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Effect of the Antiviral Drug Oseltamivir (Tamiflu) on the Bacterial Community Structure of a Surface Water Ecosystem Analyzed Using Fluorescence In Situ Hybridization

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Abstract The antiviral drug Tamiflu has received particular attention because of its recommended use against the influenza A H5N1 and H1N1 viruses. Given its resistance to degradation and its hydrophilicity the active metabolite, Oseltamivir Carboxylate (OC), is expected to enter the aquatic ecosystem from sewage treatment plants. In the present paper the bacterial community of surface water samples, treated with OC (1.5 mg L^{-1}), was characterized by Fluorescence In Situ Hybridization (FISH) in microcosm experiments. The α-, β- and γ-*Proteobacteria* increased in OC-treated versus non-treated water samples during the incubation period, suggesting these bacterial groups had an active role in OC degradation.

Keywords Pharmaceuticals · Tamiflu · Surface water · FISH

The recent influenza A H1N1 and H5N1 virus pandemics have obliged health agencies to adopt strategies to contain them. Although vaccination is the primary step in prevention, the World Health Organization (WHO) has recommended the treatment of people at risk with the antiviral drug Tamiflu (Oseltamivir Phosphate). Hundreds of millions of courses of the prodrug Oseltamivir Phosphate (chemical name (3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester) have been deployed worldwide. After administration, the prodrug Oseltamivir Phosphate (OP) is hydrolyzed to its active metabolite, Oseltamivir Carboxylate (OC). Pharmacological studies have demonstrated that over 80% of the oral dose of OC is excreted renally and fecally (Ward et al. 2005). Fick et al. (2007) highlighted that OC is not completely removed in sewage treatment plants, and, considering its low sorption into sewage sludge (low Log P), its high water solubility (Straub 2009) and its negligible degradation in river water in the absence of an active bacterial community (Accinelli et al. 2007; Saccà et al. 2009; Accinelli et al. 2010), it could potentially contaminate aquatic ecosystems. Widespread use of Tamiflu could lead to considerable pollution of surface water, leading to the development of OC-resistant strains of the viruses (Fick et al. 2007; Singer et al. 2007), and a mass administration of Tamiflu could pose a risk for drinking water safety and ecological health.

Previous studies indicated that the fate of OC in surface water is mainly governed by microbial and photochemical processes (Accinelli et al. 2007; Bartels and von Tümpling 2008). The main objective of this study was to assess the effect of OC on the bacterial community structure of a surface water ecosystem (an irrigation canal in Northern Italy) by applying the main fluorescent oligonucleotide probes with which it is possible to identify most known freshwater species (Zwart et al. 2002).

Materials and Methods

Samples were collected from a 150-km-long irrigation canal which receives water from the Po River. The main physico-chemical water characteristics were: pH 8, 13.1 mg L^{-1} dissolved oxygen (DO), and 4.7 mg L^{-1} dissolved organic carbon (DOC). The canal provides water to a 3,000 km^2 area for agricultural, urban and industrial

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uses. Sampling operations were conducted manually in April by immersing 2-L sterile glass bottles approximately 10 cm below the water surface. The water from the canal had never been exposed previously to OC.

Microcosms (2 replicates for each conditions) were set up by transferring samples (80 mL) into 250-mL sterile Erlenmeyer flasks under aseptic conditions, and treating them with oseltamivir carboxylate (OC) dissolved in 50 mM NaH_2PO_4 to give a final concentration of 1.5 mg mL^{-1}. This concentration was chosen because it was comparable with the Predicted Environmental Concentration (PEC) of common use drugs (Singer et al. 2007). Moreover, control microcosms were set up with the same amount of non-treated water. Flasks were sealed and incubated at 20°C on an orbital shaker (125 rpm) in the dark, as described in detail in a previous work (Accinelli et al. 2007).

The main chemical-physical characteristics of Tamiflu (OP) and its active metabolite OC are shown in Table 1.

In order to investigate if microbial populations can be affected by the antiviral drug and if they can be involved in its degradation, Fluorescence In Situ Hybridization (FISH) was performed on OC-treated (1.5 mg L^{-1}) and untreated sub-samples collected from degradation microcosms. The phylogenetic composition of the OC-treated and control samples was analyzed at different sampling times (0, 14, 21 and 36 days). For each condition four sub-samples (1 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. In order to separate the bacterial aggregates found in this water, a gentle sonication (10 s, 15 W using a Microson XL2000 ultrasonic liquid processor) was performed on each sub-sample, which was then filtered on a 0.2 μm polycarbonate membrane. The filters were stored at −20°C until further processing.

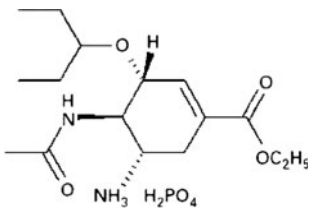
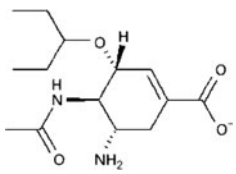
Fluorescence In Situ Hybridization (FISH) of the harvested cells, counterstained with DAPI, was performed using fluorescent probes for the identification, under the epifluorescence microscope, of the major bacterial divisions found in freshwater (Zwart et al. 2002), such as *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Planctomycetes*, the *Cytophaga-Flavobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* phylum, and Gram-positive low G + C-content bacteria. For this purpose the following Cy3-labelled oligonucleotide probes were applied: EUB338, EUB338 II, EUB338 III (for Bacteria) and inside this domain ALF1b, BET42a, GAM42a, PLA46 together with PLA886, CF319a and LGC354a. Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al. 2007).

Averages of the cells binding the bacterial probes were calculated as a percentage of the total DAPI-positive cells from 10 to 20 randomly selected fields on each filter section (corresponding to 500–1,000 stained cells). The slides were mounted with a drop of Vectashield Mounting Medium and the preparation examined and counted with a Leica DM 4000B epifluorescence microscope at 1,000× magnification.

The protocols for both FISH and bacterial abundance by DAPI counts are described in detail in our previous works (Barra Caracciolo et al. 2005, 2010; Grenni et al. 2009). The FISH experimental data are reported as the number of each bacterial group obtained from the mean of four sub-samples. We calculated this number (expressed as No. cells mL^{-1}) by multiplying the total cell abundance (previously determined by DAPI direct counts) and the percentage of cells detected by each specific probe.

Moreover, in order to know the number of actually viable bacteria, we assessed cell viability (% of live cells/live + dead), at each sampling time and for each experimental condition assessed by FISH (in two replicates), using a two-dye fluorescence bacterial viability kit (Kit Live/Dead® Bacterial Viability Kit, BacLight™), which distinguishes between viable (green) and dead (red) cells under a fluorescence microscope (Alonso et al. 2002). Then

Table 1 Chemical structure and main chemical-physical characteristics of Tamiflu (OP) and of its active form OC

Pharmaceutical	Prodrug Tamiflu (OP)	Active form OC
		
Molecular weight	312.41	284.35
Solubility (water)	>200 mg L^{-1}	>500 mg L^{-1}
Melting point	~193°C (OP-PO_3)	
Vapour pressure	~≤1.4 × 10^{-3}	~≤7.3 × 10^{-10} Pa

we calculated live cell abundance (No. live bacteria mL^{-1}) from the total cell abundance, obtained by DAPI counts, multiplied by % of live cells/live + dead.

Statistical analysis of the overall bacterial group data was done using Kruskal-Wallis One Way Analysis of Variance on Ranks, with significant differences at the $p < 0.01$ level. The comparison of data for each Bacteria group at 21 days was done using the Mann-Whitney Rank Sum Test. The PC Program used was SIGMASTAT Statistical software.

Results and Discussion

The addition of OC led to an initial decrease in the number of Bacteria cells detected by the EUB probes. However, at day 21 a significant peak ($p < 0.01$) in the Bacteria cell number was observed in the OC-treated samples (Fig. 1a). This trend was observed not only with the general probes for Bacteria, but also inside this domain ($p < 0.01$) for *α-Proteobacteria*, *β-Proteobacteria* and *γ-Proteobacteria* (Fig. 1b, c, d). In particular, the *β-Proteobacteria* group was the most abundant and constituted 40% of the Bacteria domain, suggesting an active role in the OC degradation. These results are in line with the transient decrease in live cell abundance (No. live bacteria mL^{-1}) at day 14, followed by a significant increase at day 21 in OC-treated samples (Fig. 2).

The other bacterial groups investigated by FISH were not significantly affected by the presence of OC, and represented about 1–2% of the Bacteria domain; in particular *Cytophaga-Flavobacterium* was the relatively most abundant group (the average number mL^{-1} during the experimental period was about 1.39E+04 ± 4.02E+03) followed by Gram-positive low G + C-content bacteria (about 2.8E+03 ± 1.67E+03) and finally *Planctomycetes* (about 1.22E+04 ± 7.3E+03).

The results of the analysis of the bacterial community show that the decrease at day 14 in bacterial abundance of the main phylogenetic groups in the presence of OC was transient; this initial negative effect was subsequently more than offset by a significant increase in their presence and presumably activity. This hypothesis is confirmed by the fact that in the presence of the bacterial community about 65% of the OC applied was degraded in 36 days, as shown in a previous work using the same water (Accinelli et al. 2007; Singer et al. 2008), while in the same experimental time just 5% of it was degraded in the sterilized water.

Consequently the overall results not only confirm the key role of the bacterial community in OC degradation, but also suggest which bacterial groups, i.e. *α-Proteobacteria*, *γ-Proteobacteria* and above all *β-Proteobacteria* could be directly involved. The presence of OC in the environment is a relatively recent issue, and to our knowledge no

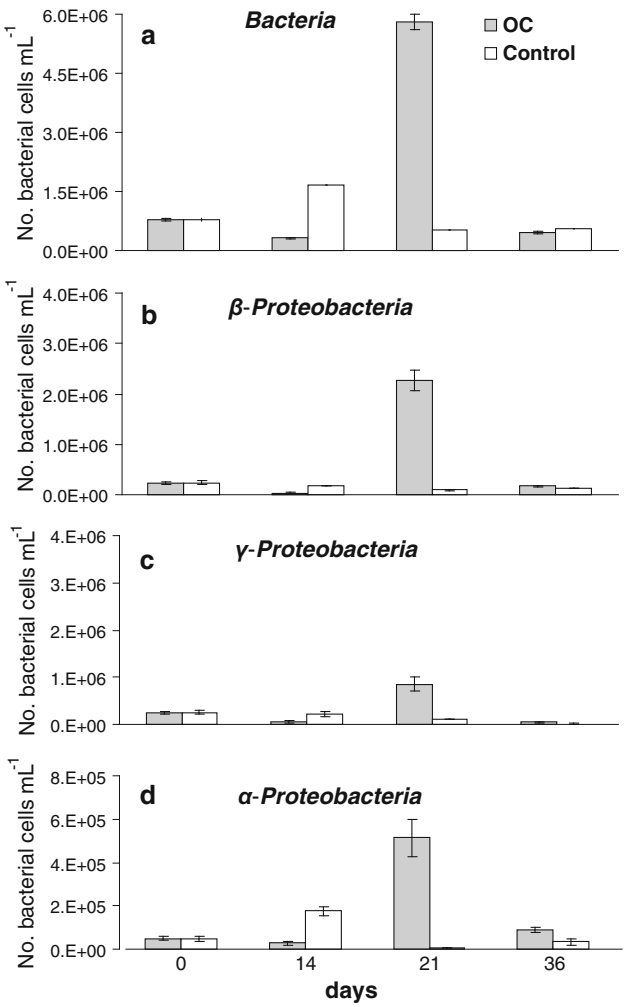


Fig. 1 Bacterial community structure detected by FISH in surface water at different times: 0, 14, 21 and 36 days, in presence of Oseltamivir Carboxylate (OC) and *Control*. *Vertical bar* represents standard errors. **a** *Bacteria*; **b** *β-Proteobacteria*; **c** *γ-Proteobacteria*; **d** *α-Proteobacteria*

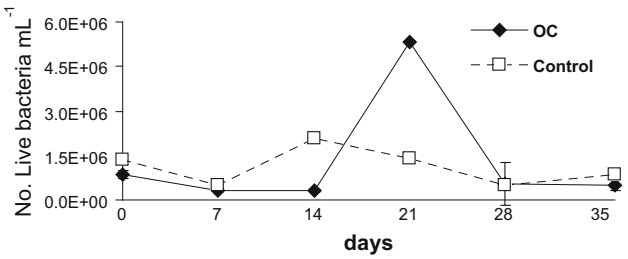


Fig. 2 Live cell abundances (No. live bacteria mL^{-1}) at different sampling times in *OC*-treated and *Control* in surface water samples. *Vertical bar* represents standard errors

OC-degrading bacteria belonging to the *β-Proteobacteria* have been identified until now.

These results encourage the performance of further studies to better investigate the bacterial metabolism

(metabolism and/or co-metabolism) of this compound and the formation of its transformation products prior to its possible mineralization. The presence/absence of bacterial populations with a natural attenuation capacity versus pharmaceuticals is a crucial factor in assessing their actual environmental fate in aquatic ecosystems.

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References

Accinelli C, Barra Caracciolo A, Grenni P (2007) Degradation of the antiviral drug oseltamivir carboxylate in surface water samples. Int J Environ Anal Chem 87:579–587

Accinelli C, Saccà ML, Fick J, Mencarelli M, Lindberg R, Olsen B (2010) Dissipation and removal of oseltamivir (Tamiflu) in different aquatic environments. Chemosphere 79:891–897

Alonso JL, Mascellaro S, Moreno Y, Ferrus MA, Hernandez J (2002) Double-staining method for differentiation of morphological changes and membrane integrity of *Campylobacter coli* cells. Appl Environ Microbiol 68:5151–5154

Barra Caracciolo A, Grenni P, Cupo C, Rossetti S (2005) In situ analysis of native microbial communities in complex samples with high particulate loads. FEMS Microbiol Lett 253:55–58

Barra Caracciolo A, Grenni P, Saccà ML, Amalfitano S, Ciccoli R, Martín M, Gibello A (2010) The role of a groundwater bacterial community in the degradation of the herbicide terbuthylazine. FEMS Microbiol Ecol 71(1):127–136

Bartels P, von Tümpling W Jr (2008) The environmental fate of the antiviral drug oseltamivir carboxylate in different waters. Sci Total Environ 405:215–225

Fick J, Lindberg RH, Tysklind M, Haemig PD, Waldenström J, Wallensten A, Olsen B (2007) Antiviral oseltamivir is not removed or degraded in normal sewage water treatment: implications for development of resistance by influenza A virus. PLoS ONE 2:e986

Grenni P, Gibello A, Barra Caracciolo A, Fajardo C, Nande M, Vargas R, Saccà ML, Martínez-Iñigo MJ, Ciccoli R, Martín M (2009) A new fluorescent oligonucleotide probe for in situ detection of s-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples. Water Res 43:2999–3008

Loy A, Maixner F, Wagner M, Horn M (2007) probeBase–an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Res 35(Database issue):D800–D804

Saccà ML, Accinelli C, Fick J, Lindberg R, Olsen B (2009) Environmental fate of the antiviral drug Tamiflu in two aquatic ecosystems. Chemosphere 75:28–33

Singer AC, Nunn MA, Gould EA, Johnson AC (2007) Potential risks associated with the widespread use of Tamiflu. Environ Health Perspect 115:102–106

Singer AC, Howard BM, Johnson AC, Knowles CJ, Jackman S, Accinelli C, Barra Caracciolo A et al (2008) Meeting report: risk assessment of Tamiflu use under pandemic conditions. Environ Health Perspect 116(11):1563–1567

Straub JO (2009) An environmental risk assessment for oseltamivir (Tamiflu®) for sewage works and surface waters under seasonal-influenza-and pandemic-use conditions. Ecotoxicol Environ Saf 72:1625–1634

Ward P, Small I, Smith J, Suter P, Dutkowski R (2005) Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J Antimicrob Chemother 55:i5–i21

Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han SK (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of freshwater lakes and rivers. Aquat Microb Ecol 28:141–155