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Degradation of Emerging Pollutants in Aquatic Ecosystems

Anna Barra Caracciolo*, Luisa Patrolecco, Martina Di Lenola, Angela Battaglia, Paola Grenni

Istituto di Ricerca sulle Acque, Consiglio Nazionale delle Ricerche (IRSA-CNR), Via Salaria km 29,300 Monterotondo – Rome. Italy
barracaracciolo@irsa.cnr.it

The present work aims to define the natural attenuation potential of a river ecosystem versus Naproxen and Gemfibrozil pharmaceutical contaminants, evaluating the role of the autochthonous bacterial community in their degradation. The biotic degradation (DT_{50}) of Naproxen and Gemfibrozil were evaluated in microbiologically active versus sterilized river water, using laboratory microcosms. Moreover, the degradations of Naproxen was also evaluated in microcosms simultaneously treated with both pharmaceuticals in order to evaluate if their co-presence could affect their environmental fate and the degradation activity of the microbial community. Each pharmaceutical was added at a concentration of 100 µg/L. Chemical analyses were performed using a HPLC-Fluorescence method recently validated in order to follow the decrease of the pharmaceuticals in microcosms. The number of live bacteria (N. live bacteria/mL) both in pharmaceutical treated and un-treated river water was also evaluated using epifluorescence microscopy.
The overall results show that no decrease in pharmaceutical concentrations was observed in the sterile water, while the bacterial community had a key role in their degradation Naproxen was in fact completely degraded after about 30 days both when alone and in co-presence of Gemfibrozil. Gemfibrozil was partially degraded and 64 % of its initial concentration is still present at day 105.

1. Introduction

During the past decade, the increasing introduction on the market of new chemicals and the development of more accurate analytical methods have added various emerging contaminants to the already large array of pollutants. Emerging contaminants are synthetic or naturally occurring chemicals that are not commonly monitored in the environment, and not yet regulated by European Community, although they have the potential to enter soil and aquatic ecosystems, causing known or suspected adverse ecological and/or human health effects. Among emerging contaminants, pharmaceuticals are a cause of concern as they have been detected in various ecosystems. Pharmaceutically active compounds are more than 4,000 molecules with different physico-chemical and biological properties (antibiotics, anticancer, anti-inflammatory, lipid regulators, antiviral drug etc.) and distinct mode of actions.
Pharmaceuticals have only recently started to be found in the aquatic environment from ng/L to µg/L, mainly due to the inefficiencies of wastewater treatment plants. Most of these micropollutants raise considerable toxicological concerns because they are intrinsically and biologically active molecules that can have sub-lethal or chronic toxic effects.

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Although pharmaceutical and therapeutic products are widely found in the natural environment, there is limited understanding of their potential unintended environmental impact and the ecological effects on receiving environments remain largely unknown.
Naproxen, a non-steroidal anti-inflammatory drug and Gemfibrozil, a fibrate drug used as a lipid regulator, have been found in several natural EU and Italian surface waters, including the river Tiber (Rome, Italy) at higher concentrations in the case of Naproxen than Gemfibrozil (Loos et al., 2009).
The present work aims to evaluate the microbial degradation of Naproxen and Gemfibrozil active ingredients in contaminated waters from the urban stretch of the river Tiber. For this purpose, different water microcosms were set up (presence/absence of microbial community) and treated with Naproxen or Gemfibrozil (100 µg/L). At different times, pharmaceutical degradation, bacterial abundance and cell viability were analysed in water samples treated versus microbiological control (no treated water samples). Moreover, the degradation of Naproxen and Gemfibrozil was also evaluated in microcosms simultaneously treated with both pharmaceuticals.

2. Materials and methods

2.1 Microcosm set-up for degradation studies

Three experimental sets, consisting of 64 destructive closed microcosms (100 mL capacity), were set up for Gemfibrozil, Naproxen and both drugs simultaneously. Aliquots from working standard solutions of the selected pharmaceuticals in acetonitrile were spiked in each single microcosm to a final concentration of 100 µg/L. Spiking was performed in a sterile cabinet and, once acetonitrile was completely evaporated at room temperature in order to eliminate any additional carbon source, 50 mL of the river water (natural or previously sterilized, see later) was added to each microcosm. In particular, some microcosms (16 replicates) were filled with natural river water (Microbiologically Active Water, MAW) and treated with the pharmaceutical (Naproxen, Gemfibrozil, Naproxen + Gemfibrozil) as previously described. Some microcosms (16 replicates) were set up with previously sterilized river water (120 °C, 20 min) and then treated with the pharmaceutical (Sterile). Other 16 replicates were not treated and filled only with river water (Control), in order to compare the effects of the pharmaceuticals on the natural bacterial community.
All microcosms were incubated at 20 °C on an orbital shaker (125 rpm) in the dark. At selected times, two destructive replicate microcosms were analysed for each condition (MAW, Sterile, Control). Two sub-samples from each single microcosm were then used for each different (chemical or microbiological) analysis in order to have four independent values for each condition. The samplings were performed 3 h (t = 0.13 d) after the treatment and at various times. All operations were conducted under sterile conditions.

2.2 Chemical analysis

Analytical determinations of the two pharmaceuticals were performed by a RP-HPLC (Varian 9012) with fluorescence detection (Perkin Elmer LS4) using an Alltech LC18 column (Alltima C18, 5 µm, 250 x 4.6 mm i.d), preceded by a guard column (4x3 mm) of the same packing material. The elution profile, at a constant flow rate of 1.0 mL/min in isocratic mode, utilised a mobile phase with acetonitrile:water (acidified to pH = 3.6 with acetic acid in order to prevent the hydrolysis of the pharmaceuticals) in a ratio 70:30 (v/v) ratio. Excitation-emission wavelengths were set as follow: λ_{exc}= 230 nm; λ_{emiss} = 302 nm for Gemfibrozil and λ_{exc} = 230 nm; λ_{emiss} = 420 nm for Naproxen. Aliquots of 50 µL of sub-sample from each treated microcosm were injected in duplicate and in these optimized analytical conditions, the chromatographic run was 10 min for the Gemfibrozil (tr = 8.54 min) and 6 min for the Naproxen (tr = 4.02 min) analysis.

2.3 Microbial analysis: total bacterial number and viability

The total bacterial number (N. bacteria/mL) was determined by direct count, in four replicates of formaldehyde-fixed subsamples (2 mL each) using 4'-6-diamidino-2-phenylindole (DAPI) as a DNA fluorescence agent (Barra Caracciolo et al., 2005a, b). 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, Germany) in order 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., 2009). We calculated the number of live bacteria (N. live bacteria/mL) from the total bacterial number, obtained by DAPI counts, multiplied by cell viability.

3. Results and Discussion

3.1 Degradation of Naproxen and Gemfibrozil in microcosm experiments

The decrease (expressed as residual percentage) vs time of Naproxen, Gemfibrozil and Naproxen in co-presence of Gemfibrozil both in the Microbiologically Active (MAW) and Sterile (Sterile) microcosms, is shown in Figure 1A, 1B and 1C, respectively.

The occurrence of the natural microbial community of the river promotes the pharmaceutical degradation. In fact, in the sterile condition no significant variation in concentration was observed. However, Naproxen was less persistent (DT_{50} about 22 d) than Gemfibrozil.

At day 36, Naproxen was completely degraded (Figure 1A), while Gemfibrozil degradation started after 45 days and 64 % of its initial concentration still persisted at day 105 (Figure 1B).

The degradation of Naproxen in co-presence of Gemfibrozil was affected in term of lag phase more than of disappearance time; when Naproxen was present alone, the degradation started after a lag phase of about 7 days (Figure 1A), while in co-presence of Gemfibrozil the lag phase was longer (about 20 days) and then the degradation occurred quickly in few days (Figure 1C). In the case of Gemfibrozil, the degradation was further slowed down and at the end of the experiment (105 d, data not shown) 87 % of its initial concentration was still present.

The greater persistence of Gemfibrozil than Naproxen in water has been recently reported by other Authors (Araujo et al., 2011) with similar values, although the role of biodegradation was not highlighted.

Naproxen was found to be biodegradable in soil (Topp et al., 2008; Lin and Gan, 2011) and in a culture medium in presence of a white-rot fungus (Rodarte-Morales et al., 2010), while Gemfibrozil was initially considered not biodegradable (Stumpf et al., 1999), but a recent study reports the capability of a microfungus to hydrolyse it (Kang et al., 2009). Our study confirms the latter result even if Gemfibrozil biotransformation occurred by bacterial populations.

3.2 Microbial analysis

The number of live bacteria (N. live bacteria/mL) in treated (Naproxen, Gemfibrozil and Naproxen + Gemfibrozil) and untreated (Control) microcosms is reported in Figure 2A, 2B and 2C, respectively.

In all three experiments, three hours after the pharmaceutical treatment ($t = 0.13$ d), the bacterial abundance was significantly lower (*t* test, $p < 0.01$) than in the Control microcosms, showing that the pharmaceuticals exerted an initial toxic effect. However, this effect was transient and after 7 days in the case of Naproxen and 10 days in that of Gemfibrozil the number of live bacteria was generally greater in the treated than control microcosms.

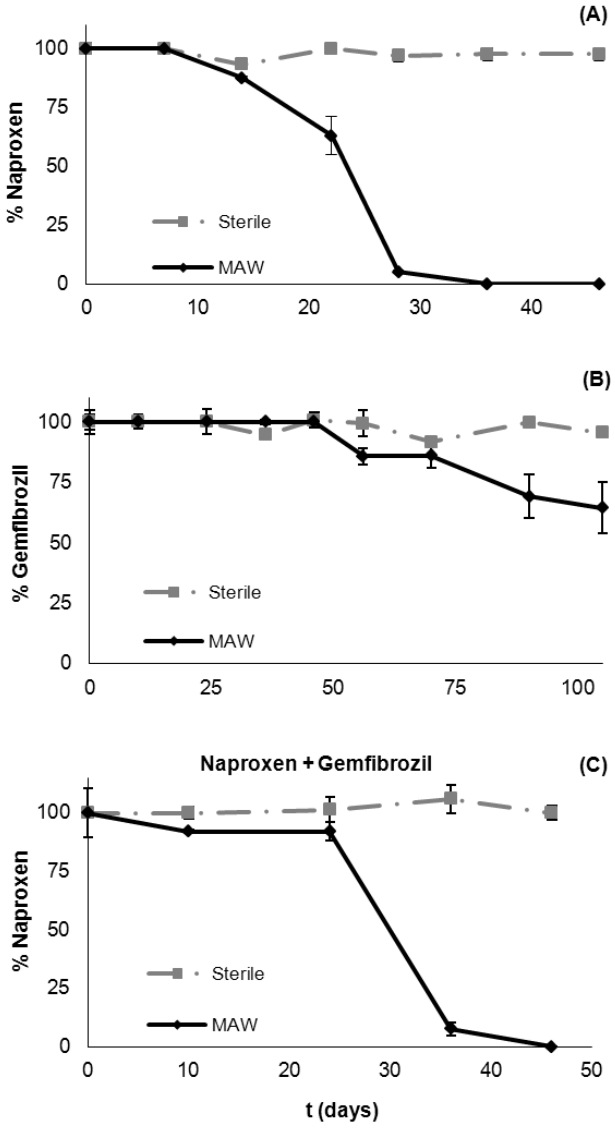

Figure 1: Percentages of Naproxen (A), Gemfibrozil (B) and Naproxen in co-presence of Gemfibrozil (C) in river water Microbiologically Active microcosms (MAW) and in Sterile vs time. The vertical bars represent the standard errors.

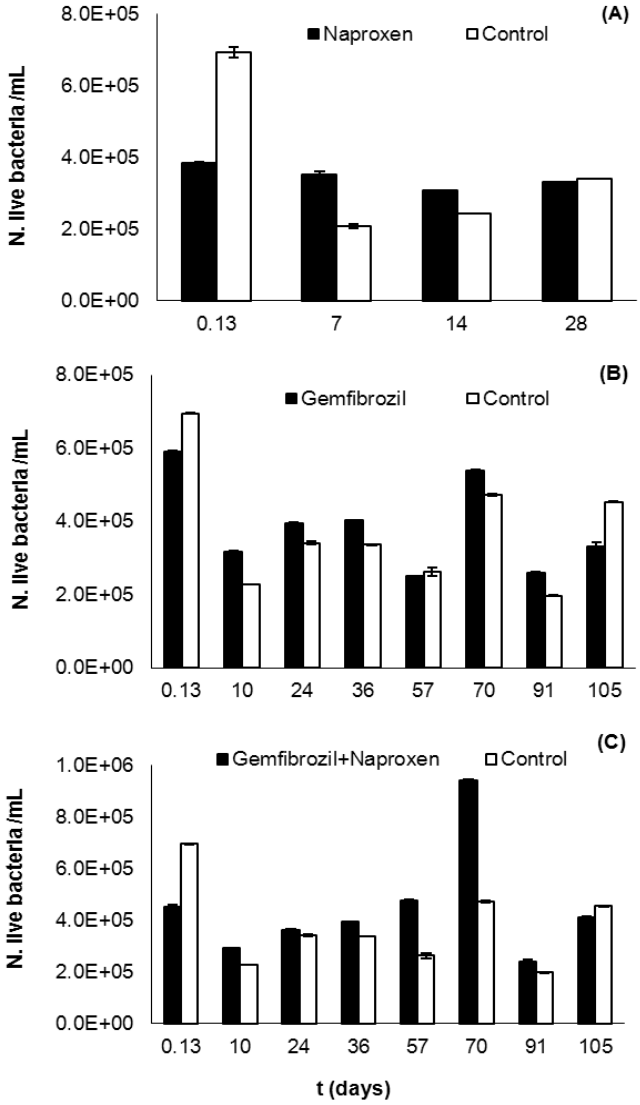

Figure 2: Number of live bacteria vs time in the microcosms treated with Naproxen (A), Gemfibrozil (B), Naproxen in co-presence of Gemfibrozil (C) and in the Control ones. The vertical bars represent the standard errors.

4. Conclusions

The results of these experiments highlight the key role of the autochthonous bacterial community of the river in the degradation of the pharmaceuticals studied. Naproxen was completely biodegraded in 30 days, while between 64 % and 87 % of Gemfibrozil still persisted after 105 days. The fact that Naproxen is generally found in the river at concentrations greater than Gemfibrozil is therefore not due to its intrinsic persistence, but to its pseudo-persistence linked to the spread in its use among the human population and the resulting release in the river waters.

Further experiments are in progress in order to better investigate the degradation pathways, the metabolite formation and specific bacterial strains involved in their metabolic and/or co-metabolic

transformations. The identification and isolation of the microbial pool and/or of specific strains involved in the pharmaceutical degradation can be useful for bioremediation purposes.

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