View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Florence Research

Alkylphenol Polyethoxylate Removal in a Pilot-Scale Reed Bed and Phenotypic Characterization of the Aerobic Heterotrophic Community

Cristiana Sacco, Anna Maria Pizzo, Emilia Tiscione, Daniela Burrini, Luca Messeri, Luciano Lepri, Massimo Del Bubba

ABSTRACT: The removal of the non-ionic surfactant Triton X-100, dosed at 30 and 300 mg/L in a pilot-scale subsurface horizontal flow reed bed, and the aerobic heterotrophic cultivable community associated with the roots and with the substrate gravel in both absence and presence of Triton X-100 were investigated. t-Octylphenol (OP) and its mono-, di- and tri-ethoxyl derivatives, among others, were found in the outlet. A mass balance allowed us to calculate that approximately 40% of the Triton X-100 metabolites OP and octylphenol polyethoxylate derivatives flowed out of the reed bed during the dosage and postdosage experiments. More aerobic heterotrophic microorganisms adhered to the roots than to the gravel. The appearance of new strains (*Aeromonas*, *Flavobacterium*, and *Aquaspirillum*) and the increased presence of others (*Pseudomonas*) during the dosage of Triton may be linked to the capacity of these bacteria to adapt to the presence of the surfactant or to use it as a nourishment. *Water Environ. Res.,* **78,** 000 (2006).

KEYWORDS: aerobic heterotrophic cultivable community, biodegradation, constructed wetlands, non-ionic surfactants, reed beds, Triton X-100 removal.

doi:10.2175/106143005X72975

Introduction

Horizontal subsurface flow (SSF-h) reed beds have been extensively used in many countries for the treatment of urban wastewater (especially from small communities) (Brix, 1998; Kern and Idler, 1999; Kowalik et al., 1995; Reed and Brown, 1995; Vymazal, 1999) and, less frequently, for industrial wastewaters (Davies and Cottingham, 1994; Chong et al., 1999; Del Bubba et al., 2004; Knight et al., 1999). Good results have been also obtained in the treatment of wastewater made synthetically by adding specified organic compounds (Machate et al., 1996). The interesting results obtained in the treatment of industrial wastewaters and selected organic compounds by using constructed wetlands are encouraging for testing the removal of chemicals of primary importance from an environmental point-of-view and for which natural purification has not yet been studied. Among these chemicals, the non-ionic surfactants alkylphenol polyethoxylates (APEOs) are extensively used for industrial activities (Ejlertsson et al., 1999). As a consequence, APEOs and their metabolites are widely diffused in the environment (Blackburn et al., 1999; Field and Reed, 1996; Lepri et al., 1997). Among their byproducts, octylphenol (OP), nonylphenol (NP), and their mono-, di-, and tri-ethoxyl derivatives are toxic to aquatic life (Field and Reed, 1996), can mimic estrogen, can be taken up by estrogen receptors (Soto et al., 1991; Tange et al., 1999), and have the potential to accumulate in the fatty tissues of aquatic organisms (Blackburn et al., 1999).

Little is known about either the structure of the community or the functioning of the several strains that are active in the removal processes of non-ionic surfactants occurring in subsurface flow (SSF) constructed wetlands (Chong et al., 1999).

Because no field studies about the removal of Triton X-100 (a commercial mixture of oligomers of 4-t-octylphenoxypolyethoxyethanol) and the identification of microbial genera involved in its biodegradation have been carried out in natural systems, in the present research, Triton X-100 was dosed in a pilot-scale SSF-h reed bed. Two different concentrations (30 and 300 mg/L) of non-ionic surfactant were tested, allowing for the simulation of mixed (municipal and industrial) wastewater and of those originating from surfactant manufacturing and textile industries or commonly encountered in soil washing and other surfactant-based remediation technologies (Zhang et al., 1999).

A further aim of this paper has been to characterize the aerobic heterotrophic cultivable bacterial community associated with the treatment of Triton X-100, because bacteria belonging to this community are generally involved in the degradation of several classes of organic compounds (Amann et al., 1995; Di Cello and Fani, 1996; Esteve-Nunez et al., 2001; Komancova et al., 2003; Perei et al., 2001; van Hervijnen et al., 2003) and, particularly, of alkylphenols and their ethoxylate (EO) derivatives (Fujii et al., 2000; Soares et al., 2003, Tanghe et al., 2000).

It should also be noted that the use of small pilot plants allows the study of the removal of very high concentrations of chemicals, such as those found in industrial wastewater.

Materials and Methods

Site Description. The investigation was carried out from February 2000 to January 2001, in a SSF-h pilot reed bed (Figure 1). The pilot system treats the outlet of an activated sludge plant that deals with civil wastewater. Table 1 shows the average composition of the wastewater after secondary treatment and of wetland effluent during the period November 1998 to November 1999, in the absence of Triton X-100 dosage. An Imhoff settling tank (ISEA, Lodi, Italy) was used to avoid possible clogging phenomena of the wastewater distribution pipe. The reed bed was created in 1997. Rhizomes of *Phragmites australis* that had an initial average density of 10/m^2 were used. The reed bed was completely occupied

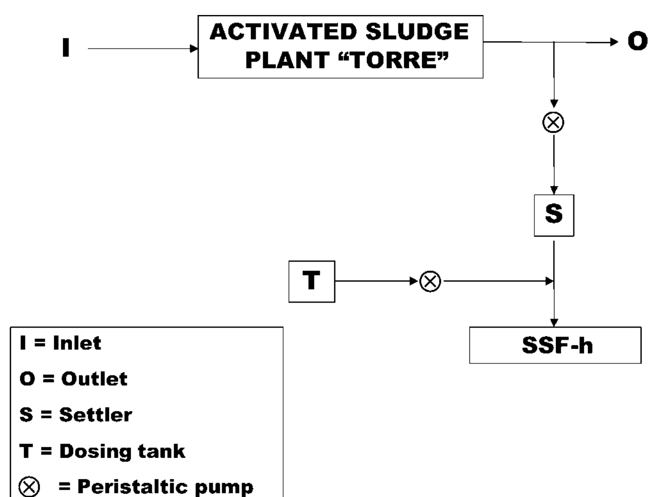

Figure 1—Operational scheme of the pilot system.

by the root zone during the experiments and was continuously in operation during the entire study period. The pilot system is made of sheets of Plexiglas (12 mm thick), placed above ground level and protected, on the outside, by black movable polyurethane panels (40 mm thick), to prevent algal growth on the interior walls of the cell. The filling medium was clean gravel (mean grain size = 8 mm), while the settling and drainage zones were both 10 cm in length. These areas were made of clean rubble (Ø = 8 cm). The depth of the media was 0.6 m. Three pipes were placed inside the bed at different distances from the inlet (40, 80, and 120 cm) and at a depth of 25 cm. The reed bed (basin slope 1%) is located in the countryside, on the outskirts of Florence (Italy). The length and width of the system were 1.52 and 0.43 m, respectively; thus, the system area was 0.65 m^2. The average flow through the system was 0.024 m^3/d (the dose was preestablished by a peristaltic pump), and the corresponding hydraulic retention time (HRT) was 5 ± 0.4 days. In July and August, when the wetland temperature was as high as 27°C, evapotranspiration phenomena were significant, and a 30 to 40% decrease of the outlet flow was observed; therefore, a corresponding HRT increase occurred. The pilot plant was sheltered by a nylon tarpaulin to reduce plant transpiration phenomena and to avoid any dilution effects resulting from rainwater.

The wetland temperature was measured continuously in situ; results were recorded every 2 hours. Daily values were the average of the 12 measurements taken within a 24-hour period.

Dosage of Triton X-100. The dosing of surfactant in the pilot system was established by aspirating an aqueous solution of the surfactant (gently stirred by a magnetic stirrer) from a jerry can, by using a peristaltic pump. The mixing of the inflowing water and homogenization occurred a short time before entry into the system.

The surfactant was added in increasing doses to acclimate the microbial community to its presence. In this way, shock situations that could affect the flora were avoided. Triton X-100 was dosed at two different concentrations: 30 mg/L for 66 days (February 26 to May 3, 2000) and 300 mg/L for 134 days (May 4 to September 15, 2000); it was therefore possible to verify the removal efficiency of the constructed wetlands by varying the concentrations of Triton X-100.

Postdosage Study. After the end of the dosage, the pilot plant was fed with the effluent of the activated sludge plant, and a weekly monitoring of the OP and octylphenol polyethoxylates (OPEOs) present in the outlet was carried out. The postdosage study was terminated 113 days after the end of the 300-mg/L dosing.

Table 1—Mean concentrations ± standard deviation (n = 13) of some chemical parameters determined during the period November 1998 to November 1999 in influent and effluent of the SSF-h pilot system fed with the outlet of an activated sludge plant.

	In	Out
TSS	19 ± 10	7 ± 4
BOD_5	20 ± 7	6 ± 3
COD_T	29 ± 3	14 ± 2
COD_S	20 ± 3	9 ± 2
Ammonium-nitrogen ($N\text{-}NH_4^+$)	5.7 ± 2.1	3.3 ± 2.6
Nitrous-nitrogen ($N\text{-}NO_2^-$)	0.27 ± 0.19	0.10 ± 0.07
Nitric-nitrogen ($N\text{-}NO_3^-$)	3.2 ± 2.5	1.2 ± 0.8

Sampling Procedure. Wastewater samples (250-mL grab) were collected during the Triton X-100 dosage at the inlet, outlet, and at different distances (40, 80, and 120 cm) from the inlet.

All samples collected before and after the surfactant dosing were taken 80 cm from the inlet (at the center of the basin). During Triton X-100 dosing, further samples were collected at 40 and 120 cm from the inlet. The sampling frequency varied in relation to the modifications brought about in the system (the addition of two different quantities of Triton X-100).

To investigate if a release of Triton X-100 metabolites occurred after the end of the dosage (postdosage study), composite 24-hour sampling of the effluent wastewater was carried out weekly by a refrigerated (4°C) sampler (Sigma, Milan, Italy).

Differentiated sampling was carried out for both the filling gravel of the basin and for the *Phragmites australis* roots; a 7-cm-diameter corer was used. Care was taken to avoid contamination of the corer.

The aerial part of a reed was cut 5 cm above ground level, and sampling was carried out by coring a root and soil portion at a depth of 25 cm and at various distances from the inlet. Then, the root and soil samples were placed in steril plastic bags, separately.

The first two samplings of gravel and roots (80 cm from the inlet of the system) were made in February 2000, when the surfactant dosage had not yet begun.

Chemical Analyses. *Sample Filtration.* Wastewater samples were filtered (within 1 hour after sampling) in duplicate through glass fiber filters (GF/F, Whatman, Maidstone, Kent, United Kingdom; porosity 0.45 μm,) previously dried at 105°C for 1 hour and weighed. After filtration, one filter was heated for 1 hour at 105°C and then weighed again to calculate the amount of total suspended solids (TSS). The other was placed in a drier until constant weight was reached, and then it was analyzed to evaluate the content of OPEOs adsorbed on particulate matter.

Extraction of Water Samples. Extraction of OPEOs and OP was carried out by adding 20 g sodium chloride (NaCl) (Prolabo, Paris, France) to 20-mL aliquots of filtered samples. These solutions were then extracted three times with 10-mL portions of chloroform (Baker, Deventer, Netherlands). The organic extracts were combined, dried over anhydrous sodium sulphate, and made up to 30 mL. The average recovery, calculated for the oligomers of Triton X-100, was 78 ± 3% (n = 5).

Extraction of Particulate Samples. Extraction of particulate matter was performed according to Desideri et al. (1988), with some modifications. The filter was placed in a 20-mL vial, and 1.5 mL

water (milliQ, Millipore, Billerica, Massachusetts), 1.5 mL of a mixture methylene chloride/*n*-hexane 1:1 v/v, and 0.5 mL methanol were added. Extraction was performed by means of magnetic stirring (15 minutes) and centrifugation (10 minutes, 2500 rpm). The methylene chloride/*n*-hexane phase was dried over anhydrous sodium sulphate. The average recovery, calculated for the oligomers of Triton X-100, was 85 $\pm$ 4% (n = 5).

Chromatographic Analyses. The extracts were analyzed using both reversed-phase high-performance liquid chromatography (RP-HPLC) and gas chromatography (GC). The RP-HPLC allowed for the determination of the OPEO oligomers, while GC was a suitable method for the analysis of OP and those OPEOs with fewer than eight EO units.

The RP-HPLC analysis was carried out on OPEO derivatives to obtain chromatographic separation of the oligomers, according to the method of Zanette et al. (1996), with some modifications, both in the derivatization and in the chromatographic elution conditions. The derivatization reaction was performed in a conical tube according to the following procedure: a 15-mL aliquot of the organic extract was evaporated to dryness under a gentle stream of nitrogen. A 50-µL volume of 1-naphthyl isocyanate (Aldrich, Munich, Germany) was then added to the residue to obtain the corresponding urethanes. The tube was heated in a water bath at 40°C for 30 minutes. The resulting mixture was dissolved in 2.5 mL acetonitrile for HPLC analysis. The analysis was carried out at ambient temperature (22 to 25°C) using a double-pump liquid chromatograph (LC-10ADVP, Shimadzu Italia, Milan, Italy) equipped with a SPD M10AVP diode array detector (Shimadzu Italia). The detection was performed at λ = 220 nm. A Class VP 4.2 chromatography data system (Shimadzu Italia) was used to make and handle the chromatograms. The samples were injected to a reversed-phase C-18 column LC-PAH (Restek, Bellefonte, Pennsylvania) by a 20-µL loop, using a 50-µL syringe (Kloehn, Las Vegas, Nevada). The flowrate was 1 mL/min. The mobile phase was a mixture of HPLC-grade acetonitrile and water (CH_3CN/H_2O) (Baker, Deventer, Netherlands). The elution conditions were as follows:

- 0- to 10-minute isocratic elution with 60% acetonitrile;
- 10- to 25-minute linear gradient from 60 to 75% acetonitrile,
- 25- to 40-minute isocratic elution with 75% acetonitrile,
- 40- to 60-minute linear gradient from 75 to 100% acetonitrile, and
- 60- to 70-minute isocratic elution with 100% acetonitrile.

Response factors for the different OPEO oligomers were the same.

Gas chromatographic analysis was carried out according to Desideri et al. (1998) by using a capillary column CP-SIL 8 CB Low-Bleed/MS (30 m $\times$ 0.25 mm internal diameter, 0.25-µm thickness) purchased from Chrompack (New York). The chromatographic peaks were analyzed with a Mega 2-computer system (Carlo Erba, Milan, Italy), using Spectra Physics software (Spectra Physics, Mountain View, California). Response factors were calculated separately for the OP and OPEO oligomers.

A confirmatory high resolution gas chromatographic/mass spectrometric analysis was performed as reported by Desideri et al. (1998). All peaks were identified by using a reference chromatogram and by comparing their mass spectra with those reported in the National Bureau of Standards and National Institute of Standards and Technology (Gaithersburg, Maryland) libraries.

Quantification Limits. The HPLC quantification limits were the same for all OPEOs and equal to 0.2 mg/L. The GC quantification limits for OP and OPEOs with 1 to 7 EO units were included in the range 0.020 to 0.045 mg/L, depending on their response factors, which decrease with increasing the length of the EO chain.

Chemical Oxygen Demand Analysis. Chemical oxygen demand (COD) was determined both on filtered (COD_S) and unfiltered (COD_T) samples according to the standard IRSA-CNR method (1981).

Identification of Total Bacterial Load and Bacterial Strains. Each sample was a pool of two portions of roots or gravel having the same fresh weight (Sartorius handy-Prokeme H110, Sartorius, Goettingen, Germany), collected in two different sites, both located 10 cm from the sides of the basin. The gravel samples were mechanically stirred for 20 minutes in a 0.8% NaCl solution in distilled water that had been added at a ratio of 1/10 v/v. The roots were gently washed in the NaCl solution and then placed in a sonicator bath (Branson Ultrasonics Corporation, Danbury, Connecticut) for 10 minutes to remove rhizosphere microorganisms (Duineveld et al., 1998). The NaCl solution was added to the samples at a ratio of 1/10 v/v.

The root and gravel suspensions were serially diluted (up to 10^{-4}). A 0.1-mL aliquot was taken from the different dilutions and spread on agar media, in triplicate. The plates were incubated at 24°C for up to 7 days. The culture media used were as follows:

- Triptone yeast (TY): maximum culture medium for the isolation of bacteria (Hatano et al., 1993).
- Triptone yeast crystal violet (TYCV): maximum culture medium for the isolation of gram-negative bacteria (Hatano et al., 1993).
- Triptone yeast triton (TYT): culture medium (TY + 30 mg/L of Triton X-100) for the isolation of bacteria that can grow in the presence of Triton X-100 (Fantroussi et al., 1999).

The different typologies of colonies were counted, and each one was identified by means of the Biolog MicroStation system (Biolog Inc., Hayward, California) (Heinaru et al., 2000; Johnsen et al., 1996; Picard et al., 2000). According to the *Biolog Reference Manual* (Biolog Inc., 1999), phenotypic identification was performed only if a similarity index value greater than 0.5 (for gram-negative aerobes at 24 hours incubation) or 0.75 (for gram-positive aerobes at 6 hours incubation) was observed. For correct use of the system, each bacterial strain was submitted to gram staining and microscopic analysis. Additional oxidase and catalase tests were also carried out. Tryptic soy agar with 5% sheep's blood (OXOID, Hampshire, United Kingdom) was used to grow each strain.

Results and Discussion

The 30-mg/L dosage of Triton X-100 began when the reed (*Phragmites australis*) bed was still in a state of winter quiescence. Vegetation of the plants began towards the end of March as the temperature increased. From the beginning, the development of the reeds was noteworthy, and no evident sign of vegetative suffering that could be associated with the surfactant was noted. In the beginning, purified yields were scarce (approximately 20%); there was a considerable presence of heavy OPEO oligomers in the final outlet. Thereafter, the situation progressively improved, and the stabilization in the oligomer composition was reached in the outlet.

The increase in the concentration of Triton X-100 to 300 mg/L had no apparent negative effect on the development of the *Phragmites*, because a vigorous growth of the reeds and an almost total absence of withered leaves were observed.

Octylphenol Polyethoxylate Composition at Different

Table 2—Average percentage composition (n = 3) of the different OPEO oligomers determined in the commercial product Triton X-100.

EO units	1	2	3	4	5	6	7	8	9	10	11	12	13
Triton X-100	0.7	1.0	1.3	3.6	7.0	12.6	15.9	16.5	12.4	10.6	8.9	5.7	3.8

Distances from the Basin Inlet. The composition of the used surfactant, as determined by HPLC analysis, is shown in Table 2. Oligomers with a number of EO units ranging from 1 to 13 were found, and the mean value was 8.1.

Based on the concentrations of OPEOs and OP found in the water and in the particulate phase, for both the 30- and 300-mg/L dosages, relative percentages of the OPEOs and OP present along the basin were calculated. Percentages of the OPEOs (EO = 1 to 7) and OP were plotted as a function of the distance from the basin inlet (see Figures 2 and 3). Only the oligomers with a number of EO units less than 7 were reported, because the oligomers with more than 7 EO units were below detection limits, even at 40 cm from the basin inlet.

Regarding the 30-mg/L dosage (Figure 2), the OPEO oligomers with 6 and 7 EO units disappeared almost completely, even at 40 cm from the inlet of the basin.

The percentages of OPEO oligomers with 4 and 5 EO units had a strongly decreasing trend at 80 cm from the inlet. In the outlet, they were only found at trace levels.

The oligomer with three units showed an almost constant trend along the first 40 cm of the basin, while, for the diethoxylate, an increasing trend was observed. Thereafter, they decreased. Nonetheless, they continued to exist in appreciable quantities along the basin; in the outlet, they were present at 2.6 and 6.6%, respectively.

The 4-t-octylphenol and its monoethoxylate derivative had an increasing trend during the first 40 cm of the basin. Their trends then diverged. The monoethoxylate reached a maximum value at 40 cm from the inlet. It decreased in a very limited manner from 40 to 120 cm, and then, in the final tract, its decreasing trend was more evident. The concentration of 4-t-octylphenol increased along the basin. This compound represented the most abundant one (70.2%) among those present in the outlet.

The trends of the relative percentages of OPEOs and OP as a function of the distance from the basin inlet, for the 300-mg/L dosage (see Figure 3), showed that the OPEOs with 6 and 7 EO units disappeared along the first 40 cm of the basin and that OPEOs with 3, 4, and 5 EO units strongly decreased in this tract of the bed; at 40 cm, such oligomers were present at lower percentages (1.5, 0.6, and 0.4 %) than that previously observed for the 30 mg/L dosage. Such behavior is in apparent contrast with the increased concentration of Triton X-100 and could be attributed to the longer acclimatization period. In addition, the higher temperatures observed during this experiment (22 to 27°C), with respect to those measured during the previous one (15 to 18°C) could be a contributing factor, which positively affected the biodegradation rate.

The diethoxylate showed a behavior quite similar to that previously observed for the monoethoxylate, reaching the maximum value at 40 cm (8.8%) and then decreasing. The trends of the monoethoxylate and 4-t-octylphenol were analogous with respect to the 30-mg/L trends; however, in this case, the monoethoxylate represented the most abundant oligomer. Monoethoxylate reached the maximum value at 40 cm (79.1%); it decreased slightly from 40 to 120 cm along the basin and in a flatter way in the final part of the basin, persisting in the outlet at a very high percentage (67.1%). For OP, a uniform increasing trend was found; in particular, a strong increase occurred in the last tract of the bed (outlet percentage 30.2), where the above-mentioned decrease of the monoethoxylate was observed.

The disappearance of and/or decrease in certain OPEO oligomers seem to be correlated to increases in others and suggests that each oligomer could be formed through shortening in the ethoxylic chain of its superior homologous and that the biodegradation rate of the oligomers with a number of EO units greater than 3 is higher than those observed for compounds with shorter EO chains. These results are in agreement with findings reported elsewhere for the

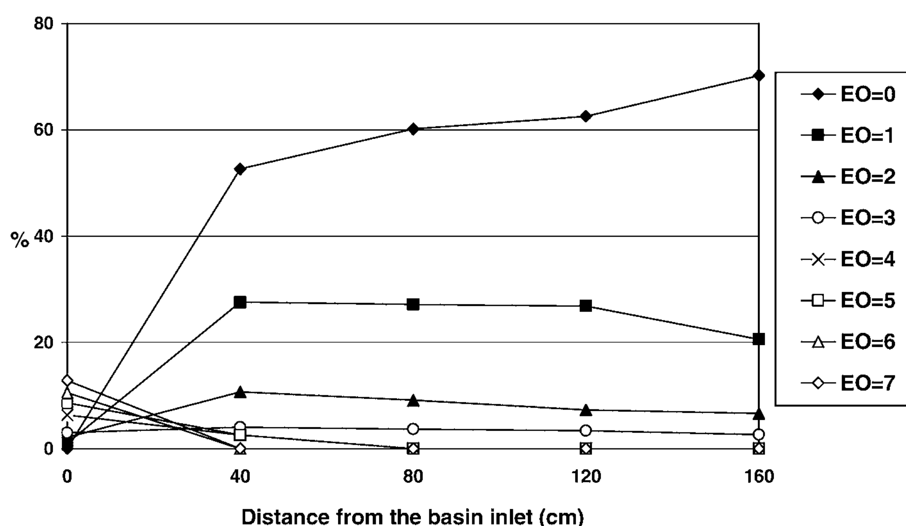

Figure 2—Mean percentages (n = 3) of OP and OPEOs found in water and particulate phases at the inlet, at various distances from the inlet and in the outlet during the Triton X-100 dosage of 30 mg/L. EO = number of ethoxylate units. Determination carried out by GC analysis. Mean total concentrations of the oligomers with 0 to 7 EO units: 0 cm = 14.92 mg/L; 40 cm = 6.15 mg/L; 80 cm = 3.71 mg/L; 120 cm = 2.44 mg/L; 160 cm = 1.91 mg/L.

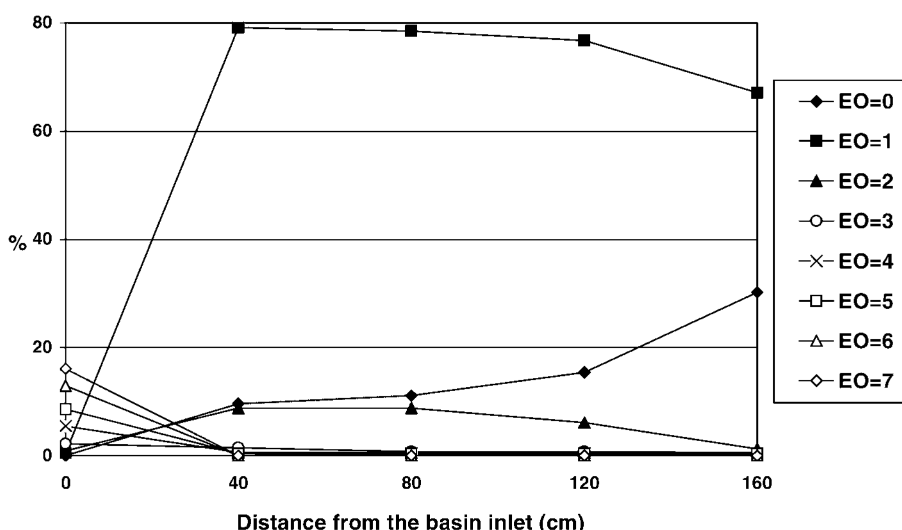

Figure 3—Mean percentages (n = 3) of OP and OPEOs found in water and particulate phases at the inlet, at various distances from the inlet and in the outlet during the Triton X-100 dosage of 300 mg/L. EO = number of ethoxylate units. Determination carried out by GC analysis. Total concentrations of oligomers with 0 to 7 EO units: 0 cm = 134.81 mg/L; 40 cm = 66.60 mg/L; 80 cm = 51.27 mg/L; 120 cm = 40.35 mg/L; 160 cm = 32.78 mg/L.

biodegradation pathways of APEOs in activated sludge treatment plants (Giger et al., 1984; Swisher, 1987; Ying et al., 2002).

Triton X-100 Removal. The sum of the concentrations (in milligrams per liter) found in liquid and particulate phases of the effluent for OPEOs and OP under normal operating conditions was very low compared to the 30- and 300-mg/L dosages of Triton X-100 (see Table 3). The mean concentration of the oligomers in the particulate phase was 16 ± 7 µg/mg (n = 9) at the 30-mg/L dosage and 256 ± 68 µg/mg (n = 22) at the 300 mg/L dosage. Because the average outlet TSS concentration was 14 ± 6 mg/L (n = 31), the particulate phase contributed only approximately 12% to the amount of OP and OPEOs found in the outlet.

These findings clearly indicate a high level of efficiency in the overall removal of the dosed surfactant, which could be attributed to biodegradation processes or may also be a consequence of sorption phenomena by the substrate gravel and organic and inorganic matter present inside the reed bed.

Analysis of the effluent wastewater after the end of the dosage (when the reed bed was fed with wastewater devoid of Triton X-100) was carried out to investigate if the sorption processes were reversible and, therefore, if a release of OPEOs and OP occurred. Mono-, di-, and triethoxylate oligomers were found in the effluent (see Figure 4). For any oligomer, a decreasing trend was observed with increasing time. The monoethoxylate showed an almost exponential decreasing trend (R^2 = 0.96), while the others decreased linearly (R^2 = 0.92 to 0.96). The most abundant compound was the monoethoxylate, which was present at significant concentrations (4 mg/L) even 113 days after the end of the dosage, when the monitoring was terminated. Diethoxylate showed concentration levels of approximately 4 mg/L during the first month of postdosage and then decreased quite homogeneously, reaching concentrations less than 1 mg/L after 97 days. Finally, triethoxylate was always present at concentrations less than 1 mg/L and disappeared from the effluent 70 days after the end of the dosage period.

The OP was abundant in the beginning of the postdosage study (approximately 8 mg/L) and persisted at concentrations higher than 5 mg/L for approximately 50 days after the end of the dosage. Its concentration was found to be less than 1 mg/L 105 days after the end of the dosage period.

A mass balance of OP and OPEOs was performed by expressing concentration data (milligrams per liter) as mass units (millimoles). Millimoles of OP and OPEOs dosed in the inlet and those present in the outlet during the entire dosage period were calculated by multiplying mean concentration values determined in the inlet and in the outlet (see Table 2) by the hydraulic loading, by the duration of the dosage experiments (66 and 134 days for the 30- and 300-mg/L dosed concentrations, respectively), and by mean molecular masses of the mixtures of oligomers present in the influent and

Table 3—Inlet and outlet concentrations (mg/L) of OPEOs and OP in the influent and effluent wastewater. The reported values are mean ± standard deviation: n = 9 and 22 for 30- and 300-mg/L dosages, respectively. bql = below quantification limits.

OPEOs oligomers	30 mg/L		300 mg/L	
	Inlet	Outlet	Inlet	Outlet
EO = 0	—	1.1 ± 0.2	—	8.4 ± 0.6
EO = 1	0.3 ± 0.1	0.4 ± 0.2	1.3 ± 0.2	22.8 ± 1.1
EO = 2	0.5 ± 0.2	0.2*	2.4 ± 0.2	0.5 ± 0.2
EO = 3	0.8 ± 0.2	bql	5.7 ± 0.8	0.2*
EO = 4	1.8 ± 0.3	bql	14.8 ± 1.2	0.2*
EO = 5	2.6 ± 0.4	bql	27.1 ± 1.4	0.2*
EO = 6	3.9 ± 0.6	bql	38.6 ± 1.3	bql
EO = 7	4.7 ± 0.5	bql	46.8 ± 1.6	bql
EO= 8	5.4 ± 0.8	bql	50.6 ± 0.8	bql
EO = 9	3.5 ± 0.4	bql	35.7 ± 1.2	bql
EO = 10	2.9 ± 0.3	bql	29.1 ± 0.9	bql
EO = 11	2.4 ±0.5	bql	21.3 ± 1.1	bql
EO = 12	1.6 ± 0.4	bql	15.3 ± 0.7	bql
EO = 13	1.2 ± 0.2	bql	9.1 ± 0.8	bql
Total	31.6 ± 4.9	1.7 ± 0.4	297.8 ± 12.2	32.3 ± 1.9

* HPLC quantification limit.

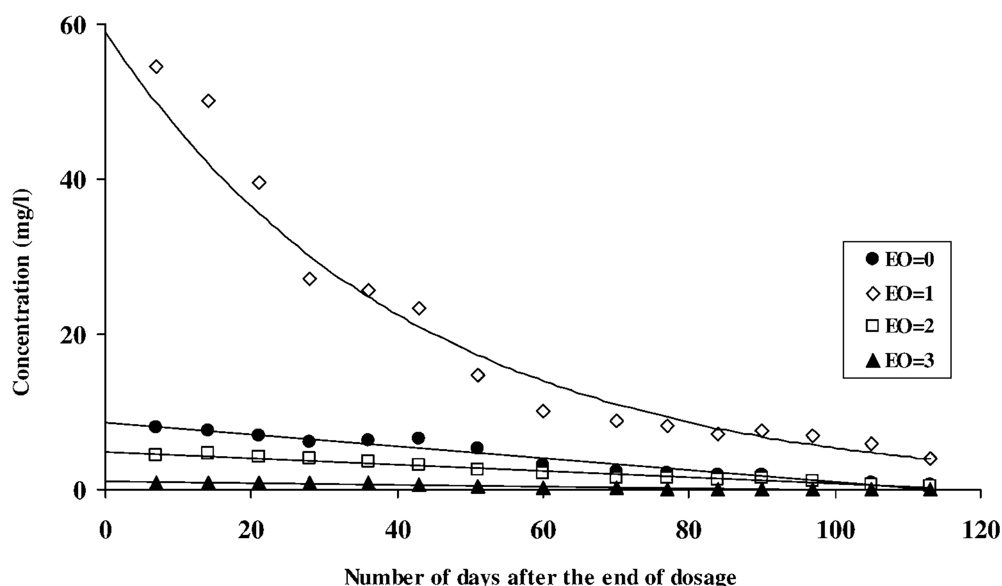

Figure 4—Sum of concentrations found in the effluent wastewater and particulate matter for OPEOs and OP during the postdosage period. EO = number of ethoxylate units.

effluent. The amounts of oligomers flowing out from the reed bed during the postdosage period were computed by expressing the postdosage concentration data reported in Figure 4 as millimoles per day. They were plotted as a function of time (figure not shown), obtaining the same trend reported in Figure 4. The regression curves best fitting these experimental data delimit an area that represents the total amounts of millimoles released by the pilot plant during the entire postdosage period. Such amounts can be easily calculated by integration of the regression equations.

The amounts of OPEO oligomers dosed in the inlet and of OP and OPEOs found in the outlet during both the Triton X-100 dosage and postdosage period are reported in Table 4. The mass of Triton X-100 dosed in the pilot-scale reed bed was approximately 1800 millimoles. The amount of OP and OPEOs globally determined in the effluent during the entire study period was approximately 730 millimoles. Thus, a total of approximately 40% of the non-ionic surfactant flowed out of the plant during the dosage and postdosage experiments. Regarding the fate of the remaining 60% of Triton X-100, the formation of metabolites different from OP and OPEOs with 1 to 3 EO units and the ultimate degradation of the surfactant can be hypothesized. In fact, APEOs carboxylated on the ethoxylic chain and/or on the alkyl chain have been identified as biotransformation products of APEOs (Di Corcia and Samperi, 1994; Di Corcia et al., 1998).

The comparison between effluent COD_T experimental data (obtained at a Triton X-100 dosage of 300 mg/L) and theoretical COD (calculated on the basis of the OP and OPEO concentrations found in the outlet) can give further important information about the fate of Triton X-100. Theoretical COD (mean 89 mg/L, range 80 to 97 mg/L) accounted for 78% of experimental COD_T (mean 114 mg/L, range 101 to 126 mg/L). Because the background COD (as estimated from the data collected during the period November 1998 to November 1999; see Table 1) accounted for 12% of experimental COD_T, it can be deduced that the effluent COD_T measured during the Triton X-100 dosage at 300 mg/L is almost completely a result of OP and OPEOs.

Table 4—Millimoles of OPEO oligomers dosed in the inlet and of OP and OPEOs found in the outlet both during the Triton X-100 dosages and the postdosage period.

	Inlet	Outlet
Triton X-100 dosage 30 mg/L	89.3	9.5
Triton X-100 dosage 300 mg/L	1709	415.5
Postdosage	—	303.5

Aerobic Heterotrophic Culturable Bacterial Load in Relation to the Dosage of Triton X-100. The graphs shown in Figures 5 (microorganisms adhered to the roots) and 6 (microorganisms adhered to the filling medium) illustrate the trend of the bacterial load in relation to the presence/absence of Triton X-100.

For all samples, the roots showed load bacterial values that were higher by at least one log factor when compared with those recorded in the substrate gravel. Within the same culture media, the samples collected before the beginning of surfactant dosing showed similar values of bacterial load. This is in agreement with the almost identical temperatures (5 to 8°C) registered during this sampling period and points out the slight variability of the results because of the procedures of sampling and quantification of microorganisms.

A decreasing trend in the number of bacteria (incubated on TY and TYCV culture media) adhered to the roots was observed in relation to the 30-mg/L dosage of Triton X-100 (see Figure 5). This decrease in the number of microorganisms may be the result of a toxic effect of surfactant towards microbial flora or of an unfavorable selection of specific strains. The microbial flora, in fact, might not yet have adapted to the presence of Triton X-100, which is a well-known antibacterial product and has been found to inhibit or reduce the biodegradation of several molecules (Stellmack et al., 1998; Van Ginkel, 1996; Willundsen and Karlson, 1998).

The number of bacteria growing on TYT culture medium before the Triton X-100 dosage was less than that measured in the other media—particularly in TY, which showed a bacterial load one log higher (Figure 5). During the 30-mg/L dosage, bacterial load TYT was increasing and more similar to that of the TY. This suggests

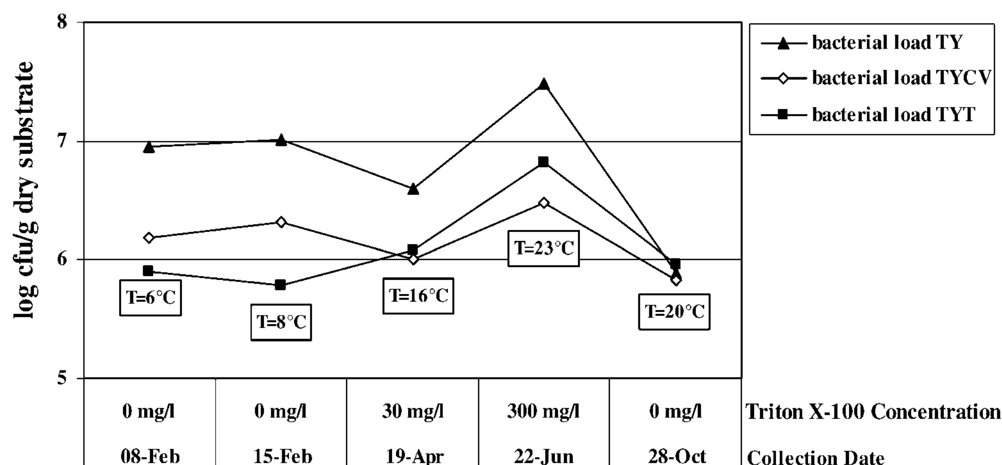

Figure 5—Trends of bacterial load (mean values of three replicates) in the TY, TYT, and TYCV culture media in relation to the dosage of Triton X-100. Microorganisms adhered to the roots at 80 cm from the basin inlet and at a depth of 25 cm.

that the number of those bacteria that can grow in the presence of Triton X-100 is increasing.

A microbial load peak (see Figure 5) was registered for bacteria adhering to the roots (independently of the culture media used) in correspondence with the 300-mg/L dosage of Triton X-100. Such a peak could be related to a positive selection of microorganisms resistant to the surfactant. The wetland temperature registered during this sampling was definitely favorable (23°C) to the growth of microorganisms and could, therefore, have contributed to the microbial load peak.

After the end of the Triton X-100 dosage, the bacterial presence on the roots decreased, even if a drop of only 3°C in wetland temperature was observed. These results confirm that a microbial flora adaptation to the surfactant took place and that its presence influenced the microbial populations of the reed bed, favoring the development of microorganisms degrading Triton X-100.

Trends quite similar to those described for aerobic bacteria adhered to the roots were observed for the filling medium (see Figure 6).

Aerobic Heterotrophic Culturable Bacterial Load at Different Distances from the Basin Inlet During the Triton X-100 Dosage. The microbial load determined during the 30-mg/L dosage of Triton X-100 at three different distances from the system inlet (at 40, 80, and 120 cm) is reported in Table 5.

The bacterial load found in association with the roots was always one or two log factor higher than that of the gravel, confirming the results obtained for the study of bacterial load in relation to the dosage of Triton X-100.

This behavior could be because of a difference in the composition of microbial populations adhered to the roots and substrate gravel and suggests that aerobic bacteria adhered to the roots could be better adapted to the presence of non-ionic surfactants than those collected with the gravel. It should also be noted that many aerobic bacterial species live in association with the roots of aquatic plants and obtain nourishment from them. Therefore, these microorganisms might have been favored within the habitat of the roots of *Phragmites australis*, which is quite rich in oxygen (Brix and Shierup, 1990).

A significant decreasing trend of the bacterial load determined on gravel was observed for every culture media used, with increasing distance from the basin inlet. This finding was probably a consequence of the toxic action of the short-chain OPEOs and particularly of OP, which showed an increase in concentration along the basin, owing to its trapping into the bed.

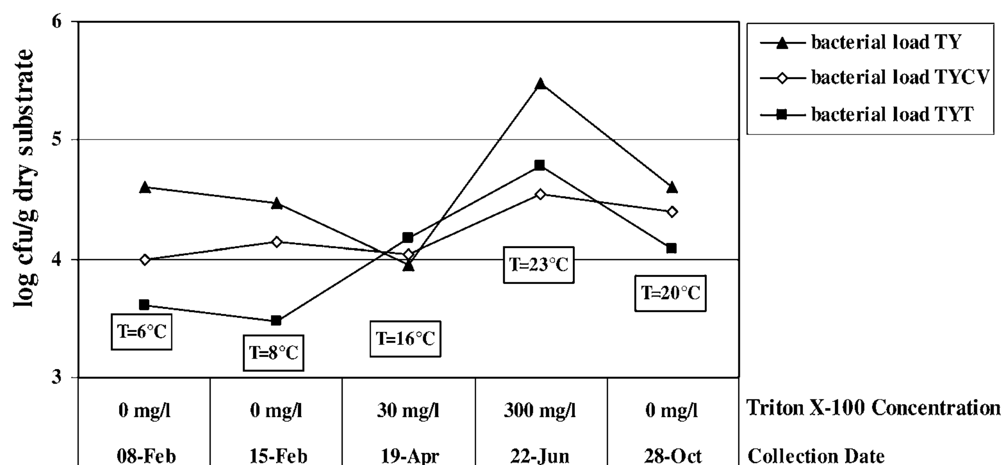

Figure 6—Trends of bacterial load (mean values of three replicates) in the TY, TYT, and TYCV culture media in relation to the dosage of Triton X-100. Microorganisms adhered to the filling medium at 80 cm from the basin inlet and at a depth of 25 cm.

Table 5—Mean (n = 3) bacterial mesophilic load (cfu × 10^4/g dry substrate) in gravel and roots at different distances from the basin inlet during the 30-mg/L dosage of Triton X-100.

Distance from the basin inlet (cm)	TYT		TY		TYCV	
	Roots	Gravel	Roots	Gravel	Roots	Gravel
40	48	4.5	76	12	65	8.9
80	120	1.5	400	0.9	100	1.1
120	70	0.15	80	0.3	20	0.1

Regarding bacteria adhered to the roots, a different trend from that observed for gravel was found (Table 5). In fact, such bacteria showed an expected growth curve, with a maximum at 80 cm from the inlet. The different behavior observed in gravel and roots samples suggests that a bacterial diversity occurred in the two matrices.

Bacterial Diversity in Relation to the Presence of Triton X-100. In Table 6, the microorganisms identified in the root samples are reported. Only the genus was taken into consideration to have an immediate picture of the modifications occurring to the aerobic heterotrophic culturable bacterial community. For "Absence of Triton X-100", we mean samples collected both before and after Triton X-100 dosage. "Presence of Triton X-100" refers to the samplings performed during the dosages (30 and 300 mg/L).

Gram-negative bacteria were identified as follows: *Sphingomonas macroboltabidus*; *Pseudomonas* (*fluorescens*, *synnxantha*, *putida*, *taetrolens*, *oleavorans*); *Agrobacterium tumefaciens/radiobacter*; *Brevundimonas vesicularis*; and *Janthinobacterium lividum*.

Several gram-positive bacteria, such as *Arthrobacter histidinolovorans*, *Actinomyces hordeovulneris*, and *Tsukamurella inchonensis* were also isolated.

Data obtained on root samples during the addition of Triton X-100 (30 mg/L) indicated modifications in the microbial flora. The species, *Flavobacterium multivorum* and *Aeromonas hydrophila*, were favorably selected and were the most numerous microorganisms during the surfactant dosage. Moreover, these strains grew well in TYT, thus indicating their resistance to Triton X-100. In fact, *Flavobacterium multivorum* and *Aeromonas hydrophila* were always found during Triton X-100 dosing. Their presence decreased from the inlet of the basin towards the outlet. This phenomenon could probably be linked to the decrease in the OPEO oligomers with EO units greater than 3. These compounds are easily metabolized by bacterial activity (Allen et al., 1999; Van Ginkel, 1996). *Aquaspirillum dispar* that was not found in absence of Triton X-100 dosage appeared in considerable concentrations during the dosage, especially at distances of 80 and 120 cm from the inlet. This finding suggests that this microorganism is resistant to the presence of OP and OPEOs with a number of EO units less than 3, which represents more than 90% of the catabolic products of Triton X-100. Two further genera were identified at 300-mg/L dosage of Triton X-100: *Achromobacter* and *Photorabdus*. The *Pseudomonas* (*fluorescens, biotypes G, D, C*; *mephitica*; and *synxantha*) seemed to resist well to the surfactant. Its bacterial load was high in the TYT plates during the Triton X-100 dosage at the two different concentrations. The bacteria were often isolated by the TYT plates.

At the end of the surfactant dosage, the bacterial flora seemed more varied when compared with the flora present at the start of the study. Several gram-positive bacteria, such as *Bacillus* spp. and

Table 6—Bacterial diversity* in root samples in relation to the absence or presence of Triton X-100 in the SSF-h pilot-scale reed bed.

Genus of microorganisms	Absence of Triton X-100	Presence of Triton X-100
Sphingomonas	+++	
Brevundimonas	+	
Actinomyces	+	
Tsukamurella	+	
Arcanobacterium	+	
Sphingobacterium	+	
Erwinia	+	
Morganella	+	
Photorabdus		++
Rahnella		+
Achromobacter		++
Aquaspirillum		+++
Enterobacter		+
Flavobacterium		+++
Escherichia		+
Empedobacter		+
Pseudomonas	++	+++
Agrobacterium	++	+
Janthinobacterium	+	+
Aeromonas	+	+++
Arthrobacter	+	+

* The number of the signs + is related to the presence of the bacterial genus (+ = 10 to $\leq 10^2$; ++ = $\geq 10^2 - \leq 10^4$, +++= $\geq 10^4$).

Arcanobacterium haemolyticum, appeared again. *Sphingomonas macrogoltabidus* and *Brevundimonas vesicularis* were found again. These strains were most probably sensitive to the toxic action of the surfactant. In fact, for *Sphingomonas paucimobilis*, a 30-mg/L dosage of Triton X-100 has been shown to reduce bacteria culturing by 100% in 24 hours (Willunsen and Karlson, 1998).

Pseudomonas genus was also present and was represented, for the most part, by the species *maltophilia*. Strains belonging to the family *Enterobacteriaceae* were present in both absence and presence of Triton X-100, even though they were found in smaller quantities in absence of the surfactant. *Erwinia chrysanthemi* and *Morganella morganii* were found after the end of Triton X-100 dosing.

Pseudomonas synxantha, *mephitica*, and *fluorescens* were strongly present during surfactant dosing, suggesting that such microorganisms could probably degrade Triton X-100. This is in agreement with the literature; in fact, a strain of *Pseudomonas* that is capable of demolishing polyethoxylate nonylphenol into a diethoxylate has been isolated (Allen et al., 1999; Maki et al., 1994).

Before the starting of Triton X-100 dosing, the bacteria isolated from the gravel samples did not belong to species different from those found in the roots. During the dosage, significant modifications occurred in the microbial flora collected on the roots, while less evident changes were observed in the gravel. This could be a result of the prevalence, in the gravel samples, of microorganisms that are characteristic of the wastewater.

Conclusions

This study showed that SSF-h constructed wetlands are effective in the treatment of Triton X-100, in which oligomers with a number of EO units greater than 5 completely disappeared, both at

30-and 300-mg/Ldosages, owing to the progressive shortening of the EO chain.

The postdosage study pointed out that sorption phenomena of OP and OPEOs with 1 and 2 EO units occurred extensively.

A mass balance approach verified that OP and OPEOs flowed out of the plant during dosage, and postdosage accounted for 40% of the dosed surfactant.

From the comparison between theoretical and experimental effluent COD data, it can be deduced that approximately 50% of the dosed surfactant gave rise to metabolites having very low COD and/ or was ultimately biodegraded. In this regard, it should be noted that ultimate biodegradation half-lives of OPEOs ranging from 1 to 4 weeks were recently measured (Staples et al., 2001) using acclimated treatment plant sludge. Such results are in agreement with the high HRT adopted in this study; in addition, the residence time of metabolites trapped in the bed can be as high as a few months.

Results of the microbiological investigations supported these findings. A microbial load peak was observed for bacteria adhering to the roots in association with the 300-mg/L dosage. Such a peak could be the result of a positive selection of microorganisms resistant to the surfactant. Strains of the *Aeromonas*, *Flavobacterium*, and *Aquaspirillum* genera, which were not found in absence of Triton X-100, appeared and were dominant during surfactant dosage. *Pseudomonas* genus, which was elsewhere found to be effective in the degradation of Triton X-100, increased their concentration during the dosage of the non-ionic surfactant. The appearance of new species and the increased presence of others should be linked to their capacity to adapt to the presence of the surfactant and suggests that these microorganisms are able to degrade Triton X-100.

More aerobic microorganisms were found adhering to the roots than associated with the substrate gravel, showing that roots of *Phragmites australis* are of great importance for the growth of the aerobic biomass potentially involved in the degradation of organic chemicals.

Acknowledgments

Credits. This study was partly carried out by the financial support of Ente Cassa di Risparmio di Firenze, Florence, Italy.

Authors. Cristiana Sacco and Emilia Tiscione are researchers at the Department of Public Health at the University of Florence, Italy. At the time of this study, Anna Maria Pizzo and Luca Messeri were students at the University of Florence, in Biology and Chemistry, respectively. Daniela Burrini is a researcher at the Water Supply Company of Florence. Luciano Lepri is a full professor of analytical chemistry and Massimo Del Bubba is a researcher at the Department of Chemistry at the University of Florence. Correspondence should be addressed to Massimo Del Bubba, Department of Chemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy; e-mail: delbubba@unifi.it.

Submitted for publication November 27, 2003; revised manuscript submitted July 17, 2005; accepted for publication August 25, 2005.

The deadline to submit Discussions of this paper is (??).

References

Allen, C. C. R.; Boyd, D. R.; Hempenstall, F.; Larkin, M. J.; Sharma, N. D. (1999) Contrasting Effects of Non-Ionic Surfactant on the Biotransformation of Polycyclic Aromatic Hydrocarbons to Cis-Dihydrodiols by Soil Bacteria. *Appl. Environ. Microbiol.,* **65,** 1335.

Amann, R., Ludwig, W., Schleifer, K. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Micr. Rev.,* **59,** 143.

Biolog Inc. (1999) *Biolog Reference Manual* (MicrologPM System User Guide). Biolog Inc.: Hayward, California.

Blackburn, M. A.; Kirby, S. J.; Waldock, M. J. (1999) Concentrations of Alkylphenol Polyethoxylates Entering UK Estuaries. *Mar. Poll. Bull.,* **38,** 109.

Brix, H. (1998) Denmark. In *Constructed Wetlands for Wastewater Treatment in Europe*, Vymazal, J., Brix, H., Cooper, P. F., Green, M. B., Haberl, R. (Eds.); Backhuys Publisher: Leiden, Netherlands, p 123.

Brix, H.; Schierup, H. H. (1990) Soil Oxygenation in Constructed Reed Beds: The Role of Macrophyte and Soil-Atmosphere Interface Oxygen Transport. In *Constructed Wetlands in Water Pollution Control*, Cooper, P. F., Findlater, B. C. (Eds.); Pergamon Press: Elmsford, New York, p 53.

Chong, S.; Garelick, H.; Revitt, D. M.; Shutes, R. B. E.; Worrall, P.; Brewer, D. (1999) The Microbiology Associated with Glycol Removal in Constructed Wetlands. *Water Sci. Technol.,* **40,** 99.

Davies, T. H.; Cottingham, P. D. (1994) The Use of Constructed Wetlands for Treating Industrial Effluent (Textile Dyes). *Water Sci. Technol.,* **29,** 227.

Del Bubba, M.; Checchini, L.; Pifferi, C.; Zanieri, L.; Lepri, L. (2004) Olive Mill Wastewater Treatment by a Pilot-Scale Subsurface Horizontal Flow (SSF-h) Constructed Wetland. *Ann. Chim. (Rome),* **94,** 875.

Desideri, P.; Lepri, L.; Canovaro, M.; Checchini, L. (1988) Recovery, Identification and Determination of Organic Compounds in Marine Sediments. In *Chemistry for Protection of the Environment,* Pawlowski, L., Mentasti, E., Lacy, W. J., Sarzanini, C. (Eds.); Elsevier: Amsterdam, p 317.

Desideri, P.; Lepri, L.; Udisti, R.; Checchini, L.; Del Bubba, M.; Cini, R.; Stortini, A. M. (1998) Analysis of Organic Compounds in Antarctic Snow and their Origin. *Int. J. Environ. Anal. Chem.,* **71,** 331.

Di Cello, F., Fani, R. (1996) A molecular strategy for the study of natural bacterial communities by PCR-based techniques. *Minerva Biotecnologica,* **8,** 126.

Di Corcia, A.; Costantino, A.; Crescenzi, C.; Marinoni, E.; Samperi, R. (1998) Characterization of Recalcitrant Intermediates from Biotransformation of the Branched Alkyl Side Chain of Nonylphenol Ethoxylate Surfactants. *Environ. Sci. Technol.,* **32,** 2401.

Di Corcia, A.; Samperi, R. (1994) Monitoring Aromatic Surfactants and their Biodegradation Intermediates in Raw and Treated Sewages by Solid-Phase Extraction and Liquid Chromatography. *Environ. Sci. Technol.,* **28,** 850.

Duineveld, B., Rosado, A., Dirk van Elsas, J., Johannes Van Veen, A. (1998) Analysis of the dynamics of bacterial communities in rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. *Appl. Environ. Microbiol.,* **64,** 4950.

Ejlertsson, J.; Nilsson, M. L.; Kylin, H.; Bergman, A.; Karlson, L.; Oquist, M.; Svensson, B. H. (1999) Anaerobic Degradation of Nonylphenol Mono- and Diethoxylates in Digestor Sludge, Landfilled Municipal Solid Waste and Landfilled Sludge. *Environ. Sci. Technol.,* **33,** 301.

Esteve-Nunez, A.; Caballero, A.; Ramos, J. L. (2001) Biological Degradation of 2,4,6-Trinitrotoluene. *Microbiol. Mol. Biol. Rev.,* **65,** 335.

Fantroussi, S.; Verschuere, L.; Verstraete, W.; Top, E. M. (1999) Effect of Phenylurea Herbicides on Soil Microbial Communities Estimate by Analysis of 16S rRNA Gene Fingerprints and Community-Level Physiological Profiles. *Appl. Environ. Microbiol.,* **65,** 982.

Field, J. A.; Reed, R. L. (1996) Nonylphenol Polyethoxy Carboxylate Metabolites of Nonionic Surfactants in U.S. Paper Mill Effluents, Municipal Sewage Treatment Plant Effluents and River Waters. *Environ. Sci. Technol.,* **30,** 3544.

Fujii, K.; Urano, N.; Ushio, H.; Satomi, M.; Iida, H.; Ushio-Sata, N.; Rimura, S. (2000) Profile of a Nonylphenol-Degrading Microflora and its Potential for Bioremedial Applications. *J. Biochem.,* **128,** 909.

Giger, W.; Brunner, P. H.; Schaffner, C. (1984) 4-Nonylphenol in Sewage Sludge: Accumulation of Toxic Metabolites from Nonionic Surfactants. *Science,* **225,** 623.

Hatano, K.; Trettin, C. C.; House, C. H.; Wollum, A. G. (1993) Microbial Population and Decomposition Activity in Three Subsurface Flow

Constructed Wetlands. In *Wastewater Treatment in Constructed Wetlands: System Design, Removal Process and Treatment Performance,* Moschiri, G. A. (Ed.); Lewis Publishers: Boca Raton, Florida, p 541.

Heinaru, E.; Truu, J.; Stottmeister, U.; Heinaru, A. (2000) Three Types of Phenol- and P-Cresol- Degrading Bacteria Isolated from River Water Continuously Polluted with Phenolic Compounds. *FEMS Microbiol. Ecol.,* **31,** 195.

IRSA-CNR (1981) Chemical Oxygen Demand, Method E-007 (In Italian); Institute of Water Research-Centre of National Research (IRSA): Rome, Italy.

Johnsen, K.; Andersen, S.; Jacobsen, C. S. (1996) Phenotypic and Genotypic Characterization of Phenanthrene-Degrading Fluorescent *Pseudomonas* Biovars. *Appl. Environ. Microbiol.,* **62,** 3818.

Kern, J.; Idler, C. (1999) Treatment of Domestic and Agricultural Wastewater by Reed Bed Systems. *Ecol. Eng.,* **12,** 13.

Knight, R. L.; Kadlec, R. H.; Ohlendorf, H. M. (1999) The Use of Treatment Wetlands for Petroleum Industry Effluents. *Environ. Sci. Technol.,* **33,** 973.

Komancova, M.; Jurcova, I.; Kochankova, L.; Burkhard, J. (2003) Metabolic Pathways of Polychlorinated Biphenyls Degradation by Pseudomonas sp. 2., *Chemosphere,* **50,** 537.

Kowalik, P.; Mierzejewski, M.; Obarska-Pempkowiak, H.; Toczylowska, I. (1995) *Constructed Wetlands for Wastewater Treatment from Small Communities.* Politecchnika Gdanska: Poland.

Lepri, L.; Desideri, P.; Del Bubba, M. (1997) Analysis of Organic Pollutants in a Wastewater Treatment Plant. *Ann. Chim. (Rome),* **87,** 317.

Machate, T.; Noll, H.; Kettrupp, A. (1996) Removal of Fluoranthene and Phenanthrene in a Constructed Wetland. *Proceedings of the 5th International Conference on Wetland Systems for Water Pollution Control*, Vienna, Austria, Sept. 15–19; International Water: London, United Kingdom, p 20.

Maki, H.; Masuda, N.; Fujiwara, Y.; Ike, M.; Fujita, M. (1994) Degradation of Alkylphenol Ethoxylates by Pseudomonas sp. Strain TR01. *Appl. Environ. Microbiol.,* **60,** 2665.

Perei, K.; Rakhely, G.; Kiss, I.; Polyak, B.; Kovacs, K. L. (2001) Biodegradation of Sulfanilic Acid by Pseudomonas Paucimobilis. *Appl. Microbiol. Biotechnol.,* **55,** 101.

Picard, C.; Di Cello, F.; Ventura, M.; Fani, R.; Guckert, A. (2000) Frequency and Biodiversity of 2,4-Diacetylphloroglucinol-Producing Bacteria Isolated from the Maize Rhizosphere at Different Stages of Plant Growth. *Appl. Environ. Microbiol.,* **66,** 948.

Reed, S. C.; Brown, D. (1995) Subsurface Flow Wetlands—A Performance Evaluation. *Water Environ. Res.,* **67,** 244.

Soares, A.; Guieysse, B.; Delgado, O.; Mattiasson, B. (2003) Aerobic Degradation of Nonylphenol by Cold-Adapted Bacteria. *Biotechnol. Lett.,* **25,** 731.

Soto, A. M.; Justicia, H.; Wray, J. W.; Sonnenschein, C. (1991) p-Nonylphenol: An Estrogenic Xenobiotic Released from ‘‘Modified’’ Polystyrene. *Environ. Health Persp.,* **92,** 167.

Staples, C. A.; Naylor, C. G.; Williams, J. B.; Gledhill, W. E. (2001) Ultimate Biodegradation of Alkylphenol Ethoxylate Surfactants and their Biodegradation Intermediates. *Environ Toxicol Chem.,* **20,** 2450.

Stellmack, P. L.; Gray, M. R.; Pickard, M. A. (1998) Bacterial Adhesion to Soil Contaminants in Presence of Surfactants. *Appl. Environ. Microbiol.,* **65,** 163.

Swisher, R. D. (1987) *Surfactant Biodegradation.* Marcel Dekker Inc.: New York.

Tanghe, T.; Devriese, G.; Verstraete, W. (1999) Nonyphenol and Estrogenic Activity in Aquatic Environmental Samples. *J. Environ. Qual.,* **28,** 702.

Tanghe, T.; Dhooge, W.; Verstraete, W. (2000) Formation of the Metabolic Intermediate 2,4,4-Trimethyl-2-Pentanol during Incubation of a Sphingomonas sp. Strain with the Xeno-Estrogenic Octylphenol (2000) *Biodegradation,* **11,** 11.

Van Ginkel, C. G. (1996) Complete Degradation of Xenobiotic Surfactants by Consortia of Aerobic Microorganisms. *Biodegradation,* **7,** 151.

van Herwijnen, R.; Wattiau, P.; Bastiaens, L.; Daal, L.; Jonker, L.; Springael D.; Govers, H. A.; Parsons, J. R. (2003) Elucidation of the Metabolic Pathway of Fluorene and Cometabolic Pathways of Phenanthrene, Anthracene and Dibenzothiophene by Sphingomonas sp. LB126. *Res. Microbiol.,* **154,** 199.

Vymazal, J. (1999) Removal of BOD_5 in Constructed Wetlands with Horizontal Sub-Surface Flow: Czech Experience. *Water Sci. Technol.,* **40,** 133.

Willunsen, P. A.; Karlson, U. (1998) Effect of Calcium on the Surfactant Tolerance of a Fluoranthene Degrading Bacterium. *Biodegradation,* **9,** 369.

Ying, G. G.; Williams, B.; Kookana, R. (2002) Environmental Fate of Alkylphenols and Alkylphenol Ethoxylates—A Review. *Environ. Int.,* **28,** 215.

Zanette, M.; Marcomini, A.; Marchiori, E.; Samperi, R. (1996) High-Performance Liquid Chromatographic-Fluorescence Determination of Aliphatic Alcohol Polyethoxylates and Polyethylene Glycols in Aqueous Samples. *J. Chromatogr. A,* **756,** 159.

Zhang, C.; Valsaraj, K. T.; Constant, W. D.; Roy, D. (1999) Aerobic Biodegradation Kinetics of Four Anionic and Nonionic Surfactants at Sub- and Supra-Critical Micelle Concentrations (CMCs). *Water Res.,* **33,** 115–124.