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RESEARCH ARTICLE

Capability of the natural microbial community in a river water ecosystem to degrade the drug naproxen

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Abstract The present work aims at evaluating the ability of the River Tiber natural microbial community to degrade naproxen in water samples collected downstream from a wastewater treatment plant. For this purpose, different water microcosms were set up (microbiologically active vs sterile ones) and treated with naproxen (100 μg/L) alone or in the co-presence of gemfibrozil in order to evaluate if the co-presence of the latter had an influence on naproxen degradation. The experiment was performed in the autumn and was compared with the same experimental set performed in spring of the same year to highlight if seasonal differences in the river water influenced the naproxen degradation. Pharmaceutical concentrations and microbial analysis (total cell number, viability, and microbial community composition) were performed at different times in the degradation experiments. The overall results show that the natural microbial community in the river water had a key role in the naproxen degradation. In fact, although there was a transient negative effect on the natural microbial community in all the experiments (3 h after adding the pharmaceutical), the latter was able to degrade naproxen within about 40 days. On the contrary, no decrease in the pharmaceutical concentration was observed in the sterile river water. Moreover, the co-presence of the two drugs lengthened the naproxen lag phase. As regards the natural microbial community composition detected by Fluorescence in situ Hybridization, Alpha and Gamma-*Proteobacteria* increased when the pharmaceutical halved, suggesting their role in the degradation. This study shows that with the concentration studied, naproxen was degraded by the natural microbial populations collected from a river chronically contaminated by this pharmaceutical.

Keywords Naproxen biodegradation · River Tiber · Natural microbial community · Gemfibrozil · Degradative bacterial groups · Microcosms · Microbial phylogenetic characterization

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Introduction

Pharmaceuticals have been detected in all aquatic compartments (Luo et al. 2014). A majority of studies on these emerging contaminants focus on concentrations detected in waste water treatment plants (WWTPs; Garcia-Rodríguez et al. 2014); however, few studies take into account the role of the river natural microbial community in drug removal and the possible impact of these chemicals on it. An abundant and varied natural microbial community is a necessary prerequisite for ecosystem self-purification processes. In fact, they control water quality and provide several ecosystem regulation services (Radke et al. 2010; Gerbersdorf et al. 2011). The degradation of organic compounds depends on abiotic and biotic processes, but only the latter are responsible for their complete degradation (mineralization) and removal from the environment.

Naproxen is one of the polar acidic pharmaceuticals most used among human populations as an anti-inflammatory and antipyretic drug without prescription (Straub and Stewart 2007; Daneshvar et al. 2010; Araujo et al. 2011). Like other human pharmaceuticals, following therapeutic administration, it mainly enters aquatic ecosystems through municipal WWTPs (more than 80 % is excreted unaltered or in its conjugated forms; Carballa et al. 2008a; Jianga and Zhoua 2013; Brozinski et al. 2013). Owing to its high use and a DT_{50} (the time required for degradation of 50 % of initial

concentration) varying from a few days to about 1 month (Straub et al. 2007; Araujo et al. 2011), naproxen is found as a micro-contaminant in receiving waters. In European surface waters, naproxen is in fact one of the most frequently detected pharmaceuticals (concentrations ranging from ng/L to μg/L; Andreozzi et al. 2003; Loos et al. 2008), including in drinking water; this causes concern for both human and ecosystem health, because it is biologically active at very low concentrations. For instance, recent studies have found that it can hamper or inhibit microbial activity in a WWTP (Kraigher et al. 2008) and also reduce the biodiversity of the natural microbial community in biofilms in a contaminated river (Proia et al. 2013). Furthermore, based on an extensive set of criteria (regulation, consumption, physical-chemical properties, toxicity, occurrence, persistence and resistance to treatment), naproxen has been classified as a high priority pharmaceutical (de Voogt et al. 2009; Schlabach et al. 2009; Ginebreda et al. 2012).

Relatively little information is available on its biodegradation pathways (such as metabolism or co-metabolism) or the role of important functional microbial groups in its degradation. Its biodegradation was detected in WWTP active sludge (Tiehm et al. 2011), in soils (Topp et al. 2008; Lin and Gan 2011), and in a culture medium in the presence of fungus (Rodarte-Morales et al. 2012; Cruz-Morató et al. 2013; Aracagök et al. 2013), but few studies focused on the role of the natural microbial community in naproxen degradation and the microbial groups possibly involved.

Another important aspect to take into account is that it is generally found in the environment together with other drugs. As an example, it has frequently been detected in the co-presence of a prescribed fibrate, the blood lipid regulator gemfibrozil (Loos et al. 2008; Rosal et al. 2010; Patrolecco et al. 2013). In fact, like naproxen, gemfibrozil use in the human population is very widespread; moreover, its environmental persistence is quite high (DT_{50}>70 days; Araujo et al. 2011; Grenni et al. 2013). Mixtures can have unknown synergistic or antagonistic effects, especially on non-target organisms, and naproxen degradation pathways could be influenced by a co-occurrence with other pharmaceuticals (Pomati et al. 2008); however, knowledge about these phenomena is so far quite scarce.

The aim of this study was to evaluate the capability of the natural microbial community of a river chronically polluted by pharmaceuticals, such as the River Tiber downstream from a WWTP, to degrade naproxen alone or in the co-presence of gemfibrozil. The microcosm approach, which makes it possible to study multiple stressors in different conditions (chemical mixtures, co-presence of nutrients and organic contaminants, and so on; Daam et al. 2011; Barra Caracciolo et al. 2013, Babut et al. 2013) was used for this purpose. Water samples collected downstream from a WWTP were used to set up the degradation experiment. Microbiologically active microcosms treated with naproxen alone (100 μg/L) or in the co-presence of gemfibrozil (100 μg/L each) were compared with chemical control microcosms (samples previously sterilized and treated with the pharmaceuticals) and with non-treated microcosms (microbiological controls). At different experimental times, the pharmaceutical concentrations were analyzed and the abundance of live microbial cells was assessed using epifluorescence microscope methods in all conditions. Moreover, the natural microbial community was analyzed with the Fluorescent in situ Hybridization method in the river water samples and in naproxen-treated microcosms immediately after the addition of the pharmaceuticals, when naproxen was halved and at the end of the experiment.

Materials and methods

River water characterization

Water samples from the River Tiber (0–20 cm from the surface) were collected in spring and autumn (April and October 2010) in a stretch flowing through the city of Rome, 382 km from its source and downstream from the Magliana wastewater treatment plant (in southern Rome). The sampling point was selected because a mixture of pharmaceuticals was found there and in particular naproxen and gemfibrozil were detected at their highest concentrations (Patrolecco et al. 2013). Some parameters (pH, O_2, temperature) were analyzed on site and others were examined in the laboratory. The samples were transported to the laboratory in a refrigerated bag (4 °C). Some subsamples were immediately used for the initial microbiological and chemical analysis; others were kept at 20 °C in the dark overnight prior to use for the microcosm setup. Aliquots of water samples were acidified and then analyzed for dissolved organic carbon content (DOC, mg/L) with high temperature catalytic oxidation (HTCO) using a Shimadzu TOC-5000A analyzer with a detection limit of 0.050 mg/L. Dissolved oxygen (DO, mg/L) was measured with an Oxi 538 microprocessor, and the pH was determined with a PHM290 model Radiometer Analytical pH meter. Naproxen and gemfibrozil were analyzed in the natural river water samples using a prior extraction in solid-phase extraction (SPE) followed by an HPLC with fluorescence detection (Patrolecco et al. 2013), as described in the chemical analysis section. The microbial community was also analyzed. In particular, total cell number, viability, and microbial community composition were measured as described in the corresponding section of the "Materials and methods."

Chemicals

Naproxen (+)-(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid and gemfibrozil, 2,2-dimethyl-5-(2,5-dimethylphenoxy)pentanoic acid, were purchased from Sigma-Aldrich (Steinheim, Germany) at ≥98 % purity. The

main characteristics of these chemicals are reported in Table 1. Stock solutions (500 mg/L) of each pharmaceutical were prepared by dissolving an adequate quantity of its standard in acetonitrile and were kept at −20 °C. The working standard solutions (50 mg/L) were achieved by dilution of stock solutions with acetonitrile and stored at 4 °C. HPLC-grade acetonitrile was purchased from VWR (Radnor, PA, USA). Acetic acid (glacial) was supplied by Carlo Erba (Milan, Italy). Water for chromatography was purified (18 MΩ/cm quality) by a Milli-Q system (Millipore, Bedford, MA, USA).

Naproxen degradation experiment in microcosms

The experimental set consisted of closed destructive microcosms (100 mL capacity), filled with River Tiber water collected in the autumn. It was set up as follows:

- Some microcosms (16 replicates) were treated with naproxen at a concentration of 100 μg/L (Naproxen).
- Some microcosms (16 replicates) were treated simultaneously with naproxen and gemfibrozil at the same concentration (100 μg/L each), (Nap+Gem).
- Some microcosms (16 replicates) were filled with previously sterilized river water (120 °C, 20 min) and then treated with naproxen, 100 μg/L (Sterile).
- Some microcosms (16 replicates) were filled with previously sterilized river water (120 °C, 20 min) and then treated with naproxen and gemfibrozil at the same concentration (100 μg/L each).
- Sixteen replicates were filled with non-treated river water (Control) and used as microbiological controls.

Moreover, pH and dissolved oxygen concentrations were measured at each sampling time in 16 other naproxen-treated and naproxen plus gemfibrozil-treated microcosms used exclusively for this purpose.

Aliquots from standard working solutions (in acetonitrile) of the two pharmaceuticals were spiked in each single microcosm. Then, in order to eliminate any additional carbon source, acetonitrile was completely evaporated at room temperature from each microcosm and 50 mL of the river water (natural or previously sterilized) added.

All microcosms were incubated at 20 °C on an orbital shaker (125 rpm) in the dark in order to avoid possible photodegradation phenomena (Tixier et al. 2003; Vieno et al. 2005).

The samplings were performed 3 h after the spiking with the pharmaceuticals and at selected times until the complete degradation of naproxen. At each sampling, two destructive replicate microcosms were analyzed for each condition (Naproxen, Sterile; Nap+Gem, sterile; Control). Two subsamples from each single microcosm were then used for each different (chemical or microbiological) analysis in order to have four independent values for each condition. All operations were conducted under sterile conditions.

In order to pinpoint if river water seasonal differences could have influenced the degradation of naproxen, the results of this experiment were compared with those of another previously performed with River Tiber water collected at the same point in spring of the same year. In the spring experiment, the same autumn experimental condition was repeated and naproxen (100 μg/L initial concentration) was studied alone (Grenni et al. 2013).

Table 1 Main characteristics of naproxen and gemfibrozil

	Naproxen NSAID	Gemfibrozil Lipid Regulator
MF	$C_{14}H_{14}O_3$	$C_{15}H_{22}O_3$
MW	230.26	250.33
CAS	22204–53–1	25812-30-0
Solubility mg/L	27	5
pK_a	4.2[a]	4.6[b]
Log K_{ow}	3.18[a]	4.77[a]
Partition Kd	10.8[c]	19.3[d]
Photolysis $t_{1/2}$ (h)[e]	14.8–91.4	1.1–2.5
PNEC μg/L[f]	2.62	1.18
Removal in STP (%)	66±7[c]	15-78[g]

NSAID: Nonsteroidal anti-inflammatory

[a] Beausse 2004

[b] Westerhoff et al. 2005

[c] Carballa et al. 2008b

[d] Radjenovic et al. 2009

[e] Lin and Reinhard 2005

[f] Martín et al. 2012

[g] Ying et al. 2009

Chemical analysis

The concentrations of naproxen and gemfibrozil in the natural river water samples and from the microcosm degradation experiments were analyzed following a procedure described in Patrolecco et al. (2013). Briefly, an SPE pre-concentration and purification procedure was performed using polymeric Strata-X extraction cartridges. The detection was done by a RP-HPLC (Varian 9012) with a fluorescence detection (Perkin Elmer LS4) analytical step, using an Alltech LC18 column (Alltima C18, 5 μm, 250×4.6 mm i.d), preceded by a guard column (4×3 mm) of the same packing material. The elution profile, at a constant flow rate of 1.0 mL/min in the isocratic mode, utilized a mobile phase with acetonitrile:water (acidified to pH=3.6 with acetic acid) in a 70:30 (*v*/*v*) ratio. Excitation–emission wavelengths

were set as follows: λ_{exc}=230 nm; λ_{emiss}=420 nm for naproxen and λ_{exc}=230 nm; λ_{emiss}=302 nm for gemfibrozil.

The concentrations of both pharmaceuticals were determined by direct injection of samples (50 μL aliquots of subsamples were injected in duplicate) into the HPLC with fluorescence detection, set up under the same analytical conditions described above (Limit Of Quantification, LOQ: naproxen, 60 ng/L; gemfibrozil, 10 ng/L).

Total cell number, viability, live cell abundance

In order to examine the microbial community in the natural river water collected in the two seasons and to verify if the adding of the pharmaceuticals to the microcosms influenced it, the total microbial number and cell viability were assessed. In particular, the total cell number was determined by direct count, in four replicates of formaldehyde-fixed (2 % final concentration) subsamples (2 mL each), using 4'-6-diamidino-2-phenylindole (DAPI) as a DNA fluorescence agent (Barra Caracciolo et al. 2005). Cell viability (% live cells/live+dead) was assessed in four non-fixed replicates (2 mL each) using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-Aldrich), to distinguish between viable (green) and dead (red) cells under a fluorescence microscope (Leica DM 4000B Leica Microsystems GmbH, Wetzlar, Germany), as reported in a previous work (Grenni et al. 2009a). We calculated the live cell abundance (No. live cells/mL) from the total microbial number, obtained by DAPI counts, multiplied by cell viability.

Microbial community composition by Fluorescence in situ Hybridization

The phylogenetic composition of the natural microbial community was analyzed both in the samples collected in spring and autumn and in the water samples from the degradation microcosm experiments by using Fluorescence in situ Hybridization (FISH), as described in detail in Barra Caracciolo et al. (2005). For each sample (natural or treated water), two subsamples (2 mL each) were fixed (1:1) with a solution composed of phosphate-buffered saline (130 mM NaCl; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; 2 % formaldehyde; 0.5 % Tween 20 and 100 mM Sodium Pyrophosphate). Each subsample was then filtered on a 0.2 μm polycarbonate membrane. The filters were stored at −20 °C until further processing.

Fluorescence in situ Hybridization of the harvested cells, counterstained with DAPI, was performed using fluorescent probes for the identification, under the epifluorescence microscope, of the major microbial groups found in freshwater (Hahn 2006; Pernthaler 2013). In particular, the following groups were searched for: *Archaea* and *Bacteria*, Alpha-, Beta- and Gamma-*Proteobacteria*, *Planctomycetes*, the Cytophaga-Flavobacterium cluster of the Cytophaga-Flavobacter-Bacteroides phylum, and Gram-positive bacteria with high or low G+C content. For this purpose, the following Cy3-labeled oligonucleotide probes were applied: ARCH915 (*Archaea*), EUB338I-II-III (*Bacteria*) and inside this domain ALF1b (Alpha-*Proteobacteria*), BET42a (Beta-*Proteobacteria*), GAM42a (Gamma-*Proteobacteria*), PLA46 together with PLA886 (*Planctomycetes*), CF319a (Cytophaga-Flavobacter-Bacteroides), HGC69A and LGC354a (Gram-positive bacteria with high or low G+C content, respectively). Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al. 2007).

The slides were mounted with a drop of Vectashield mounting medium (Sigma-Aldrich), and the preparation was examined and counted with a Leica DM 4000B epifluorescence microscope at ×1,000 magnification. The cells binding each probe were estimated as a proportion of the total DAPI-positive cells (% positive cells vs DAPI or No. positive cells vs DAPI) in at least 20 different random fields on each filter section (corresponding to 500–1,000 stained cells).

Results

River Tiber water characteristics at the sampling point

The main characteristics of the water samples collected in spring and autumn are reported in Table 2. The physical-chemical properties showed some differences due to the season (such as temperature, DO, DOC). In particular, the autumn DOC value was higher than the spring one. Naproxen was found in both seasons at a concentration about four times higher than gemfibrozil.

The total microbial number and cell viability were higher in the autumn than in the spring samples in line with the higher DOC and temperature values (Eiler et al. 2003). However, the microbial community composition, expressed as % positive cells for each oligonucleotide probe vs DAPI (Fig. 1), was quite similar in the two seasons. In fact, in both samplings, the *Archaea* domain (Table 2) was detected at very low percentages and the *Bacteria* domain ranged from 60 to 75 % of the DAPI-stained cells, with the higher values in autumn (Table 2). Inside the *Bacteria* domain, Beta-*Proteobacteria* and *Planctomycetes* were the dominant groups in terms of relative percentages in the natural River Tiber water in both seasons (Fig. 1).

Degradation of naproxen in microcosm experiments

The pH and oxygen values were monitored in the microcosms at each sampling time and neither varied significantly (*t* test

Table 2 River water characterization at the sampling point

	Spring	Autumn
Temperature (°C)	12.9	18
pH	7.7	7.8
DO (mg/L)	11.4	7.5
DOC (mg/L)	2.0±0.050	3.5±0.035
Naproxen (ng/L)	240±9	270±12
Gemfibrozil (ng/L)	50±7	78±13
Total microbial number (No. cell/mL)	$1.31\times10^{6}\pm1.3\times10^{5}$	$3.16\times10^{6}\pm1.1\times10^{5}$
Cell viability (%)	27±5.3	75.36±2.6
Live cell number (No. live cell/mL)	$3.54\times10^{5}\pm1.1\times10^{4}$	$2.36\times10^{6}\pm1.0\times10^{5}$
Bacteria:		
No. Positive cells vs DAPI	$8.07\times10^{5}\pm1.2\times10^{4}$	$2.36\times10^{6}\pm1.3\times10^{5}$
(% positive cells vs DAPI)	(61.6±1.6)	(74.6±2.8)
Archaea:		
No. Positive cells vs DAPI	$1.44\times10^{4}\pm1.0\times103$	$1.90\times10^{4}\pm1.4\times10^{3}$
(% positive cells vs DAPI)	(1.1±0.3)	(0.6±0.1)

not significant) either in the naproxen or in the naproxen plus gemfibrozil-treated microcosms. The pH remained at a value of about 8.00±0.08 in both the experiments. The oxygen concentration was always close to a saturation level and was, therefore, never a limiting factor. Finally, there was also no significant variation in the DOC measured at the end of the experiments compared to its initial value.

The naproxen decrease (expressed as a residual percentage of the applied compound) vs time in the spring and autumn experiments is shown in Fig. 2a–c, for the microbiologically active (Naproxen) and sterilized (Sterile) microcosms.

The presence of the river natural microbial community always promotes naproxen degradation. In fact, the pharmaceutical was completely degraded within 46 days in all experiments (Fig. 2). On the contrary, no significant variation in concentration was observed in the sterile condition.

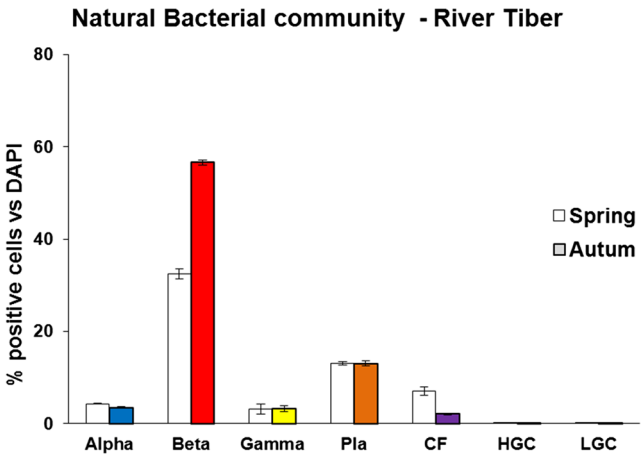

Fig. 1 Microbial community composition (% positive cells for each oligonucleotide probe vs DAPI) detected by Fluorescence in situ Hybridization in the River Tiber Spring and Autumn samples. *Alpha*: Alpha-*Proteobacteria*; *Beta*: Beta-*Proteobacteria*; *Gamma*: Gamma-*Proteobacteria*; *Pla*: *Planctomycetes*; *CF*: *Cytophaga-Flavobacterium* cluster phylum CFB; HGC and LGC: Gram-positive bacteria with high or low G+C content. The *vertical bars* represent the standard errors

In spring microcosms (Fig. 2a), naproxen degradation started after 20 days and a DT_{50} value of about 27.6±0.1 days was detected.

In autumn, when naproxen was alone (Fig. 2b), the degradation started after 7 days (DT_{50}: 22±2.5 days), while in the presence of gemfibrozil (Fig. 2c), the lag phase was longer, with the degradation starting after 24 days and then occurring quickly in a few days, with a DT_{50} of about 30±1.1 days. In the latter experiment, gemfibrozil was detected, at all sampling times and at the end of the experiment (46 days), at 100 % of the initial concentration in both microbiologically active and sterilized microcosms.

Live cell abundance

Live cell abundance (No. live cells/mL) in the degradation experiments in naproxen-treated and non-treated (Control) microcosms are reported in Fig. 3. In accordance with the initial water samples (Table 1), the live cell abundance was higher in the autumn than spring microcosms. In all the experiments, 3 h after addition of naproxen (0.125 days) the live cell abundance was significantly lower than in the control microcosms (*t* test, $p<0.01$), showing that the pharmaceuticals exerted an initial toxic effect. However, this effect was transient and after about 7 days the live cell abundance was generally greater in the presence of the drugs than in the control microcosms.

Microbial community composition

The microbial community composition was analyzed in the naproxen degradation experiments 3 h after the

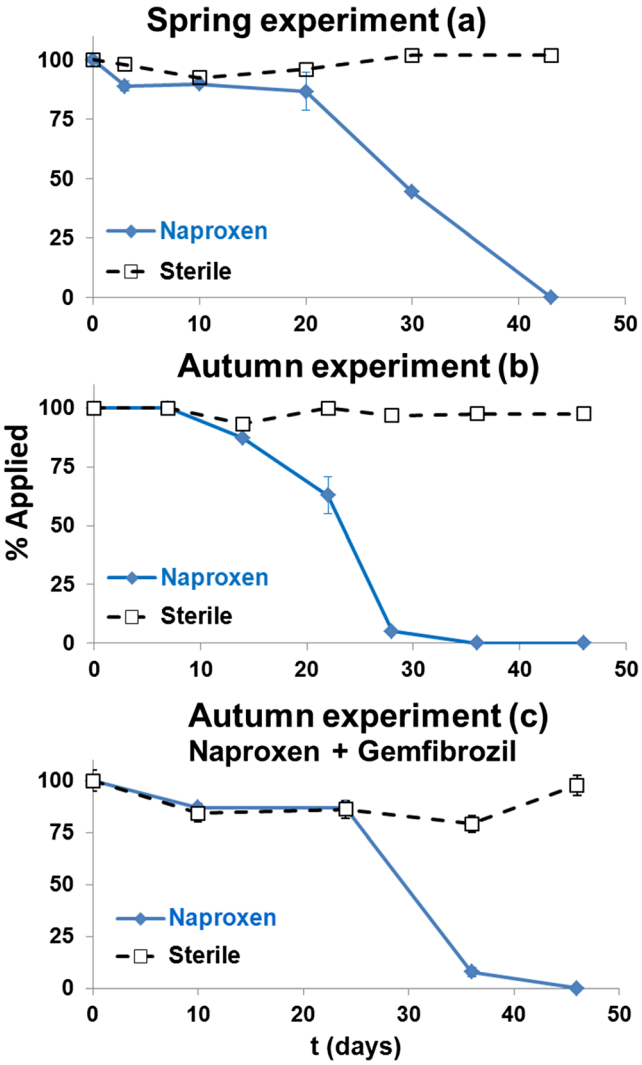

Fig. 2 Residual percentages of naproxen in river water microcosms (*Naproxen*) and in sterile ones vs time in the three experiments: spring (**a**), autumn (**b**), and autumn in co-presence of gemfibrozil (**c**). The *vertical bars* represent the standard errors

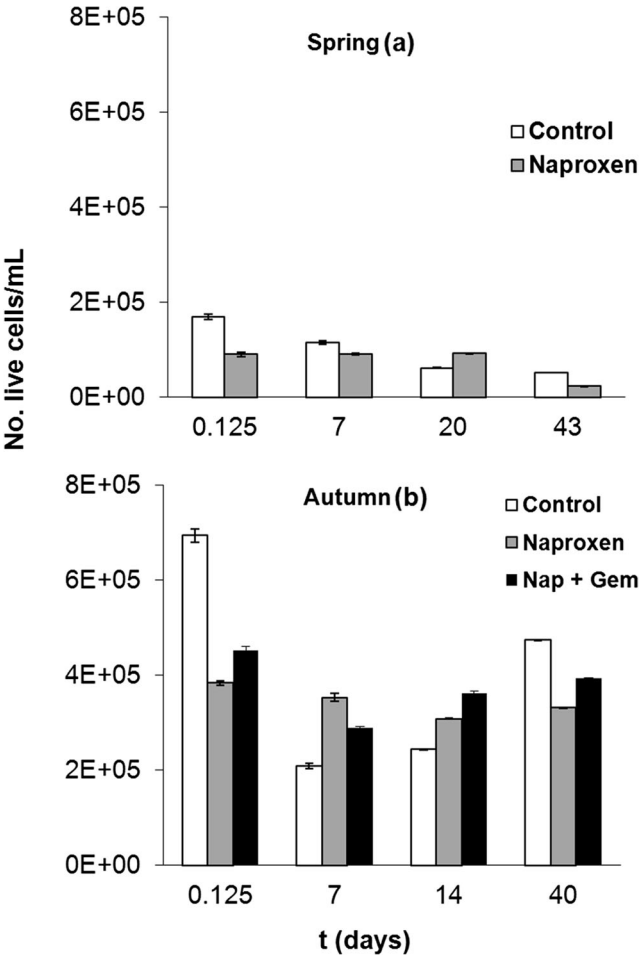

Fig. 3 Number of live cells (No. live cells/mL water) detected in the three experiments, in spring with microcosms treated with naproxen (**a**) and in autumn with naproxen or naproxen plus gemfibrozil (**b**). Treatments: Naproxen, water microcosm treated only with naproxen; *Nap+Gem*: microcosm treated with naproxen and gemfibrozil; *Control*: non-treated water microcosm. The *vertical bars* represent standard errors

pharmaceutical addition (0.125 days), when the naproxen halved (Fig. 4) and at the end of the experiments.

During the experimental time, the *Archaea* percentages were lower than in the initial water samples in the experiments in both seasons and in all conditions (0 to 0.6 %). The percentage of *Bacteria* decreased in naproxen-treated microcosms after 3 h in both experiments (Spring: control: 44.2±1.5 %; naproxen: 38.7±1.7 %; Autumn: control: 74.6±1.6 %; naproxen: 47.3±3.3 %). This result was particularly evident in the autumn microcosms (*t* test significant, $p<0.01$) in which Beta-*Proteobacteria*, although remaining the dominant group, diminished in percentage terms in the naproxen (30.4±1.9) vs control (56.6±0.6) microcosms.

The comparison of the microbial community structure in the non-treated microcosms (control) vs naproxen-treated microcosms when naproxen halved (Fig. 4) shows changes in the abundance and shifts in the dominance of some bacterial groups. In particular, a significant increase in Alpha- and Gamma-*Proteobacteria* groups was observed in both the spring (Fig. 4a) and autumn (Fig. 4b) experiments in the naproxen-treated microcosms (*t* test, $p<0.01$).

Finally, at day 40, when the pharmaceutical was completely degraded, there were no significant differences between the microbial community composition in the control and treated microcosms in all the experiments (data not shown).

Discussion

Although naproxen is not generally reported to be a persistent compound (Straub et al. 2007), it has been found in several EU and Italian natural surface waters. In the River Tiber, it is commonly found as a micro-contaminant together with gemfibrozil (Loos et al. 2008; Patrolecco et al. 2013) and their

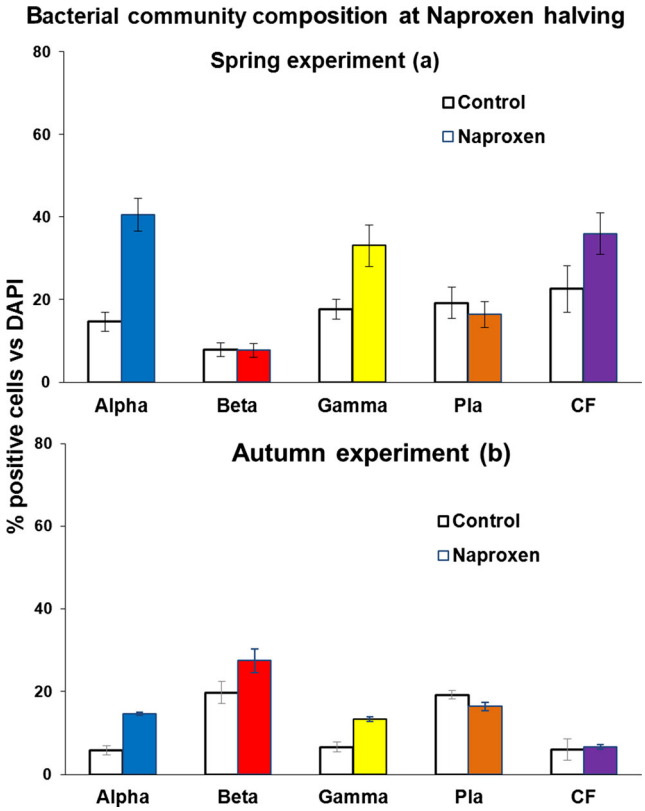

Fig. 4 Bacterial community composition (% positive cells vs DAPI) detected by Fluorescence in situ Hybridization at the naproxen DT_{50} sampling time in the spring (**a**) and autumn (**b**) experiments, detected in the treated microcosms (*Naproxen*) and in the control ones (*Control*). *Alpha*: Alpha-*Proteobacteria*; *Beta*: Beta-*Proteobacteria*; *Gamma*: Gamma-*Proteobacteria*; *Pla*: *Planctomycetes*; *CF*: *Cytophaga-Flavobacterium* cluster phylum CFB. The *vertical bars* represent standard errors

concentrations exceed the 10 ng/L value proposed as a limit by EMEA (CHMP 2006). Our study confirms that naproxen and gemfibrozil can be considered chronic contaminants of the River Tiber. The fact that naproxen is always found in higher concentrations than gemfibrozil, although naproxen has a fairly high removal rate (Bueno et al. 2012; Grenni et al. 2013; Grossberger et al. 2014), confirms that naproxen is chronically discharged into the river from WWTPs more than gemfibrozil, owing to its higher use among the human population. Consequently, naproxen can be considered a pseudo-persistent compound (Radke et al. 2010).

Although some differences were found in physical-chemical properties due to the season in the River Tiber water samples (e.g., temperature, dissolved organic carbon and oxygen), the bacterial community composition did not vary between the spring and autumn samples (Fig. 1) in terms of the percentage split between the different phylogenetic groups (e.g., Beta-*Proteobacteria*>Planctomycetes>*Cytophaga-Flavobacterium* cluster) but only as regards their relative abundance, indicating that it does not depend on the time of year. In fact in autumn, when there was more DOC and a higher water temperature (Table 1), the live cell abundance was also higher than in spring, as generally found in water ecosystems (Kirchman et al. 2004; Ruiz-González et al. 2013).

The overall results from the degradation experiments make it possible to confirm that the River Tiber microbial community was able to degrade naproxen at the concentration used for the experiments (100 μg/L) thanks to the presence of a bacterial population adapted to its presence. In fact, naproxen degradation was observed only in the microbiologically active microcosms (Fig. 2), pointing to the role of the microbial community in its degradation. Data from literature on the capability of natural microbial communities to biodegrade naproxen are quite scarce so far (Quintana et al. 2005; Mascolo et al. 2010). Naproxen degradation in surface water has been reported in a few studies (Tixier et al. 2003; Araujo et al. 2011) with similar values (DT_{50} of about 20–30 days) to those of our microcosm experiments, although the role of the natural microbial community in degradation of naproxen was not examined. Its biodegradation by some microbial consortia, including two microfungi of the *Cunninghamella* and *Aspergillus* genera was found only in an aerobic soil (half-life from 17 to 69 days; Grossberger et al. 2014).

Photolytic degradation is reported to be an important abiotic degradation process for naproxen, although it is not able to degrade it thoroughly (Tixier et al. 2003; Vieno et al. 2005). In any case, we can exclude this abiotic process in our experiments because we performed them on purpose in the dark. Moreover, naproxen photolytic degradation in a river like the Tiber may not occur in some seasons because of the amount of suspended particulate (Andreozzi et al. 2003), which is very high all along this river owing to rainfall, and in particular in its last stretch (Patrolecco et al. 2006).

At the start of the experiments, naproxen had an immediately detrimental effect on the overall microbial community in terms of live cell abundance (Fig. 3). This transient effect is ascribable to a decrease in Beta-*Proteobacteria* in the autumn experiment. In such case, a toxic effect on the bacterial populations involved in key ecosystem functioning cannot be excluded. As an example, Beta-*Proteobacteria* include ammonia-oxidizing bacteria involved in the nitrogen cycle (Schweitzer et al. 2001; Elifantz et al. 2005) and a toxic effect on this bacterial group was found in a WWTP with naproxen present (Wang and Gunsch 2011). These initial detrimental effects, however, were transient suggesting not only that the natural microbial community was able to recover from the toxic effect of the chemical, but also that some bacterial populations were involved in the naproxen degradation. The natural microbial community was, therefore, able to provide an ecosystem regulation service, i.e., removing naproxen from water, presumably thanks to the occurrence of microbial populations adapted to its presence. In fact, the increase in the

microcosm experiments in Alpha and Gamma-*Proteobacteria* at DT_{50s} (about four to five fold in autumn to ten fold in spring) confirms this hypothesis and suggests the degradation role of these groups. At the end of the experiments, when naproxen was completely degraded, no significant differences between treated and control microcosms were observed. This result may be ascribable to the depletion of the substrate (naproxen), which promoted Alpha and Gamma-*Proteobacteria* growth.

We also performed enrichment cultures (in which 1 mg/L naproxen was used as the sole carbon source) using water samples collected from the microcosms (at DT_{50}) in order to isolate a specific bacterial strain able to grow on the pharmaceutical, in accordance with previous experiments (Grenni et al. 2009b). Although the microbial pool from microcosms in the liquid culture supplemented with naproxen was able to grow on it (data not shown), the single isolates were not able to grow on it as the sole carbon source. The latter result suggests that naproxen degradation can be due to microbial populations that degrade the chemical in succession, as reported for naproxen in an activated sludge (Quintana et al. 2005).

The fact that naproxen degradation was faster in autumn than in spring in microcosms where it was alone (Spring: 20 day lag phase, DT_{50} 27.6±0.1 days; Autumn: lag phase 7 days, DT_{50}: 22±2.5 days, Fig. 2 a, c) was probably due to the higher live cell abundance and higher percentage of bacterial community composition (Barra Caracciolo et al. 2010), in line with a higher DOC content in the autumn. In fact, the live cell abundance was about 75 % higher in this season than in spring (Table 2).

The degradation of naproxen with gemfibrozil co-present (Fig. 2c) was affected in terms of lag phase more than of disappearance time; in fact, the lag phase was longer (about 20 days instead of 7 days, Fig. 2b and c) indicating that the natural microbial community was affected by the mixing of the two pharmaceuticals and consequently its homeostatic capability was slowed down. The occurrence of mixtures should be taken into consideration when degradation studies are performed, including for a more reliable environmental risk assessment (Babut et al. 2013).

This study demonstrates that naproxen is biodegradable and its complete disappearance occurs within 30–40 days when the natural river microbial community is present. However, if it is being continuously released into the river, owing to its high use among the human population (pseudo-persistence), biodegradation rates are not sufficient to remove it completely.

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