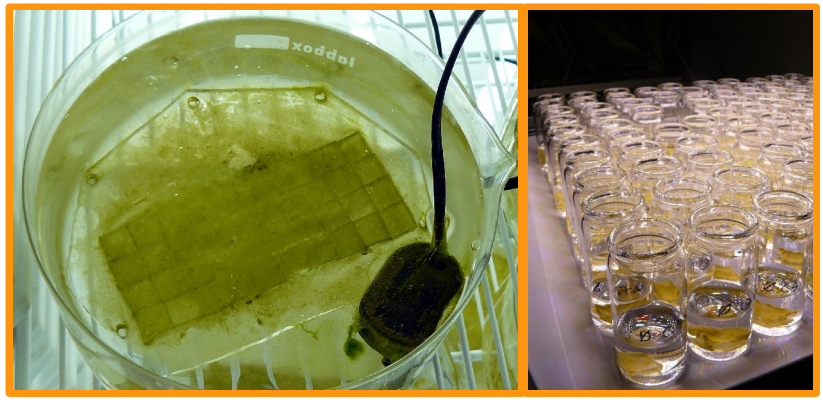
Fluvial biofilms: a pertinent tool to assess β-blockers toxicity



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Abstract

Among increasingly used pharmaceutical products, β-blockers have been commonly reported at low concentrations in rivers and littoral waters of Europe and North America. Little is known about the toxicity of these chemicals in freshwater ecosystems while their presence may lead to chronic pollution. Hence, in this study the acute toxicity of 3 β-blockers: metoprolol, propranolol and atenolol on fluvial biofilms was assessed by using several biomarkers. Some were indicative of potential alterations in biofilm algae (photosynthetic efficiency), and others in biofilm bacteria (peptidase activity, bacterial mortality). Propranolol was the most toxic β-blocker, mostly affecting the algal photosynthetic process. The exposure to 531 µg L^{-1} of propranolol caused 85% of inhibition of photosynthesis after 24 h. Metoprolol was particularly toxic for bacteria. Though estimated No-Effect Concentrations (NEC) were similar to environmental concentrations, higher concentrations of the toxic (503 µg L^{-1} metoprolol) caused an increase of 50% in bacterial mortality. Atenolol was the least toxic of the three tested β-blockers. Effects superior to 50% were only observed at very high concentration (707 mg L^{-1}). Higher toxicity of metoprolol and propranolol might be due to better absorption within biofilms of these two chemicals. Since β-blockers are mainly found in mixtures in rivers, their differential toxicity could have potential relevant consequences on the interactions between algae and bacteria within river biofilms.

Keywords: biofilm, β-blocker, biomarkers, multivariate approach.

1. Introduction

Drugs consumption has steeply increased during the last decade and as a consequence the amount of pharmaceutical products that reach freshwater ecosystems has increased. Among these emerging toxicants, β-blockers are widely used in therapy against hypertension or heart failure. As a documented example, from 100 to 250 tonnes of β-blockers are consumed each year in Germany (Cleuvers 2005). The arrival of these products may lead to chronic contamination, with unknown impacts on aquatic ecosystems. Moreover, the degradation of these pharmaceutical products is highly variable depending on the molecule, but also on the type of sewage treatment. Ternes (1998) found that propranolol was removed up to 96% and metoprolol up to 83% through Sewage Treatment Plants (STP), while Vieno et al. (2006) found an 11% removal of metoprolol and a 76% removal of atenolol through a STP in Finland. In any case, β-blockers are not completely removed from sewage effluents and consequently concentrations at the ng L^{-1} range can be found in rivers and littoral waters of Europe and North America. Huggett et al. (2003) reported a concentration of propranolol of up to 1.9 μg L^{-1} in North America. In the River Llobregat (Spain), some β-blockers (propranolol, metoprolol, atenolol and sotalol) have been detected, with maximum values of 60, 180, 670 and 1820 ng L^{-1}, respectively (Muñoz et al. 2009).

In spite of the potential toxicity of some β-blockers, particularly for fish and algae, few studies have been performed on the impact of β-blockers on the aquatic ecosystem. The growth of the fish *Oryias latipes* (medaka) was reduced and its levels of testosterone and plasma estradiol significantly changed after 14 days of exposure to 0.5 mg L^{-1} of propranolol. A reduction of the number of laid eggs was also observed after an exposure of 4 weeks to 0.5 and 1 μg L^{-1} of propranolol (Huggett et al. 2002). Using the *Daphnia magna* immobilization test, the *Lemna minor* and *Desmodesmus subspicatus* growth inhibition tests to assess the toxicity of propranolol, metoprolol and atenolol, Cleuvers (2005) found that *Desmodesmus* was the most sensitive, with an EC_{50} of 0.7 mg L^{-1} for propranolol. Escher et al. (2006) illustrated the phytotoxicity of

4 β-blockers in a non-target effect study based on the inhibition of the photosynthesis of green algae. These single-species tests highlighted the specific acute toxicity of β-blockers towards green algae. However, algae are part of multi-species communities in the environment and a multi-species test involving algae should be the next step (OECD guidelines) in assessing the toxicity of β-blockers. In this paper, therefore, the toxicity of β-blockers has been assessed on fluvial biofilms.

Fluvial biofilms are communities mainly composed of diatoms and green algae as well as of cyanobacteria, bacteria, protozoa and fungi, all embedded in an extracellular matrix (Sabater and Admiraal 2005). Fluvial biofilms are present in different river microhabitats (rocks, sediments, organic debris), and represent an interface between the flowing water and the stream bed (Romaní et al. 2004; Sabater et al. 2002). These characteristics make biofilms pertinent bioindicators of environmental perturbations within the aquatic ecosystem. Several studies have highlighted the biofilm sensitivity to a large panel of toxicants such as heavy metals (Guasch et al. 2002; Ivorra et al. 2002; Pinto et al. 2003), herbicides (Guasch et al. 2003; Gustavson et al. 2003; Leboulanger et al. 2001; Pesce et al. 2008; Schmitt-Jansen and Altenburger 2005b), and pharmaceuticals (Lawrence et al. 2005). Due to this sensitivity, fluvial biofilms can be used as early warning systems for the detection of the effects of toxicants on aquatic systems (Sabater et al. 2007).

Disturbances occurring in the ecosystem would first lead to biochemical and physiological changes within the biofilm that could evolve to community changes if perturbations persist. While changes in community composition have been linked to persistent pollution of river sites (Tornés et al. 2007), the biochemical and physiological changes indicate an initial stress response, and can be used as early indicators of ecosystem damage. Biofilm complexity provides a large panel of functional and structural endpoints to assess toxicity of emerging pollutants. In this study, a biomarker approach has been used to investigate the effect on the whole biofilm community of a 24 h exposure to the three β-blockers metoprolol, atenolol and propranolol.

Because the toxicity mode of action of β-blockers on algal communities is unknown, the set of biomarkers should account for a global status of biofilm, and needs to include their two most important compartments (algae and bacteria). Photosynthetic efficiency was selected as a classical biomarker of autotrophic biofilms (Brack and Frank 1998; Fai et al. 2007; Schmitt-Jansen and Altenburger 2008a). The sensitivity of the different groups of primary producers (cyanobacteria, green algae and diatoms) was estimated through their specific photosynthetic efficiencies (Zhang et al. 2008). To assess toxicity on bacterial compartment, bacterial mortality was determined and the effects on the heterotrophic activity of biofilms (and therefore on the bacteria–-algal relationships; Francoeur and Wetzel 2003) were estimated by means of the extracellular enzyme activities (e.g. peptidase). Furthermore the oxidative stress response was measured as a reflection of the general stress level. Oxidative stress is due to the accumulation of reactive oxygen species (ROS) during metabolic processes, which is enhanced by the presence of heavy metals, herbicides among others (Pinto et al. 2003; Zbigniew and Wojciech 2006). To prevent injuries from ROS accumulation, cells develop antioxidative systems composed of different enzymes and pigments, which can be used as biomarkers of stress from toxicant exposure (Tripathi and Gaur 2004; Tripathi et al. 2006). Catalase is an antioxidant enzyme which scavenges hydrogen peroxide. Geoffroy et al. (2004) observed that catalase activity was a more sensitive biomarker than photosynthetic activity after exposition of *Scenedesmus obliquus* to the herbicide flumioxazin. Most of these biomarkers are functional since the short-time (24 h) exposure is mainly expected to affect the function of biofilms rather than their structure. Altogether, these endpoints compile a set of biomarkers encompassing general metabolic pathways (photosynthetic efficiency, peptidase, bacterial mortality) and stress response mechanism (oxidative stress), allowing responses occurring at molecular level (oxidative stress, peptidase) and at community level (bacterial mortality, photosynthetic efficiency of each algal group) to be detected.

This study aims to illustrate the potential of a multi-species system combined with a biomarker approach to assess toxicity of β-blockers. To do so, the following points are questioned:

1. Does the set of biomarkers allow the toxicity impact on fluvial biofilms of the three analysed β-blockers (same type of chemicals) to be differentiated ?
2. Which β-blocker is the most toxic ?
3. Which biomarker is the most sensitive ?

2. Material and methods

2.1. Chemicals

Pure propranolol hydrochloride (CAS: 3506-09-0), atenolol (CAS: 29122-68-7) and metoprolol tartrate (CAS: 56392-17-7) were purchased from Dr. Ehrenstorfer GmbH® (Germany).

2.2. Biofilm colonization

Biofilm communities were grown on glass substrata installed in crystallizing dishes containing 1.5 L of tap water which had been previously passed through a carbon filter to eliminate chlorine. The original biofilm inoculum was obtained from the river Llémena (NE Spain; Serra et al. 2009a). An aquarium pump allowed constant circulation of water in order to simulate flowing water. The dishes were incubated at 19 ºC and under a 12/12 h day–night cycle, with a photon flux density of about 120 µmol photons m^{-2} s^{-1}. The water was changed twice a week and phosphate was added to a final nominal concentration of 158 µg L^{-1}.

2.3. Diatom community composition

Biofilm samples for diatom examination were collected randomly from 3 crystallizing dishes. Samples were digested with hydrogen peroxide and mounted in permanent slides. Identification and counting was performed as described by Ricart et

al. (2009). Shannon–Wiener diversity index (Shannon and Weaver 1963) was calculated by means of the OMNIDIA package (version 2).

2.4. Acute exposure experiments

Acute toxicity tests were performed on 3 week-old biofilms. Each glass substrata was incubated in a vial containing 10 mL of colonization medium and the corresponding toxicant concentration. Samples were incubated during 24 h under the same conditions as the colonization, using a single-speed orbital mixer (KS260 Basic, IKA®) to maintain constant agitation. Two samplings were performed after 6 h and after 24 h of exposure. Four replicates were used for each endpoint and each concentration (and controls). Four concentrations were tested for each toxicant. Concentrations of propranolol and metoprolol were: 0.9, 90, 900 and 9000 µg L^{-1}. Atenolol concentrations were 0.9, 900, 9000 and 900 000 µg L^{-1}. Concentrations were chosen based on the literature (Munoz et al. 2009; Huggett et al. 2003), atenolol being described as less toxic than propranolol and metoprolol (Cleuvers 2005). Abiotic controls consisted of water samples without incubated biofilms. Real concentrations of the 3 β-blockers were analysed in water of both biotic and abiotic samples, to distinguish between toxicants degraded by photolysis and those being adsorbed and/or absorbed by the biota (Liu and Williams 2007; Piram et al. 2008).

2.5. Photosynthetic efficiency

The chlorophyll fluorescence measurements were carried out by means of a PhytoPAM (Pulse Amplitud Modulated) fluorometer (Heinz Walz GmbH). Four colonised glass substrata for each treatment were collected at random after 6 and 24 h for in vivo chlorophyll fluorescence measurements. These were performed at room temperature and in a dark chamber. The distance between the optical fiberoptics and the sample surface was set at 2 mm. The fluorescence signal was determined by the emitter-detector unit (PHYTO-EDF). Maximal photosynthetic efficiency of PSII (maximal PSII Quantum Yield) was obtained after a 20 min dark-adaption of samples and effective PSII quantum yield (efficiency of PSII) after light-adaptation.

Calculations were done following Genty et al. (1989). The deconvolution of the fluorescence signal into the contributions of the three algal groups and cyanobacteria is based on the internal reference excitation spectra of a pure culture, which has been previously validated for periphyton communities by Schmitt-Jansen and Altenburger (2008a). The maximal and the effective photosynthetic efficiencies were estimated based on the fluorescence signal recorded at 665 nm and given as relative units of fluorescence. The relative contribution of the different algal groups was also used to obtain the effective photosynthetic efficiency for each of them, using the fluorescence signal linked to green algae, cyanobacteria and diatoms.

2.6. Bacterial mortality

Live and dead bacteria were counted with epifluorescence microscopy after double staining with the LIVE/DEAD® Bacteria Viability Kit L7012 (BacLightTM). Two nucleic acid stains composed this kit: the SYTO 9 which stains all cells (excitation/emission 480/500 nm) and the propidium iodide (excitation/emission 490/635 nm) which penetrates cells that have damaged membranes. Sample preparation, staining and counting were done as described by Ylla et al. (2009) using pre-sterilized medium for dilution.

2.7. Peptidase activity

Potential extracellular activity of leucine-aminopeptidase enzyme (EC3.4.11.1) was measured spectrofluorometrically using fluorescent-linked substrate L-leucine-4--methyl-7-coumarinylamide (Leu-AMC) as described by Ricart et al. (2009) and Romaní et al. (2004).

2.8. Protein extraction

Biofilms were removed from the glass substrata with a sterile silicone cell scraper (Nunc, Wiesbaden, Germany) and centrifuged at 2300 × g and 10ºC for 5 min to remove the excess of water. The pellets were frozen immediately in liquid nitrogen and stored at -80 ºC until the enzyme assays were started. Samples were homogenized for 3 min on ice by adding 0.9 mL of homogenization buffer containing

100 mM potassium phosphate buffer (pH 7.4), 100 mM KCl, 1 mM EDTA and 10% (w/v) PVPP (Polyvinylpolypyrrolidone) for 100 mg of wet weight of biofilms. Homogenates were then centrifuged at 10 000 × g and 4ºC for 30 min. Supernatant was used as the enzyme source, the protein content of supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.9. Catalase activity

Catalase activity was measured spectrophotometrically at 240 nm according to Aebi (1984). 800 µL reaction mixture was obtained adding potassium phosphate buffer (pH 7.0; 80 mM final concentration); H_2O_2 (20 mM final concentration) and the enzyme extract (10 µg of protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25 ºC for 4 min. Catalase activity was calculated as µmol H_2O_2 mg protein^{-1} min^{-1} using the extinction coefficient of H_2O_2: 0.039 cm^2 µmol^{-1}.

2.10. Analysis of β-blockers in water

After 6 and 24 h of exposure, water from 4 biotic and 4 abiotic samples was filtered through a 0.45 µm nylon filter (Whatman, England) and kept at −20 ºC until HPLC analyses. Control and 0.9 µg L^{-1} samples (nominal concentration) were concentrated prior analyses through tC18 cartridges (Sep-Pak® Vac 3 cc tC18, Waters, Ireland). Cartridges were conditioned with 5 mL of methanol and 5 mL of water before sample enrichment. Then, cartridges were washed with 5 mL of water and dried during 20 min. Elution was done with 8 mL of methanol, eluted samples were then dried under nitrogen stream and reconstituted with 1 mL methanol/water (1:3, v/v).

Samples were analysed by liquid chromatography. The HPLC system consisted of a binary HPLC Pump (1525 Waters), an autosampler (717 Plus Waters) and an UV-detector (Dual λ Abs. Detector 2487 Waters). The methods used were adapted from Delamoye et al. (2004). Flow rate was set at 1 mL min^{-1} and wavelength detection at 227 nm. For controls and samples of a nominal concentration of 0.9 µg L^{-1}, separation was carried out on 3.5 µm C18 column (Symmetry 4.6 mm × 75 mm)

maintained at 30 ºC. Injection volume was 20 µL. Elution was performed with a gradient of acetonitrile and phosphate buffer (10 mM, pH = 3.8) as described in Table 1.

Table 1. Gradient of HPLC elution (in percentage) for the different samples.

Samples concentration	Time (min)	% Acetonitrile	% Phosphate buffer
Control 0.9 µg L^{-1}	0	10	90
	3	10	90
	3.5	20	80
	8	40	60
	12	40	60
	12.5	10	90
	15	10	90
From 90 µg L^{-1} to 900 000 µg L^{-1}	0	15	85
	3	15	85
	3.6	40	60
	10	40	60
	11	15	85
	15	15	85

For samples of nominal concentration between 90 and 900 000 µg L^{-1}, separation was carried out on a 5 µm C18 column (Sunfire 4.6 mm × 150 mm) maintained at 30 ºC. Injection volume was 100 µL. Elution was performed with a gradient of acetonitrile and phosphate buffer (10 mM, pH = 5.00) as described in Table 1. For each β-blocker, the detection limits were 71 µg L^{-1} for propranolol, 42 µg L^{-1} for atenolol, and 20 µg L^{-1} for metoprolol. A value of half the detection limit was attributed to samples whose concentration was below detection limit.

2.11. Data analyses

All statistics analyses were done using R 2.6.2 (R Development Core Team 2008).

The use of multi-species communities implies higher variability than those accounted for by the single-species tests. Thus, two different approaches were used to explore the data. First, No-Effect Concentration was determined independently for each β-blocker and each biomarker to quantify the impact of each toxicant. Measured β-blockers concentrations and all biomarkers responses were used. Biomarker responses corresponding to concentrations below detection limit were used as controls, and then a linear regression was done in the linear range of the remaining points. The linear range was the result of a compromise to maximize the number of points and r^2 (regression coefficient) and to obtain a significant regression ($p < 0.05$). NEC was defined as the intercept between the mean of controls and the linear regression, range for each NEC was determined through inverse regression as described by Liber et al. (1992) and Draper and Smith (1981). When no significant linear regression could be obtained, potential differences between controls and treatments were analysed using analysis of variance (ANOVA), effects were *post hoc* analysed with a Tukey test. For all the analyses, statistical significance was set at $p < 0.05$.

Next, a partial Redundancy Direct Analysis (Vegan package, Oksanen et al. 2008) was performed to integrate the variability due to growth conditions and biofilm formation. Among the ordination method, RDA allows constraining the arrangement of the response variables along the axes. This analysis included the exposure results to the three β-blockers. The biomarkers with too many missing values were excluded from the analysis. The following biomarkers were then used: catalase activity, peptidase activity, life-dead bacterial ratio, photosynthetic efficiency of cyanobacteria and photosynthetic efficiency of green algae. samples containing missing values were also discarded from the analysis (28 samples on a total of 120). Biomarkers responses were scaled. As the gradient of nominal concentration used was very large, concentrations were expressed as ranks (from 0 for control to 5 for the highest concentration). The time of exposure was set as a covariable to integrate results of 6 and 24 h of exposure.

3. Results

3.1. Biofilm colonization

After 3 weeks of colonization, the biofilm had a diatom community composed of 36 species (mean value of three replicates). The most dominant taxa were *Achnanthes minutissima*, *Cymbella microcephala*, *Navicula atomus var. permitis*, *Nitzschia dissipata*, *Nitzschia frustulum* and *Navicula seminulum*. Shannon–Wiener diversity index was 3.7 ± 0.6.

Physical and chemical conditions were stable during the colonization. Water conductivity was at 477 ± 9 µS, pH: 8.46 ± 0.09, dissolved oxygen concentration: 9.11 ± 0.13 mg L^{-1}, and water temperature: 20.6 ± 0.2 ºC (n = 28 for all parameters). Water used during this experiment has been previously characterized by Serra et al. (2009b) for NO_3 (1.68 ± 0.14 mg L^{-1}), NO_2 (0.07 ± 0.01 mg L^{-1}) and NH_4 (<0.1 mg L^{-1}) among others. Then, in this experiment, only phosphorus concentration was measured, the concentration had a mean value of 46.8 ± 2.1 µg L^{-1} (n = 24) just after water changes and phosphorus addition, but declined to low levels (1.5 ± 0.2 µg L^{-1}; n = 20) just before water changes. However complete depletion in phosphorus was never observed during colonization.

Moreover, the biofilm used in these experiments had a normal bacterial mortality with a live/dead bacteria ratio of 48 ± 6%. Mean peptidase activity was 332.8 ± 73.8 nmol AMC cm^{-2} h^{-1} and mean catalase activity was 28.5 ± 10.9 µmol H_2O_2 mg $protein^{-1}$ min^{-1}. The photosynthetic efficiency and capacity of these biofilms were of 0.30 ± 0.05 and 0.47 ± 0.05. The respective photosynthetic efficiencies of cyanobacteria, diatoms and green algae were 0.25 ± 0.05, 0.32 ± 0.11 and 0.33 ± 0.06.

3.2. Measured β-blockers concentrations in water

Propranolol and metoprolol concentrations were below detecttion limits in controls and samples of nominal concentration inferior to 900 and 90 µg L^{-1}, respectively. No atenolol could be detected neither in controls nor in samples of the

lowest nominal concentration (0.9 µg L^{-1}). The concentration of propranolol and metoprolol in water was at least two times lower in the biotic samples than in the abiotic samples, but the concentration of atenolol was similar in the two types of samples (Fig. 1). Moreover, propranolol and metoprolol concentrations were also lower (up to 17 times) than the nominal concentrations in the abiotic samples for all concentrations tested, with an even larger difference at the highest concentration (Fig. 1A and B). The concentration of atenolol in the abiotic samples was close to the nominal one, except for the intermediate concentration (Fig. 1C).

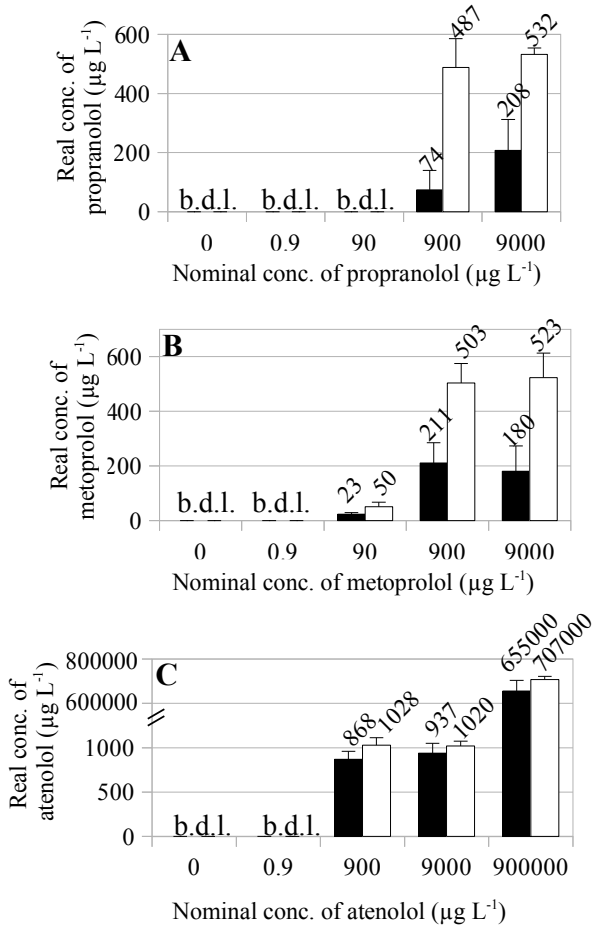

Figure 1. Concentrations of propranolol (A), metoprolol (B) and atenolol (C) of biotic (black bars) and abiotic (white bars) samples after 24h of exposure. On the horizontal axis, nominal concentrations in µg L^{-1} of each β-blocker are shown.

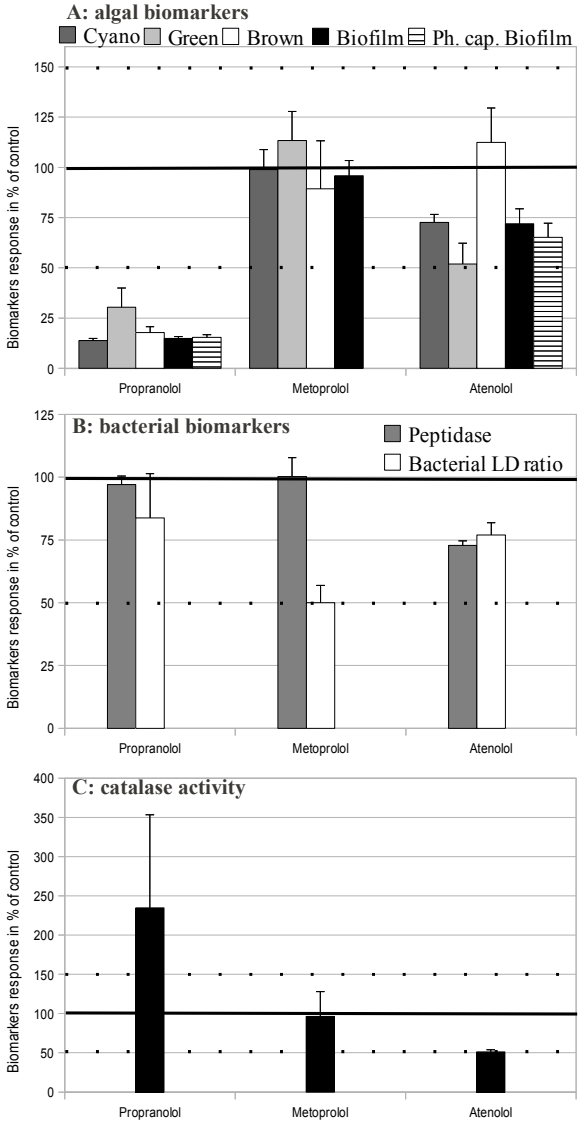

Figure 2: Response of the different biomarkers, expressed in percentage of control, after 24h of exposure to 531 µg L^{-1} of propranolol, 522 µg L^{-1} of metoprolol and 707 000 µg L^{-1} of atenolol. Graph A represents the response of algal biomarkers: photosynthetic efficiency of cyanobacteria (dark grey), green algae (grey), brown algae (white) and all biofilm (black) and photosynthetic capacity of all biofilm (horizontal hatches). Graph B represents bacterial biomarker: peptidase activity (grey) and live/dead ratio of bacteria (white). Graph C represents the catalase activity (black). In all graphs error bars depict the standard error; a plain black line indicates the control (100%) and dotted lines indicated +/- 50% of activation/ inhibition compared to control.

3.3. Effect of the highest β-blockers concentration

After 24 h of exposure, the two highest concentrations tested caused clear effects. However, the three β-blockers tested affected the various biomarkers differently; in most of the cases, the effects observed increased with concentration, here mean data expressed in percentage of control obtained after 24 h of exposure to the highest concentration are presented (Fig. 2).

The algal component was mainly affected by propranolol and atenolol while metoprolol had only a transitory effect on cyanobacteria. Indeed, after 6 h of exposure, the cyanobacteria photosynthetic efficiency was inhibited at 20 ± 4% in samples exposed at the two highest concentrations of metoprolol (503 and 522 µg L^{-1}; data not shown). However, these observations were not confirmed after 24 h of exposure (Fig. 2A). The highest concentration of propranolol (531 µg L^{-1}) caused 86 ± 1% of inhibition of photosynthetic efficiency for cyanobacteria, 70 ± 10% for green algae and 82 ± 3% for diatoms. The photosynthetic capacity of the whole biofilm was inhibited at 85 ± 1% (Fig. 2A). The highest concentration of atenolol (707 000 µg L^{-1}) significantly inhibited the photosynthetic efficiency of cyanobacteria by 27 ± 4% and of green algae by 48 ± 10% (Table 2, Fig. 2A). The photosynthetic efficiency of diatoms was not significantly affected by atenolol exposure (Table 2). Photosynthetic efficiency and capacity of the biofilm was also significantly inhibited by the highest concentration of atenolol (28 ± 7% and 35 ± 7% of inhibition; Fig. 2A).

The bacterial component of biofilm was mainly affected by metoprolol and atenolol rather than by propranolol. After 6 h the peptidase activity was affected by both propranolol and atenolol (data not shown) while after 24 h exposure, only atenolol had a significant impact on peptidase activity (Table 2). The highest concentration of this β-blocker inhibited 27 ± 2% of the peptidase activity (Fig. 2B). Bacterial mortality was significantly enhanced after a 24 h exposure to metoprolol or to atenolol (Table 2). At the highest concentration, metoprolol caused an inhibition of 50 ± 7% of the Live-Dead bacterial ratio. Exposure to the highest atenolol concentration increased

bacterial mortality by 23 ± 5%. Propranolol caused a moderate increase in bacterial mortality (16 ± 18%; Fig. 2B, Table 2). Despite a great increase observed in the catalase activity of samples exposed to propranolol, this antioxidant response was not significant (Fig. 2C; Table 2). Atenolol and metoprolol antioxidant responses were similar. The highest atenolol concentration significantly inhibited 49 ± 3% of the catalase activity after 24 h of exposure (Fig. 2B, Table 2). Six hours of exposure to 503 µg L^{-1} of metoprolol caused a significant inhibition of catalase activity by 37 ± 6% (data not shown); however, after 24 h of exposure no significant changes in catalase activity were observed in any of the concentrations tested (Table 2).

3.4. Determination of the No-Effect Concentrations (NEC)

The impact of the pollutants tested was quantified by means of No-Effect Concentrations as well as by their upper and lower limits (Table 2). Metoprolol affected mainly bacterial mortality while no significant effects on photosynthetic efficiency were found (Table 2). Biomarker responses of biofilms exposed to metoprolol were highly variable; the range obtained for NECs values are the widest observed in this experiment. NECs of propranolol for peptidase activity and photosynthesis-related endpoints ranged between 293 and 300 µg L^{-1} after 6 h, and between 479 and 489 µg L^{-1} after 24 h of exposure (Table 2).

NECs of atenolol for peptidase and catalase activity and photosynthetic biomarkers had wider ranges and higher variability between endpoints than for propranolol, differing also between algal groups and times of exposure (Table 2). Responses of biofilms exposed to propranolol were the least variable. After 6 h of exposure, effects on peptidase activity and photosynthetic efficiency of cyanobacteria showed similar NEC values (Table 2). After 24 h of exposure, no impact was detected on peptidase activity, and NECs values remained similar for the photosynthetic efficiencies of the different algal groups and for photosynthetic capacity.

Table 2. NEC values in µg L^{-1} and corresponding range for each biomarker after 6h and 24h of exposure to metoprolol (M), propranolol (P) and atenolol (A).

		Peptidase act.		Catalase act.		Life-Dead bacterial ratio		Photosynthetic efficiency								Photo-synthetic capacity	
								Cyanobacteria		Green algae		Brown algae		Biofilm			
	Exp. time	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC	p	NEC
M	6h	n.s.	n.s.	***	45 [$7.6x10^{-140}$; $1.8x10^{46}$]	n.s.	0.64 [$2.2x10^{-7}$; 53.5]	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.d.	n.d.
	24h	n.s.	n.s.	n.s.	n.s.	***	0.04 [$4x10^{-126}$; $4.7x10^{14}$]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P	6h	***	341 [108;665]	n.s.	n.s.	n.s.	n.s.	***	300 [266;327]	***	n.s.	n.s.	n.s.	***	293 [248;326]	n.d.	n.d.
	24h	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	484 [475;491]	***	479 [383;515]	**	489 [451;512]	***	484 [478;489]	***	485 [476; 493]
A	6h	***	1473 [281; $5.81x10^{3}$]	n.s.	n.s.	n.s.	n.s.	**	1495 [0.9; $44x10^{5}$]	***	69 [0.04; $8.52x10^{3}$]	n.s.	n.s.	**	742 [0.06; $1.3x10^{5}$]	n.d.	n.d.
	24h	***	827 [0.8; $1.04x10^{5}$]	***	908 [39; $1.18x10^{4}$]	**	n.s.	**	590 [0.03; $1.8x10^{5}$]	**	2960 [2;$4.3x10^{5}$]	n.s.	n.s.	**	652 [0.3; $4.5x10^{4}$]	***	707 [2; $2x10^{4}$]

The p column indicates the p-value obtained from an ANOVA analysis of the biomarkers response (n=4 for each concentration)
n.s. : non-significant result with a p-value > 0.05 for ANOVA or for linear regression of NEC calculation
*n.d. : non-determined responses; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001*

3.5. Multivariate analysis of biofilm response to β-blockers exposure

The ordination of the response of the biomarkers along the gradient of β-blockers is described by the RDA (Fig. 3). The first 2 axes explained 27% of the variance observed among all the samples (from the three experiments). The first axis was mainly driven by the gradient of propranolol (score of 0.85), while the second axis was related to metoprolol (score of -0.89). The photosynthetic efficiency of cyanobacteria and green algae had high negative scores on the first axis, indicating that the photosynthetic efficiency decreased with increasing concentrations of propranolol. Catalase activity also increased along the gradient of propranolol (Table 3). The live--dead bacterial ratio (LD) got the highest positive score in the second axis. Hence the diminution of the live-dead bacterial ratio (higher bacterial mortality) increased with metoprolol concentrations. The peptidase (Pep) and catalase (Cat) activities also decreased with increasing concentrations of metoprolol (Table 3). The third axis (not represented) was mainly driven by the gradient of atenolol (score of -0.96). This axis accounted for only 1.5 % of the variance; no clear response of the biomarkers could be highlighted (Table 3). The percentage of variance observed due to the time of exposure was relatively low (4.3%), indicating that few changes occurred between 6h and 24h of exposure.

Table 3. Scores of the different biomarkers on the RDA and PCA axes.

	RDA1	RDA2	RDA3	PC1	PC2	PC3
Pep	-0.175	0.641	-0.462	1.148	-0.738	0.673
Cat	**0.937**	0.501	0.299	-0.108	-1.318	-1.082
LD	0.154	**1.150**	0.088	0.456	0.945	-0.869
Y Bl	**-1.383**	0.169	0.272	0.500	-0.034	0.509
Y Gr	**-0.962**	0.313	-0.002	1.551	0.188	-0.482

Cat corresponds to catalase activity, Pep to peptidase activity, Y to the photosynthetic efficiency of cyanobacteria (Bl) and of green algae (Gr). Highest scores on the two first RDA axes are indicated in bold.

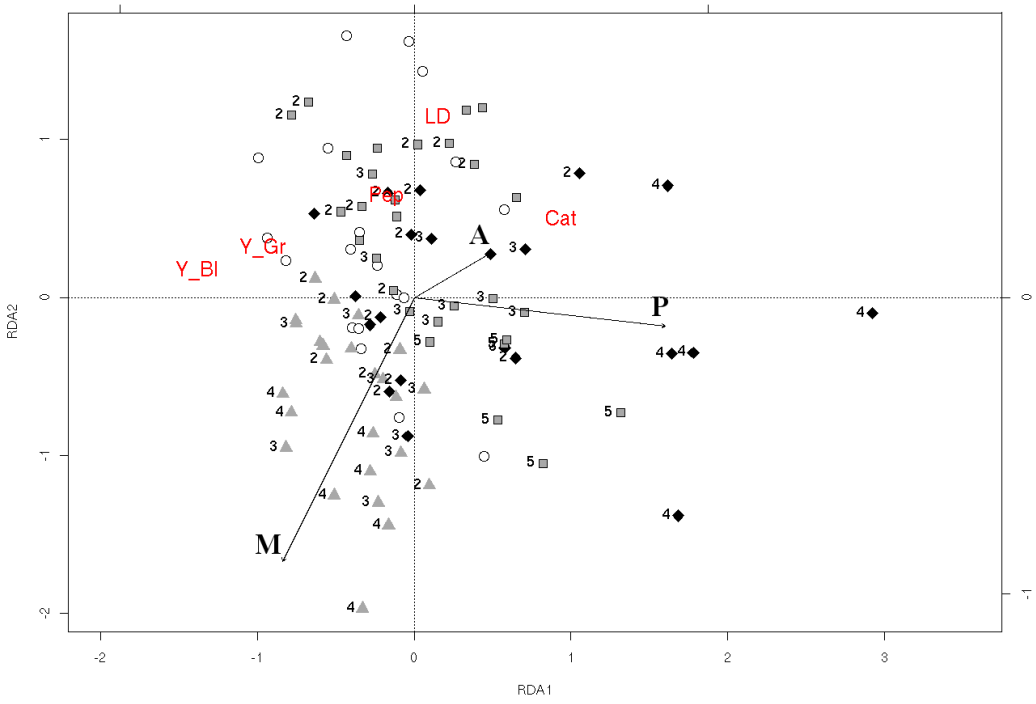

Figure 3. Results of RDA. The gradient of the different toxicants are shown by the arrows: P for propranolol, M for metoprolol and A for atenolol. Symbols corresponds to the different samples: o: control, ♦: samples exposed to propranolol, ▲: to metoprolol, ■: to atenolol. The different biomarkers are indicated in red: Cat for catalase activity, Pep for peptidase activity, Y corresponds to the photosynthetic efficiency of the cyanobacteria (Bl) and the green (Gr) algae respectively. The concentrations ranks 2,3,4,5 are indicated for each sample and correspond to the nominal concentrations of 90, 900, 9 000 and 900 000 µg L^{-1} of β-blocker.

4. Discussion

The whole set of functional and structural biomarkers of fluvial biofilms showed that impacts of the three β-blockers tested were specific. The multivariate analysis separated those samples exposed to propranolol from others exposed to metoprolol or atenolol (Fig. 3). Furthermore, at the highest concentration each β-blocker affected different endpoints (Fig. 2). This result is relevant considering that these compounds are part of the same class of pharmaceuticals, and these molecules are expected to act in a similar way as in humans in all organisms containing β-adrenergic receptors. The mode of action in humans can, in fact, give clues for assessing toxicity on these organisms (Owen et al. 2007). However, the mode of action of these pharmaceuticals in the environment is probably different from that described on human beings. The differences observed in algae and bacteria illustrate the need to produce a different classification that could be used when assessing toxicity of pharmaceuticals on the environment.

HPLC measurements highlighted that differences occurred between nominal and real concentrations for all β-blockers. Concerning atenolol, real concentrations are 9 times lower than the nominal concentration at 9000 µg L^{-1} but are similar to nominal ones at 900 and 900 000 µg L^{-1}. These observations are in contradiction with previous studies in which this compound has been described as generally stable at concentrations up to 10 000 µg L^{-1} (Liu and Williams 2007) and so suggest an operating error. For propranolol and metoprolol, an important difference between nominal and real concentrations is observed at all concentrations, indicating pollutant degradation. The degradation may be due to the light, that causes half-lives (in STP water, under UV radiation) to be around 3–4 h for propranolol and 20–48 h for metoprolol (Piram et al. 2008). Liu and Williams (2007) also observed that the kinetics of propranolol and metoprolol differ with the concentrations, which could explain the similar concentrations obtained at 900 and 9 000 µg L^{-1}. Moreover, biotic pathways of degradation may also occur as biotic and abiotic concentrations differ; the interaction

etween biotic and abiotic pathways of degradation could then lead to similar final concentration of pollutant in the water.

These results illustrate the importance to measure real concentrations in the water and the difficulties to know the real exposure. Indeed, the measure of a toxicant concentration during an assay is a snapshot of the concentration and do not integrate the total concentration during the exposure. In fact, NEC values were derived in our study from measured concentrations of β-blockers in water minimizing therefore an overestimation of toxicity. Degradation and absorption are dynamic processes that occur along all the assay, a kinetic study would have reflected better the exposure of the biofilm. Another option to determine the level of exposure is to determine the internal concentration of pollutant in the biofilm, this method is especially relevant in case of non-metabolised pollutant as metals (Guasch et al. 2009).

Within the biofilm, propranolol affected mainly the algal compartment through inhibition of the photosynthetic process. After 6 h of exposure, cyanobacteria were more sensitive to propranolol than diatoms or green algae, but after 24 h exposure all groups were affected to the same extent, and the highest concentration (531 $\mu g\ L^{-1}$) caused the inhibition of both photosynthetic efficiency and capacity, indicating irreversible damages on the photosynthetic apparatus. Multivariate analysis indicated that high propranolol concentration was associated to high catalase activity. However, significant effects were not detected by the ANOVA and no NEC values could be calculated for this biomarker. This apparent contradiction can be related to the high variability of catalase activity in samples exposed to propranolol. Liu and Williams (2007) have already suggested that the formation of intermediate radicals, which are highly reactive and cause oxidative stress, occurs during the degradation of propranolol. An analysis of the impacts of propranolol on the different antioxidant responses of biofilm could further support our results. Propranolol had little effect on the bacterial compartment of the biofilm, only a transitory effect on peptidase activity could be detected after 6 h of exposure. In conclusion, propranolol affects first the photosynthetic efficiency of cyanobacteria, and later causes irreversible damages to all

photosynthetic groups, which may cause oxidative stress and transitory bacterial response. As algae are essential for primary production in river ecosystems, toxicity of propranolol is of special concern and should be studied more extensively to derive ecotoxicological parameters (NOECs or EC_{50}).

Metoprolol was mainly toxic for bacteria. As shown by the RDA, high concentrations of metoprolol were related with bacterial mortality. The estimated NEC values for bacterial mortality were in the ng L^{-1} range, well within the realistic environmental concentrations. Though caution is required concerning these results (r^2 of regression analysis was below 0.5, and the range for calculated NECs was very large), they indicate the potential chronic effect of metoprolol on biofilm communities.

Atenolol toxicity was very low indeed, even at the highest concentration tested (707 000 µg L^{-1}). In addition, atenolol toxicity was not specific for any of the biomarkers measured, but affected both algae and bacteria. Within the algal compartment, green algae and cyanobacteria were affected by atenolol exposure, while diatoms appeared to be resistant to this toxic. The atenolol effect on the bacterial compartment was expressed in the increase in bacterial mortality and the decrease in peptidase activity at the two highest concentrations. A decrease in peptidase activity implies a reduction in the bacterial ability of hydrolysing peptides of high molecular weight and can be both due to a direct impact of atenolol on the enzymatic activity, but also to the decrease in photosynthetic efficiency that could indirectly affect the bacterial activity (Francoeur and Wetzel 2003). That atenolol at these high concentrations causes global stress both in algae as well as in bacteria was also expressed by the inhibition of catalase activity after 24 h of exposure. Catalase inhibition may be caused by a high level of H_2O_2 due to oxidative stress.

Atenolol was the less toxic of the three β-blockers tested. slight atenolol effects were only observed at the mg L^{-1} range while 531 µg L^{-1} of propranolol caused 85% of inhibition of photosynthesis efficiency of biofilm. These results were consistent with the findings of Cleuvers (2005) who classified atenolol as non-toxic for aquatic

organisms. According to NEC values, metoprolol was the most toxic of the 3 β-blockers tested. However, at the highest concentrations tested (503 µg L^{-1} for metoprolol and 522 µg L^{-1} for propranolol), propranolol determined a greater effect than metoprolol. Therefore propranolol would be the most toxic of the three β-blockers tested, consistently with the conclusions of the risk assessment performed by Cleuvers (2005) on *Daphnia magna* and *Desmodesmus subspicatus*.

The higher toxicity of propranolol and metoprolol could be related to a better absorption by biofilms of these β-blockers. Indeed, higher concentrations of propranolol and metoprolol were found in abiotic samples than in biotic samples while concentrations were similar in these two types of samples for atenolol. Moreover the higher log Kow of propranolol and metoprolol (3.37–3.48 and 1.88–2.28, respectively) than of atenolol (0.16–1.95) support this hypothesis. Therefore, propranolol and metoprolol toxicity might be caused by direct effects on internal metabolism whereas atenolol toxicity might be caused by interactions at the cell periphery.

Propranolol and atenolol are in the lower range of toxicity in comparison to others pharmaceuticals. Their respective NOEC for photosynthetic efficiency of all biofilm were of 484 and 652 µg L^{-1}. Fent et al. (2006) reviewed the NOEC values of 9 different pharmaceuticals (acetylsalicylic acid, salicylic acid, diclofenac, ibuprofen, naproxen, propranolol, clofibric acid, carbamazepine and fluoxetine) for different aquatic organisms to range between 0.001 and 1000 µg L^{-1}. The most toxic of these pharmaceuticals for phytoplankton was the neuroactive compound fluoxetine (NOEC = 0.001 µg L^{-1}) while the less toxic was the anti-inflammatory aproxen (NOEC ≈ 1000 µg L^{-1}). Metoprolol toxicity towards bacteria (≈50% of mortality after 24 h exposure to 523 µg L^{-1}) was also lower than those of some antibiotics (phenazone, amoxicilin and erythromycin). Indeed these antibiotics caused more than the 50% decrease of bacterial adhesion of a complex microbial community after 2 days incubation at 5 µg L^{-1} (Schreiber and Szewzyk 2008). However the toxicity of most of these pharmaceutical compounds has been tested on algae or bacteria and not on the whole biofilm communities, making comparison difficult. Moreover tests at

community level often indicate subtle effects that may become relevant during chronic exposure. For example, Lawrence et al. (2008) showed that a chronic exposure of river biofilm to 10 μg L^{-1} of the antimicrobial agent chlorhexidine leaded to significant changes in periphyton community composition while conventional toxicological tests with cyanobacteria, algae and protozoa did not reveal any significant effect at concentrations up to 100 μg L^{-1}.

In this study, the use of biofilms as multi-species systems, and a wide array of biomarkers have proved useful to distinguish qualitatively the effects of the three β-blockers. Since β-blockers are mainly found as mixtures in rivers, this species--specific toxicity could have potential consequences on interactions between algae and bacteria and furthermore on the whole aquatic ecosystem. Mixtures of β-blockers in the environment are usually found at low concentrations and may especially affect the bacterial compartment of biofilms. Nevertheless, high concentration pulses can affect dramatically the algal compartment. This experiment investigated No-Effect--Concentration after short-term exposure. However, it is unknown whether or not long--term exposure to concentrations lower than NEC would affect fluvial biofilms and so analyses of chronic effects of β-blockers (at low concentrations) on biofilms would be of great interest.

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