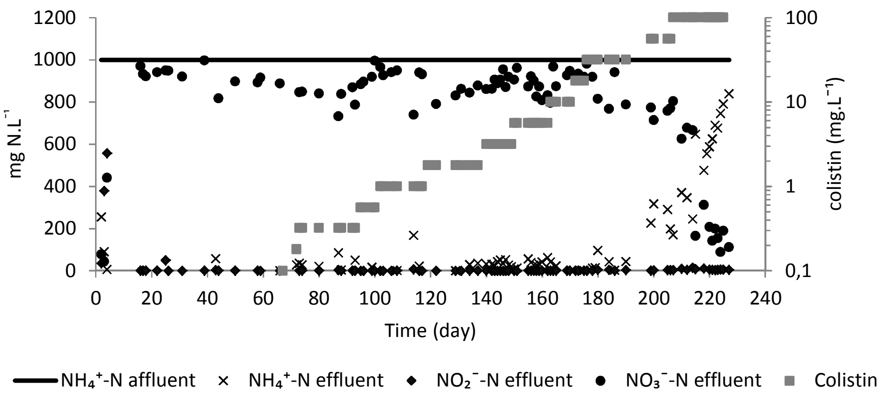

Figure 1: Effects of chronic colistin exposure upon nitrification. Profiles of influent NH_4^+ and effluent NH_4^+, NO_2^-, and NO_3^- during the experiment with colistin long term exposure.

Chronic toxicity of colistin over methanogens

After a long period at steady-state condition (~8 months), the UASB reactor was submitted to gradual log increments of colistin concentration at fed medium during a period of 175 days. Methanogenic biomass proved to be very tolerant to prolonged colistin exposure. Even at the higher concentration tested (100 mg colistin.L^{-1}), COD reduction in effluent and biogas mean production was poorly modified.

A **B**

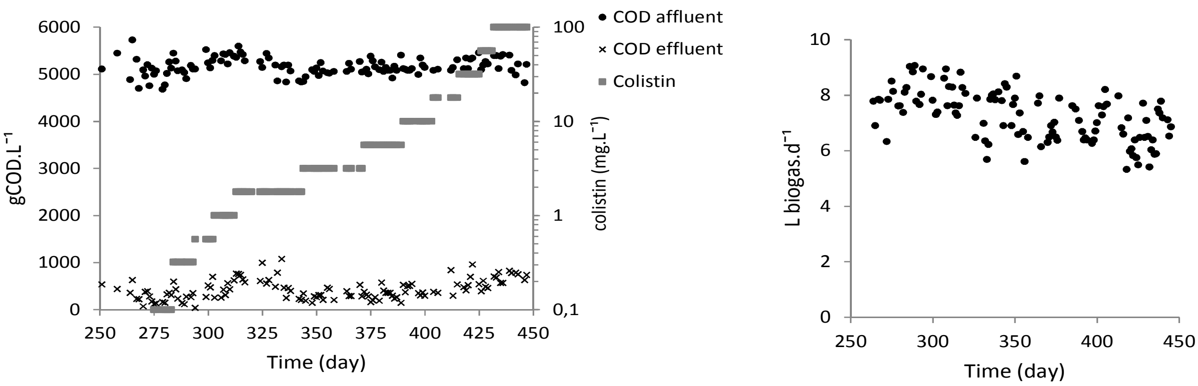

Figure 2: Effects of chronic colistin exposure upon methanogenesis. Profiles of affluent and effluent COD (A) and biogas production (B) during the experiment with long-term colistin exposure.

Conclusion and perspectives

Methanogenesis has high tolerance to colistin, not showing relevant inhibition even at the highest concentrations tested. The highest concentration tested is much higher than the one expected to be found in the environment and swine wastewater. Colistin concentration at these places remains unknown, but concentrations of other antibiotics found occur generally below 1 mg.L^{-1}, rarely occuring at higher concentrations.

Nitrifying bacteria presented lower tolerance to colistin than methanogenic microorganisms, and in some situations, colistin concentration in wastewater can theoretically reach potentially inhibitory levels.

Although heterotrophic bacteria, including denitrifying ones, often show better adaptation capabilities and tolerance to toxicants than nitrifiers and methanogens, the possibility of a higher sensitivity of another microbial group to colistin cannot be ruled out. Additional work is necessary for a comprehensive understanding of antimicrobial effects of colistin over the swine wastewater treatment process.

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