PESTICIDE BIOFILM RESISTANCE STUDY IN CONSTRUCTED WETLANDS

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Abstract

Experimental study was carried out to determine the effects of three pesticides, diuron, alachlor and endosulfan in biofilm bacterial viability in two pilot-scale constructed wetlands, one of them exposed to known concentrations of these three pesticides. Undisturbed mature biofilms (Siporax® cylinders) from the two wetlands, pre-treated and non-pre-treated with pesticides, were collected and were exposed individually to these three pesticides. To estimate bacterial viability in Siporax® cylinders was used a new colorimetric approach based on the reduction of tetrazolium sodium salts such as XTT. The results of this study indicates that pesticides impact and activity bacteria inhibition on non-pre-treated biofilms were higher than on previously treated biofilms, although, both showed tolerance and resistance to biocides.

Keywords: biofilm, pesticide, constructed wetland, XTT

Introduction

Bacteria have the ability to adhere to surfaces (solid particles and/or plant roots in wetlands) and form a biofilm. By their physical and physiological nature, biofilms are responsible for most of the essential transformations and decomposition of contaminants in aquatic environments and play an important role in the bioconcentration of those contaminants, however the sorption mechanisms in biofilms are poorly understood (Lawrence *et al.*, 2001). The understanding of biofilm dynamics requires knowledge of bacterial growth-kinetics in these environments.

XTT (sodium 3,3´-[(phenylamino) carbonyl]-3,4-tetrazolium]Bis(4-methoxy)-6-nitro)benzene sulfonic acid hydrate) has been used to estimate cell viability and proliferation in bacterial suspension (Hatzinger *et al.*, 2003). XTT is reduced to a water-soluble formazan during bacterial aerobic metabolism, which can be easily measured in cellular supernatants by colorimetric (Kuhn *et al.* 2003), such that the amount of formazan produced is proportional to the live bacteria biomass (Roslev & King, 1993).

The assay developed by Pérez *et al.*, 2010, based on XTT reduction using Siporax® cylinders, offers a compact, inexpensive and easy system that allows direct measurement of biofilm formation from water systems and to determine the respiring bacterial viability in these biofilms.

According to this background, the aim of our work was to investigate the effects of contaminants in bacterial biofilm viability in the wetlands. To understand these mechanisms, biofilm viability study in the presence of contaminants and their capacity to withstand high concentrations of contaminants in the wetlands are important aspects to determinate.

Methods

Biofilms were produced during 20 days in two pilot scale horizontal subsurface constructed wetlands (HSCW) denominated H4 and H6. The experimental conditions in both HSCW were: 5 mm gravel bed planted with Thypa latifolia *(broadleaf cattail) and fed by gravity with 20 l/d of synthetic wastewater plus 1,5 l of municipal wastewater. Each HSCW had 6 piezometers of Siporax® cylinders, fine-pore filtering materials with a large surface area that can be colonized by microorganisms (each cylinder has 0,9 m^2 of specific surface and 20-50 µm amplitude among its micropores) (Pérez et al., 2010). Additionally, a solution with diuron, alachlor and endosulfan (PESTANAL analytical standard, Sigma-Aldrich Biotechnology LP, Spain) was added every day into the H6 feed tank to a final concentration of 0,5 ppm of each pesticide/litre of synthetic wastewater. The H4 wetland didn't treated with these three pesticides.*

Cell viability was evaluated at different time points (0, 7, 14 and 21 days) of assay, using a commercially available kit (Cell Proliferation Kit II XTT – #11 465 015 001; Roche Diagnosis, Mannheim, Germany) according to method described previously by Perez et al.*, 2010. Briefly, Siporax® colonized cylinders (H4 and H6) were loaded in a 24-well plate and were exposed individually to three pesticides, diuron, alachlor and endosulfan, to final concentration of 1,5 ppm, during 10 hour at 25°C. Formazan was continuously quantified spectrophotometrically at 490 nm during this period, using a micro plate reader ELISA (GENios-Tecan, Männedorf, Switzerland). Sterile Siporax® cylinders were used as negative controls, and colonized cylinders with glucose solution was used as positive controls.*

Results and discussion

To evaluate the biofilm respiration and to stimulate bacterial growth was carry out a first assay where were incubated Siporax® sterile cylinders and Siporax® colonized cylinders in a 24-well plate loaded with saline or glucose solutions containing XTT and was compared their formazan production. As shown the Figure 1, bacterial activity on biofilms was easily detected through the formazan production after 1,30 h of assay and only Siporax® colonized cylinders showed an OD490nm increase. It is clear that there is a higher bacterial activity when added an additional carbon source and higher absorbance readings shown higher formazan production.

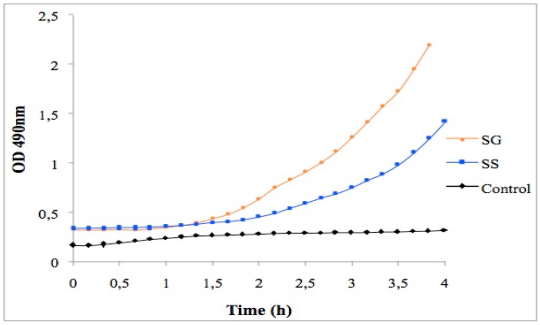


Figure 1. Evaluation of bacterial respiration in Siporax® cylinders. SG represent formazan production in Siporax® colonized cylinders loaded with glucose solution; SS represent formazan production in Siporax® colonized cylinders loaded

As shown Figure 2 at day 0 of assay (green curves) there is a similar biofilm tolerance to each biocide spikes in both constructed wetlands, H4 and H6. Over the experimental time course, biofilm pesticide tolerance was enhanced in H6 (the best example is Figure 2a*) and in general the metabolism reactivation time in H4 took longer.*

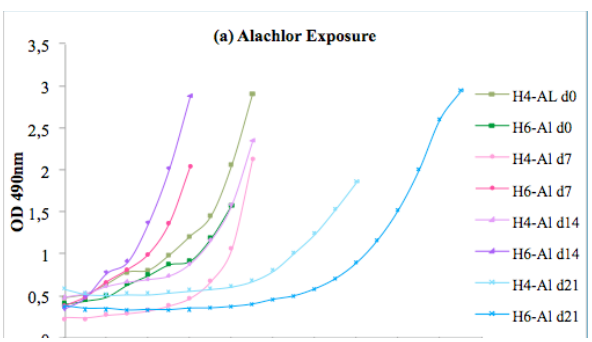
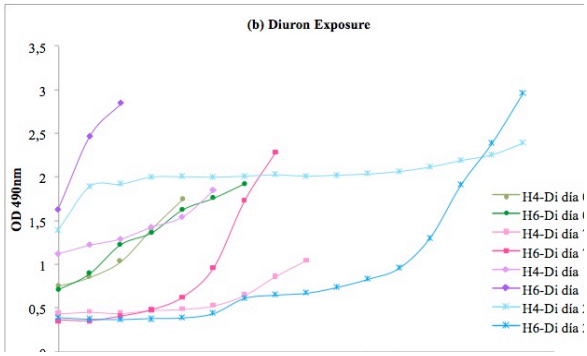


Figure 2. Biofilm respiration in the presence of different



biocides (a- alachlor, b-diuron). Formazan production was assessed spectrophotometrically at 490 nm measuring cell viability every hour during 10 hours at 25°C in a microplate reader. Samples were taken at 0, 7, 14 and 21 days of exposure in each HSCW. We didn't show the standard deviation of the mean for better visual comprehension of the figure.

Throughout the experimentation time the viability in both HSCW is clearly different (see Figure *2b*), so biocides affected the biofilm respiration at different degrees and the effect dose-response is clearly seen.

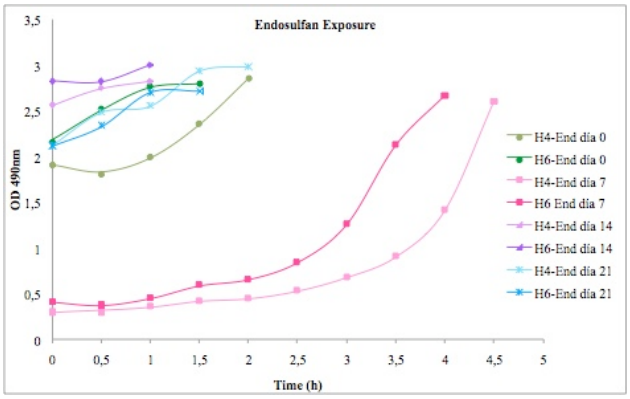


Figure 3. Biofilm respiration in the presence of Endosulfan. Formazan production was assessed spectrophotometrically at 490 nm measuring cell viability every hour during 10 hours at 25°C in a microplate reader. Samples were taken at 0, 7, 14 and 21 days of exposure in each HSCW. We didn't show the standard

The results of this study also showed that endosulfan had minimal effects in both HSCW biofilm (H4 and H6) compared to the others two toxics, diuron and alachlor (Figure 3).

The biocide concentration used in this study (0,5 ppm) showed a low toxic effect on bacterial biofilm viability.

Conclusions

The assays carried out proved that XTT is a reliable technique to be use as a simple colorimetric indicator of the metabolic activity and an effective tool to estimate bacterial viability without breaking biofilm structure. Expose the biofilm to external contamination could affect its survival; our study showed that the biocides addition generates an inhibitory effect delaying bacterial activity. Comparing two wetlands, these inhibitory effects were lower in H6 previously treated with toxics.
The present study is the first one to use XTT method to study and evaluation the effect of compounds, with different biocide activity, into bacterial viability in bacteria present in wetlands without the need to separate the micro-organisms from its supporting material.
This work is a preliminary study and more studies should be done to support the obtained results.

References

Hatzinger, P. B., Palmer, P., Smith, R. L., Peñarrieta, C. T., Yoshinari, T. 2003. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *J. Microbiol. Methods*, **52**, 47–58.

Lawrence, J. R., Kopf, G., Headley, J. V., Neu, T. R. 2001. Sorption and metabolism of selected herbicides in river biofilm communities. *Can. J. Microbiol.*, **45**, 634–641.

Kuhn, D. M., Balkis, M., Chandra, J., Mukherjee, P. K. and Ghannoum, M. A. 2003. Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. *J. Clinical Microbiol.*, **41**(1), 506–508.

Pérez, L. M., Álvarez, B. L., Codony, F., Fittipaldi, M., Adrados, B., Peñuela, G., Morató, J., 2010. A new microtitre plate screening method for evaluating the viability of aerobic respiring bacteria in high surface biofilm. *Letters in Appl. Microbiol.*, **51**, 331–337.

Roslev, P. & King, G.M. 1993. Application of a tetrazolium salt with a water–soluble formazan as an indicator of viability in respiring bacteria. *Appl. and Environ. Microbiol.*, **59**(9), 2891–2896.