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LAS degradability by marine biofilms derived from seawater in Spain and Sweden

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ABSTRACT

Marine biofilms were established on glass beads with or without deliberate pre-exposure to LAS (20 μg/L) in Spain (Cadiz) and Sweden (Kristineberg). The ability of each community to mineralize LAS (100 μg/L) was then assessed in biometers at four experimental temperatures (between 6 and 21 °C). Genetic diversity and biomass of the biofilms were assessed by genetic fingerprinting (DGGE) and direct bacterial counts. With biofilms from Sweden, where LAS was not detected in seawater ($n=3$), deliberate pre-exposure to LAS resulted in lower genetic diversity and higher mineralization rate constant; however, with biofilms from Spain, where 6.4 ± 3.9 μgLAS/L ($n=3$) was measured during the colonization, pre-exposure did not affect the bacterial community. Bacterial acclimation therefore appeared to have been induced at environmental concentrations <6 μgLAS/L. Environmental pre-exposure was not a pre-requisite for featuring the full consortia of LAS degraders in the biometers. The mineralization rate was described using an Arrhenius equation at experimental temperatures within the typical annual range; however, they departed from this model below this range.

1. Introduction

Linear Alkylbenzene Sulfonate (LAS) is a synthetic surfactant commonly used in detergents. Some 340 kilotons of this high production volume (HPV) chemical were consumed in Europe in 2005 (HERA, 2007). Growth of LAS usage worldwide is expected to average about 1.7% per year during 2008–2013 (Modler et al., 2009). Because it is removed very efficiently in waste water treatment plants (León et al., 2006), and subsequently in river waters (Takada et al., 1994; McAvoy et al., 2003; Whelan et al., 2007), LAS concentrations in estuarine and coastal waters are typically below 50 μg/L where sewage treatment systems are installed (Matthijs and Stalmans, 1993; Gonzalez-Mazo et al., 1998; Lara-Martín et al., 2008). Higher concentrations, up to 2500 μg/L, have been detected in coastal waters close to untreated discharge outlets (Gonzalez-Mazo et al., 1998). Temara et al. (2001) reported a predicted no-effect-concentration (PNEC) of 31 μgLAS/L for marine pelagic communities.

Marine biodegradation is typically assessed in closed test systems using water, with or without sediment, as an inoculum, according to OECD 306 recommendations (1992). The lifetime of a closed biodegradation unit will end when the degrader community loses its viability. This will eventually occur due to unfavorable growth conditions such as predation (grazing), and depletion of: (1) the volume of the water sample; (2) essential nutrients; or (3) primary carbon substrates (Painter, 1995; Torang and Nyholm, 2005). Therefore, the duration of the lag phase prior to biodegradation must be shorter than the system lifetime to allow detection of the potential activity of the microbial community, and thus to avoid false negative outcomes. To mitigate the problems of poorly reproducible biodegradation lag phases, two approaches have been considered: the use of inocula deliberately pre-exposed to the test compound or the use of biodegradation units with high biomass. Regulatory authorities have generally disliked the use of pre-exposed inocula in screening tests (Torang and Nyholm, 2005). Their concern is the creation of "superbugs" or unnaturally "over-adapted" inocula. On the other hand, using a high biomass in the test units has the advantage of increasing the probability that the bacterial community added gets acclimated to the compound within the test lifetime. In fact, since there can be no immigration into a closed system, system lifetimes are likely to be highly dependent on the initial biomass. The recently developed biofilm approach is based on using biofilms colonized on glass beads. This approach uses a convenient system with a high biomass, that is approximately three orders of magnitude larger than in a similar volume of seawater (Mauffret et al., 2009).

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Using this approach the effects of the pre-exposure and of the bacterial density can thus be studied separately.

In such closed test conditions, the half-life of LAS primary degradation ranges from <1 to 21 days, with most frequent values between 2 and 7 days (Terzic et al., 1992; Vives-Rego et al., 2000; León et al., 2004). In an earlier study, no biodegradation ($<$ 5%) was observed after 21 days in closed test system at 5 °C with an inoculum from a warm region, the South of Spain (Sales et al., 1987). Furthermore, while bacterial pre-exposure to LAS has been reported to enhance the biodegradation but not to modify its extent (Larson and Payne, 1981; León et al., 2004), others authors have observed that pre-exposure of the microbial communities is a pre-requisite for LAS biodegradation in closed test systems (Shimp, 1989; Terzic et al., 1992). In these studies, it is difficult to delineate the effects on the biodegradation outcomes of the lack of pre-exposure when a highly diluted inoculum, as typically found in pristine areas, is tested. There is thus no conclusive position to date on the strength of the bacterial pre-exposure effect on the extent of biodegradation reached at the steady state in closed system. The above illustrates the high variability typically obtained in closed systems (Painter, 1995; Ingerslev et al., 2000).

In the present study, marine biofilms derived from seawater in Spain and Sweden were used to examine LAS biodegradation in two relatively extreme conditions in Europe. In Sweden, most detergents (eco-labeled) do not contain LAS, while in Spain, LAS is commonly used in detergents. Few studies report the degradation of a HPV chemical in an area where it has not been commonly used, such as LAS in Sweden. Our specific objectives are to assess (1) the inherent capability of a microbial community to degrade a new synthetic molecule, and the acclimation process, including the concentration either environmental or experimental (i.e. deliberately imposed) needed to acclimate the community to that specific compound, so called threshold concentration; and (2) the effects of temperature on the biodegradation capability of biofilms derived from colder and warmer regions.

2. Materials and methods

2.1. Chemicals

The cold LAS (Marlon A390) used for acclimation during the colonization had a purity of 89.2%, contained 7% Na_2SO_4, and had an average chain length of 11.6 carbons with the following chain length distribution for C10–LAS, C11–LAS, C12–LAS, C13–LAS, and C14–LAS: 5–10%, 40–45%, 35–40%, 10–15%, and <1%, respectively. It was supplied by the manufacturer Chemische Werke Huls. The radiolabeled LAS isomer [^{14}C]C12-6φ-LAS, i.e. dodecane-6-benzene sulfonate (MW=348 g/mol), spiked in the biometer during the biodegradation monitoring, was uniformly labeled in the benzene ring. It had a specific activity of 17.1 mCi/mmol and a chemical purity >99% as determined by rad-Thin Layer Chromatography (rad-TLC). It was obtained from Procter and Gamble (USA). Uniformly labeled [^{14}C]glucose (Amersham International plc, England) was used as a positive control. It had a specific activity of 273 mCi/mmol and a purity of 98.8%, according to the manufacturer (HPLC on Aminex HPX 87-C column). All chemicals and solvents used for LAS extraction and microbial analyses were of analytical grade.

2.2. Bacterial colonization and biodegradation experiment

The biofilm approach as described in Mauffret et al. (2009) was used to collect the inoculum and to perform the biodegradation assessment. Briefly, in the general approach, acid-washed glass beads (0.4 mm diameter, 6×10^{-3} m^2/g) were placed in 1 L columns connected to an open flow-through system of seawater (15 mL/min). After the colonizations, the colonized beads, together with seawater (10 L) sampled at the colonization site, were immediately transported to the laboratory (1 day) (P&G, Brussels, Belgium) and used in the biodegradation assays after 2 to 6 days of storage at 4 °C for the beads from Sweden and Spain, respectively. Colonized beads (35 mL) and seawater (75 mL) were placed in 250 mL closed biometers and [^{14}C]C12-6φ-LAS was added (100 μg/L). Nutrients were added to each unit (C:N:P 100:10:2 (atom:atom:atom), as NH_4Cl and KH_2PO_4). A Liquid Scintillation Counting vial filled with KOH 1.5 N (1 mL) was placed in the arm of the biometer as a CO_2 trap. During 59 days, radioactivity as parent molecule or metabolites (LASeq), dissolved and volatile CO_2 were measured at regular intervals by Liquid Scintillation.

For the present study, colonizations were run from October 2006 to January 2007 in Fiskebäckskil, Sweden (Kristineberg Marine Research Station, KMRS) and in the Cadiz Bay, Spain (Centro de Investigación y Formación Pesquera y Acuícola, CIFPA). Two columns were connected to the same open flow-through system of seawater at each site. Into the first column, LAS dissolved in MilliQ water (20 μgLAS/L in the column) was continuously pumped to form deliberately pre-exposed (PE) biofilms. MilliQ water was pumped into the second column, at the same rate, to form the control biofilms (not deliberately pre-exposed: NPE). Seawater temperatures were recorded (Fig. 1) and salinity was 35.0 ± 0.5 in the KMRS and 34.4 ± 3.7 in the CIFPA. Seawater (500 mL) was sampled, on a monthly basis, at the inlet of the columns, and formalin 3% (v/v) was added to the samples. LAS in seawater was then measured via HPLC according to Gonzalez-Mazo et al. (1997) ($n=3$ months, $n=3$ replicates/months). During the colonization in Spain, 3 samples of beads (2 mL, ca. 120 beads), taken from the first top 1 mm layer of the columns, were collected on a daily basis during one week, and with a reduced frequency thereafter. Beads with biofilms were fixed with 3% formalin (v/v) and stored at 4 °C until epifluorescence counting ($n=3$). On day 1 and at the end of the colonization period, additional aliquots of beads were sampled from the top 1 mm layer of the columns, and placed in liquid nitrogen and stored at −80 °C until PCR–DGGE analyses ($n=5$).

The biometers were placed in temperature-controlled baths at either 6, 12, 16 °C or at room temperature (21 °C), protected from light and shaken by hand twice a day. Tests were run in duplicate. Additionally, three control biometers were run with biofilms from each place of origin and of each pre-exposure status, at the 4 test temperatures: (1) the abiotic controls were run with [^{14}C]C 12-6φ-LAS (100 μg/L) and weekly addition of $HgCl_2$ (4 mg/L); (2) the controls with autoclaved beads and non-autoclaved seawater were run with [^{14}C]C12-6φ-LAS (100 μg/L); and (3) the positive controls were run with [^{14}C]glucose (15 μg/L). All the units (80 in total) were run for 59 days. After 0, 17, and 59 days, selected samples of beads were collected in the biometers and fixed with 3% formalin

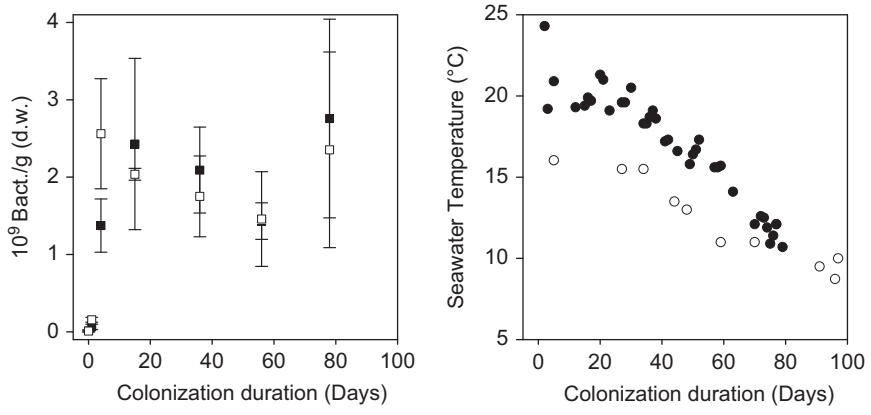

Fig. 1. Bacterial counts in the biofilm pre-exposed (PE, □) and not pre-exposed (NPE, ■) to LAS during the colonization in Spain (left panel) and seawater temperature in Spain (CIFPA, ●) and in Sweden (KMRS, ○) from 1 October 2006 to 18 January 2007 (right panel).

(v/v) until the bacterial counting or stored at −80 °C until genetic diversity assessments.

2.3. Analysis of bacterial characteristics

Bacteria attached to the glass beads were counted by epifluorescence microscopy according to Mauffret et al. (2009).

DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) were performed according to a protocol initially developed for sediment samples (Gillan, 2004) and adapted in the present study to glass bead samples. DNA was extracted by an *in situ* lysis from 0.5 g of glass beads ($n=2$–3). Proteins were then precipitated using phenol–chloroform–isoamyl alcohol, and DNA was isolated using ethanol precipitation. After drying, DNA was dissolved in 50 μL of PCR water. A touchdown PCR amplification procedure was performed using the Red'y'StarMix PCR kit (Eurogentec®) and the bacterial primers GM5F-GC-clamp and 518R (1 μM in the PCR mix). Two microlitre of the DNA extract were added to 48 μL of the PCR mix. PCR was performed using 20 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C down to 55 °C for 45 s, and primer extension at 72 °C for 30 s. Ten additional cycles were carried out at an annealing temperature of 55 °C. For the last extension step, reactions were incubated for 10 min at 72 °C. The 48 PCR products (45 μL/sample) were then loaded in 6 DGGE gels over 3 runs. DGGE was performed with a Bio-Rad DCode system with a denaturing gradient of 25–75% denaturants (100% denaturants: 7 M urea and 40% [v/v] formamide). Electrophoresis was performed at 75 V for 16 h and gels were then stained for 1 h in ethidium bromide and scanned under UV illumination using the Gel Doc 2000.

2.4. Kinetic analysis

The CO_2 production data were fitted to the first-order model

$$y = y_0 + (y_{max} - y_0) * (1 - \exp^{-k*t}) \quad (1)$$

where y, y_0, and y_{max} are the percentage of $^{14}CO_2$ produced at time t, at $t=0$ and at the steady state respectively; k is the first order mineralization rate constant (day^{-1}). Half-life was assessed based on the first order kinetic rate and defined as the time needed to reach 50% mineralization after the lag phase. Lag phase ended once the mineralization started to rise from the baseline.

Temperature effect on the mineralization rates was modeled using the Arrhenius equation in the natural log form

$$\ln(k) = \ln(A) - \frac{E_a}{RT} \quad (2)$$

where k is the first order rate constant in Eq. (1) (day^{-1}); A is a frequency factor (day^{-1}) expressing the empirical dependence of the rate coefficient on temperature; T is the temperature (K); R is the gas constant (8.314 J mol^{-1} K^{-1}); and E_a is the activation energy (J mol^{-1}).

2.5. Genetic diversity analysis

The number of DGGE bands per profile was taken as an indicator of the number of operational taxonomic units in each sample (i.e. the taxon richness S). The relative intensity of each DGGE band was used to calculate the Shannon–Weaver biodiversity index (H)

$$H = -\sum_{i \to n} \left[\frac{n_i}{N} * \log_{10} \frac{n_i}{N}\right] \quad (3)$$

where N is the total band intensity on the profile and n_i is the intensity of the band i.

2.6. Statistics

All the statistical analyses and regressions were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Extra sum-of-square F tests were run with the bacterial colonization, the CO_2 production and the Arrhenius plots to compare the colonization rates, mineralization rates and the activation energies, respectively. One-way ANOVA with Tukey's Multiple Comparison post-hoc tests were performed with the bacterial counts, the taxon richnesses (S) or the Shannon–Weaver biodiversity indexes (H), to compare the biomass and the genetic diversity in the different bacterial communities after the colonization and during the biometer experiment. Significance was inferred at $p<0.05$.

3. Results and discussion

3.1. Biofilm colonization and effect of transport and storage on the bacterial number

Colonization kinetics were monitored in Spain (Fig. 1). Initial bacterial density on the glass beads was 10^6 bact./g of beads (d.w.), possibly due to contact with the air after the acid-washing of the beads. After 4 days in the colonization system, the bacterial growth reached an apparent steady state of $1–3 \times 10^9$ bact./g (d.w.). Pre-exposure to LAS did not affect the colonization rate ($p>0.05$). This suggested that an active biofilm suitable for further biodegradation assessment could be obtained with the present colonization set-up in less than one week. This would greatly reduce the maintenance cost and effort of setting-up the colonization system. This is especially interesting when colonization is performed in a host institution. However, our data do not

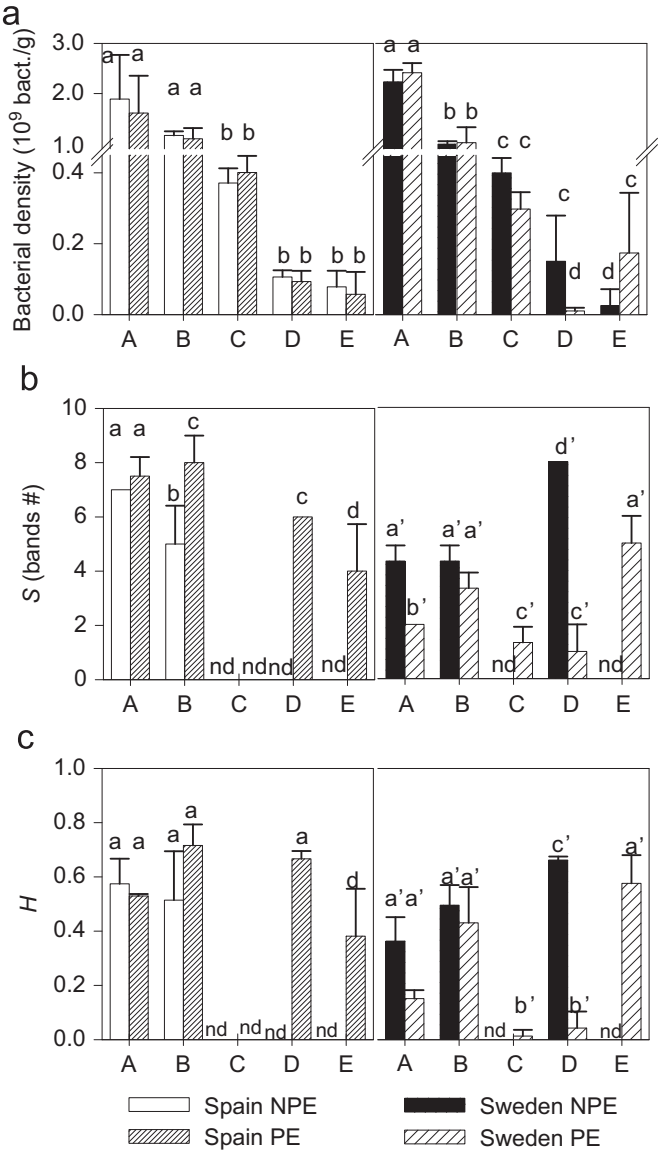

Fig. 2. Biofilm bacterial counts (a), taxon richness (S) (b), and Shannon Weaver biodiversity index (H) (c), all values at the end of the colonization period (A); after the transport and at the start of the biodegradation study (B); after 17 days at 21 °C (C), after 59 days at 21 °C (D) and after 59 days at 6 °C (E) in the biometer units. Lowercase letters indicate homogeneous subgroups after Tukey's test, $p<0.05$ ($n=3$).

indicate whether pre-exposure to LAS for one week results in an acclimated biofilm. The acclimation phase duration can be of few hours up to several months, depending on the chemical, its concentration and the bacterial communities (Alexander, 2001).

At the end of the colonization, final bacterial biomasses on the beads were similar in Sweden and in Spain, with 1.6 and 2.4×10^9 bact./g d.w., respectively (Fig. 2(a) A). Bacterial biomass on the colonized beads was also similar to biomass obtained in previous colonizations performed at the CIFPA (Spain) (Mauffret et al., 2009). After transport and storage, all bacterial biomasses declined to $1.0–1.2 \times 10^9$ bact./g d.w. (Fig. 2(a) B). Consequently, initial bacterial content added in all the tested biometer units ranged from 5.1 to 5.9×10^{10} bact./unit, as recommended in Mauffret et al. (2009).

3.2. Effect of transport and storage on the bacterial diversity

Genetic diversity of the biofilms from Spain was higher than those from Sweden ($p > 0.05$ before transport, $p < 0.05$ after transport). Transport and storage affected the relative genetic diversity found between the different treatments, although they did not significantly affect the genetic diversity of each biofilm individually (Fig. 2(b), (c) A, B). Indeed, genetic diversity of the NPE and PE biofilms from Spain, initially similar ($p > 0.05$) were different after transport ($p < 0.001$). Genetic diversity of the NPE and PE biofilms from Sweden, initially different ($p < 0.05$), were similar after transport ($p > 0.05$). No statistical relationship between the storage time (3 or 7 days) and the changes in diversity was observed in the present study. After up to 7 days spent in transport and storage, the biofilms were still metabolically active and their relative mineralization capabilities were better related to the relative biofilm genetic diversity measured before transport (Figs. 2 and 3). In contrast, Nyholm and Kristensen (1987) reported that biodegradation capability of suspended bacteria in seawater was strongly affected during storage and therefore recommended that seawater should be used within few hours after collection for biodegradation studies (Nyholm and Kristensen, 1987; León et al., 2004). Further studies are required to identify the parameters affecting the diversity and the density in the biofilms during transport and storage. These could include the length of the storage period and/or the absence of seawater flow, and thus the flow of new microbes and chemical elements. Such information would be required to refine guidance on transport conditions for future assays.

3.3. LAS recovery in the biodegradation units

The recovery of the added radioactive compound, in the present study, includes the fraction recovered as parent compound LAS, as dissolved and volatile $^{14}CO_2$, and as dissolved LASeq in the seawater (Table 1). This recovery ranged from 68% to 102% (average: $82 \pm 9\%$) and increased with the temperature (Table 1). In a similar system, Mauffret et al. (2009) performed additional washings at the end of the test period, i.e. acidification and methanol washings of the unit, to recover the ^{14}C-LASeq sorbed onto the glassware, precipitated and incorporated into the biomass. After these washings, these authors recovered 14–20% of the added radioactive compound. If we assume this additional recovery, mean mass balance in the present study would range from 96% to 102%. Mass balance was enhanced by the temperature possibly due to an increase of LAS solubility at higher temperature or to a higher LAS desorption when mineralization increased, to maintain the LAS distribution equilibrium in the units.

In the abiotic controls, treated with $HgCl_2$, less than 5% CO_2 was produced (Fig. 3(a)) indicating that little abiotic degradation

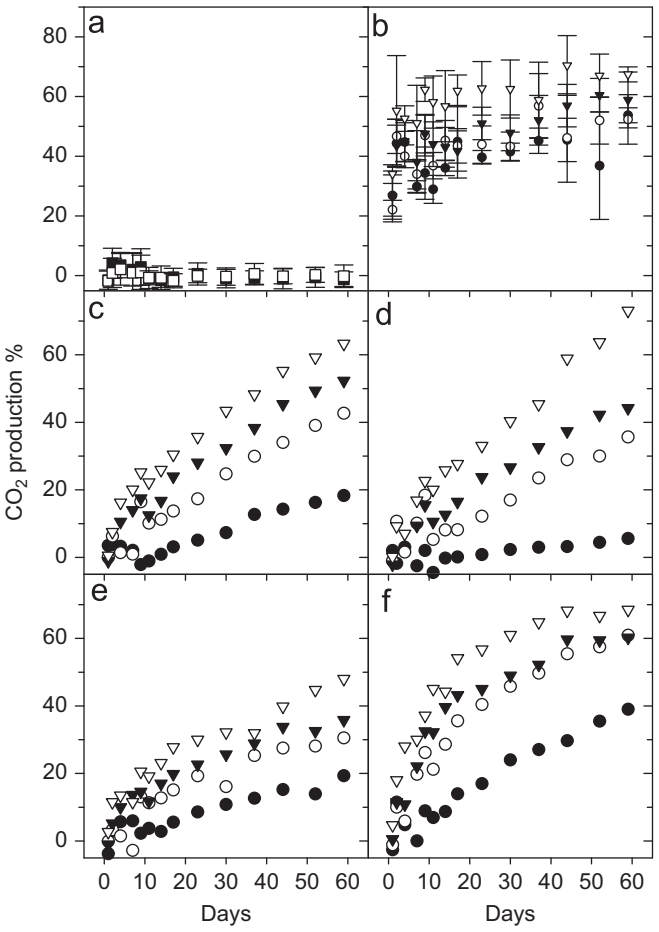

Fig. 3. LAS mineralization in the abiotic (■), autoclaved (□) (a) and glucose (b) control units; and the biotic units at 6 °C (●), 12 °C (○), 16 °C (▼) and 21 °C (▽) (c–f). For the abiotic and autoclaved control units, data for the NPE and PE beads colonized in Spain and in Sweden, at 6, 12, 16 and 21 °C were pooled ($n=16$ units) and average values and standard deviations (SD) are shown. For the glucose control unit, data for the NPE and PE beads colonized in Spain and in Sweden were pooled ($n=4$ units) and average values and SD are shown. For the biotic units, average value per unit is shown ($n=2$ units).

occurred in the test system. Likewise in the controls with autoclaved beads, less than 5% CO_2 was measured after 59 days (Fig. 3(a)), indicating that the high surface area of the glass beads did not bring together the chemical and the suspended bacteria in the biometer, which would have enhanced LAS biodegradation. Furthermore, the mineralization capability of the suspended bacteria appeared limited, as observed previously in biometers with seawater only (10^6 bact./mL) (Mauffret et al., 2009). Biofilms (ca. 10^9 bact./mL beads, Table 1) were thus required in order to effect significant mineralization in the present test system under each temperature and each pre-exposure condition. The importance of biofilm in LAS biodegradation has also been observed in the field (Takada et al., 1994; Boeije et al., 2000).

In the glucose control, 54–67% of the added glucose was converted to CO_2 (Fig. 3(b)) indicating that the colonized biofilms were metabolically active in all the test conditions. Glucose mineralization significantly increased with temperature but was independent of the origin of the bacteria and their pre-exposure to LAS.

LAS mineralization measured at the end of the experiment ranged from 10% to 73% (Fig. 3(c)–(f)). Since the initial bacterial content was similar in all the biometers (ca. 5×10^{10} bact./unit), the differences in biodegradation capability were thus not dependent on the initial biomass. Separately, the genetic diversity of the biofilms was significantly different in Spain and Sweden, which

Table 1
Kinetic parameters describing LAS and glucose mineralization in the biometers.

Origin	Pre-exposure	Temp. (°C)	Total recovery (%)	Final $ThCO_2$ (%)	Lag phase (days)	First-order kinetic			
						r^2	k (10^{-3} d^{-1})	Half-life (days)	
								$t_{1/2}$	(C−I 95%)
LAS									
Sp	PE	6	76.5 ± 1.1	5.6	> 59	n.d.			
Sp	PE	12	81.7 ± 4.5	35.7	11	0.975	6.9 ± 0.2	100	(93–108)
Sp	PE	16	88.4 ± 5.2	44.2	7	0.949	10.6 ± 0.5	66	(59–72)
Sp	PE	21	90.3 ± 11.1	73.1	1	0.974	19.4 ± 0.7	36	(33–39)
Sp	NPE	6	72.9 ± 2.5	18.3	17	0.821	3.1 ± 0.3	221	(184–278)
Sp	NPE	12	84.4 ± 0.8	42.7	9	0.955	9.4 ± 0.4	74	(67–82)
Sp	NPE	16	88.9 ± 3.5	52.3	4	0.962	13.6 ± 0.5	51	(47–56)
Sp	NPE	21	101.1 ± 2.9	63.3	1	0.938	19.0 ± 1.0	36	(33–41)
Sw	PE	6	68.3 ± 0.5	39.0	9	0.975	8.3 ± 0.3	83	(78–90)
Sw	PE	12	75.4 ± 0.4	60.9	4	0.922	19.6 ± 1.1	35	(31–40)
Sw	PE	16	73.8 ± 0.3	60.2	1	0.760	23.0 ± 2.2	30	(25–38)
Sw	PE	21	89.9 ± 5.5	68.5	< 1	0.694	34.0 ± 3.7	20	(17–27)
Sw	NPE	6	71.5 ± 0.7	19.3	14	0.951	3.5 ± 0.1	200	(182–220)
Sw	NPE	12	79.2 ± 1.3	30.5	9	0.920	7.1 ± 0.4	98	(87–111)
Sw	NPE	16	79.5 ± 2.6	35.8	2	0.816	9.2 ± 0.7	75	(65–89)
Sw	NPE	21	90.2 ± 1.2	48.0	< 1	0.839	13.0 ± 0.9	53	(46–63)
Glucose									
Sp/Sw	PE/NPE	6	87.3 ± 6.3	53.7	< 1			n.d.	
Sp/Sw	PE/NPE	12	92.6 ± 9.6	52.3	< 1			n.d.	
Sp/Sw	PE/NPE	16	93.6 ± 8.4	58.9	< 1			n.d.	
Sp/Sw	PE/NPE	21	109.7 ± 12.9	67.4	< 1			n.d.	

n.d.: not determined due to low correlation with first order kinetic. Sp: Spain. Sw: Sweden. PE: pre-exposed. NPE: non pre-exposed.

was expected given the difference in the environmental conditions of the two areas.

3.4. Effect of temperature variation on the bacterial community

Higher temperature enhanced the LAS mineralization kinetics and reduced the mineralization half-life from > 59 to 20 days (Table 1). At 21 °C, 63–73% of the LAS was mineralized with the biofilms from Spain (NPE and PE) and from Sweden (PE). At low temperature (6 °C), LAS mineralization by the biofilms from Spain was very limited (Fig. 3(a)). This is in line with previous observations in the laboratory (Sales et al., 1987; León et al., 2004, 2006) and field studies (Takada and Ogura, 1992). In contrast, mineralization by the biofilm from Sweden reached 39% at 6 °C. Such a low temperature was not recorded during the colonization run in either Spain or Sweden (Fig. 1). Nevertheless, the seawater temperature ranged from 8 to 25 °C over the year preceding the colonization (2005–2006) at the CIFPA (Spain) (Naranjo J., CIFPA, personal communication), and from 1 to 21 °C at the Fiskebäckskil Station (Sweden) (Eriksson M., KMRS, personal communication). It is likely that the bacterial communities are metabolically active at temperature ranges to which they are typically exposed. Since the colonization was performed in October–January, the biofilms had probably not been exposed to such low temperature (6 °C) over the preceding 6 months, suggesting that their ability to work under low temperature conditions is maintained within the community over ≥ 6 months. Therefore, the temperature effect on biodegradation is dependent on bacteria origin, and outcomes cannot be extrapolated from one site to another site with no consideration of the annual temperature range. Similarly, in estuarine waters (Croatia), bacteria from the surface water were able to degrade LAS at 14 and 23 °C, whereas in deeper waters (6 m), where the thermocline might reduce temperature variations, bacteria were only active at 23 °C (Terzic et al., 1992).

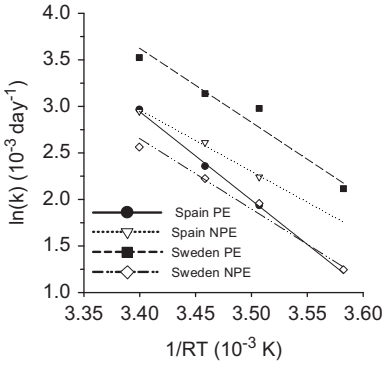

Fig. 4. Effect of temperature variation within the typical annual range on LAS mineralization by marine biofilms. Spain PE: $\ln(k1) = -80{,}060*1/RT + 35$; $r^2 = 0.998$, Spain NPE: $\ln(k1) = -82{,}440*1/RT + 37$; $r^2 = 0.947$, Sweden PE: $\ln(k1) = -62{,}310*1/RT + 29$; $r^2 = 0.952$, Sweden NPE: $\ln(k1) = -59{,}520*1/RT + 27$; $r^2 = 0.980$.

Within the respective annual water temperature ranges, 8–25 °C in Cadiz (Spain) and 1–21 °C in Fiskebäckskil (Sweden), the LAS mineralization rate doubled every 6.6 ± 1.1 °C and followed a classic Arrhenius relationship ($r^2 \geq 0.95$) (Fig. 4), independently of the biofilm origin or pre-exposure status. Activation energy was also independent of the biofilm samples ($p > 0.05$) and ranged from 59 to 82 kJ mol^{-1}. This value was in the typical range for chemical reactions (42–188 kJ mol^{-1}) (Finar, 1975). When the point at 6 °C in Spain is added, the regression quality decreases ($r^2 = 0.90$) and the activation energy approximately doubles: $E_a = 149$ kJ mol^{-1}.

3.5. Effect of acclimation on the bacterial communities

In the tests conducted with beads colonized in Spain, where 6.4 ± 3.9 μgLAS/L ($n = 3$ months, $n = 3$ replicates/month) was measured in seawater, pre-exposure to LAS had no significant effect either on the genetic diversity before transport (Fig. 2) or on

the biodegradation capability (Fig. 3(c), (d)). It is thus likely that the low level of LAS detected in the field (ca. 6 μg/L) resulted in the bacterial community being acclimated to LAS. Conversely in the tests with beads colonized in Sweden, where no LAS was detected in seawater (<1 μg/L) ($n=3$ months, $n=3$ replicates/month), mineralization ability increased significantly and genetic diversity decreased significantly following pre-exposure to LAS. The CO_2 production rate by PE biofilms was about 2.5 faster than by the NPE biofilms ($p<0.05$, Fig. 3(e), (f)). It is likely that the bacterial community has been modified during the pre-exposure to form a community with a higher enhanced ability to use LAS as a source of carbon. Although we cannot completely exclude toxicity from the LAS added in the column (20 μgLAS/L), to specific non-degrading strains in the community, the increased capacity to degrade LAS might originate from a selection pressure for LAS degrading capabilities. According to the Pollution-Induced Community Tolerance (PICT) concept (Blanck et al., 1988), communities become tolerant to toxicants through elimination of sensitive strains and the concomitant selection for tolerant strains. Separately, as seen in biodegradation tests, direct toxic effects on specific LAS degraders occur at concentrations that are several orders of magnitude higher (10 mgLAS/L, (Swisher, 1987)). Fingerprinting techniques, such as DGGE, reveal the appearance or extinction of operational taxonomic units, allowing the detection of effects of a specific compound at the entire community level. In contrast, the effects observed in typical biodegradation tests can only be related to the active degraders.

Since the PE and NPE communities from Spain showed the same mineralization capacity, the acclimation threshold is probably below the background concentration in LAS, i.e. ≤ 6 μg/L. Moreover, background concentrations in coastal waters near areas where typically LAS is used in detergents, such as Spain (Gonzalez-Mazo et al., 1998; Lara-Martín et al., 2008), Italy (Marcomini et al., 2000), Belgium (Matthijs and Stalmans, 1993) and Japan (Takada and Ogura, 1992) are in the <1–50 μg/L range. This would suggest that most bacterial communities in the coastal waters of LAS-consuming countries are acclimated to LAS. According to Wiggins et al. (1987), the acclimation phase is longer when the chemical concentration is lower. It could therefore be extrapolated that long-term exposure to low LAS concentration could lead to effective acclimation in the field. The present study is based on environmental concentrations derived from 3 sampling periods in two locations. Although they are representative of the field variability for the present study, further experiments applying the present approach are recommended to determine more precisely the concentration range threshold for acclimation.

Despite the fact that LAS was not detected in seawater in Sweden, the NPE biofilms showed approximately 50% LAS mineralization. Bacterial pre-exposure did not seem to be a prerequisite for featuring the full LAS degrading consortium in a biodegradation test with approximately 5×10^{10} bact./unit. Pre-exposure in Sweden, however, enhanced the biodegradation kinetics and reduced the lag phase (Table 1), as observed in several previous studies (Larson and Payne, 1981; Shimp, 1989; León et al., 2004). On the other hand, LAS degradation was not detected in the unit with seawater and autoclaved beads (10^6 bact./mL), suggesting that the bacterial density was too low to allow the community to acclimate to LAS within the timeframe of the experiment. Similarly, Shimp (1989) observed up to 42% mineralization of ^{14}C13-LAS in closed units with estuarine exposed effluent with 2340 Most Probable Number (MPN)/mL, and <10% in not pre-exposed estuarine waters with approximately 3 orders of magnitude less bacteria (5 MPN/mL). The present data suggests that, when an adequate bacterial density is added to a closed test system, pre-exposure is not necessary for LAS biodegradation and LAS degradability seems to be a ubiquitous capability of marine microbial communities.

3.6. Changes in the bacterial community during the biodegradation tests

Bacterial density decreased significantly after 17 and 59 days in the biometer units in which LAS mineralization was not observed, but also in biometer units in which LAS was still mineralized (Fig. 3), independently of bacteria origin and pre-exposure (Fig. 2(a)). This suggests that stringent conditions occurred in the biometers, possibly due to the limited carbon source compared to the initial biomass i.e. low food:biomass ratio. The bacteria used LAS as a carbon source for respiration, which is necessary to maintain their viability, but the amount of added LAS was probably not sufficient to sustain their growth over the period of the study. Assuming that 10^{-12} g of substrate is required to form one bacterial cell, which is often approximated in the field (Alexander, 2001), the 7.5×10^{-6} g LAS added to each biometer would produce 7.5×10^{6} bacteria, which would indeed not sustain bacterial growth in the system.

Genetic diversity was also reduced during the biodegradation tests (Fig. 2). This loss could be distinguished in two cases, and linked to the number of bacteria and the mineralization of LAS. The first case is the relatively serious loss of diversity in the PE biofilm from Sweden at 21 °C, of 3- and 10-times, respectively, for richness (S) and biodiversity index (H) ($p<0.05$) (Fig. 2D Sweden). In addition, the biomass decreased by 100-times after 59 days in these units (Fig. 2(a) D). Losses in diversity and biomass were probably due to depletion of the carbon source, since an apparent steady state for LAS mineralization was observed (Fig. 3). In contrast, both S and H were modified by a factor of less than two in the PE biofilms from Spain and Sweden at 6 °C, and the PE biofilm from Spain at 21 °C (Figs. 2D and E Spain, and 2D Sweden). Bacterial biomass in these biometer units decreased by between 6- and 20-times, after 59 days. In this case, LAS mainly remained in solution after 59 days and was thus available for mineralization, which appeared to limit the loss of bacterial diversity and biomass in the biometer units. Although bacterial activity was low at 6 °C, it was apparently not totally inhibited.

3.7. The biometer test system and the biofilm approach

The mineralization kinetics and extents at 21 °C in Spain reported in the present study are similar to the values previously obtained with beads that had been colonized at the same site in winter 2005 and in summer 2006 (Mauffret et al., 2009). This showed the reproducibility of the biofilm approach, which appears to be a useful procedure for decreasing the variability observed in typical marine biodegradation test systems, such as variability in lag phase duration and failure to acclimate, which are common problems in many biodegradation test systems (Painter, 1995; Torang and Nyholm, 2005; Mauffret et al., 2009).

One of the main advantages of the biofilm approach is that a large sample of the marine biomass is introduced into a small test system that can be handled in the laboratory. This approach holds promise for biodegradation assessment as a useful tool for determining the biodegradation capability and its geographical variability present in the marine environment, and thus for reducing the risk of false negatives. Some may be concerned by the possibility of producing false positives and unrealistically high degradation outcomes. The mineralization half-lives reported in the present study at 21 $\pm$C ($t_{1/2}=20$–53 days, Table 1) were longer than the values previously reported at similar temperatures (20–24 °C) in PE estuarine water with or without sediment

addition ($t_{1/2}$=7 days (Shimp, 1989)), and in PE or NPE river water with or without sediment ($t_{1/2}$=1–14 days (Larson and Payne, 1981)). The extent of mineralization reported in these previous studies is similar to that found in the present study; the values are all within the range of the respective positive glucose control. Therefore, the biofilm approach does not appear to produce values for biodegradation kinetics and extents that are excessively higher than those obtained in classical biodegradation tests with seawater. Additional studies on the system efficiency, with different chemicals, including non-biodegradable reference chemicals, are required to reach firmer conclusions.

4. Conclusions

By using an innovative test system, namely the biofilm approach, this study has refined the understanding of biodegradation processes of a high production volume surfactant under different conditions. The selected sites contrasted in local levels of usage of the compound and temperature conditions. The study provides an estimate of the threshold environmental concentration for bacterial acclimation to LAS (<6 μgLAS/L), and has determined the effect of pre-exposure to this chemical on the microbial community in seawater samples collected in two sites—one with moderate LAS contamination and the other with little or no LAS contamination, in terms of diversity, cell number and mineralization capability. The study has also illustrated the contrasting effects of temperature on bacteria collected in warm and cold areas. The present study contributes improved knowledge of biodegradation mechanisms of synthetic chemicals in marine environments.

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References

Alexander, M., 2001. Biodegradation and Bioremediation, Second Edition Academic Press.

Blanck, H., Wängberg, S.Å., Molander, S., 1988. Pollution-Induced Community Tolerance—A New Ecotoxicological Tool. In: Pratt, J.J.C.a.J.R. (Ed.), Functional Testing of Aquatic Biota for Estimating Hazards of Chemicals. American Society for Testing and Materials, Philadelphia, pp. 219–230.

Boeije, G.M., Schowanek, D.R., Vanrolleghem, P.A., 2000. Incorporation of biofilm activity in river biodegradation modeling: a case study for linear alkylbenzene sulphonate (LAS). Water Res. 34, 1479–1486.

Finar, I.L., 1975. Organic Chemistry. Vol. 1. The Fundamental Principles. In: Longman (Ed.), London, p. 66.

Gillan, D.C., 2004. The effect of an acute copper exposure on the diversity of a microbial community in North Sea sediments as revealed by DGGE analysis––the importance of the protocol. Mar. Pollut. Bull. 49, 504–513.

Gonzalez-Mazo, E., Forja, J.M., Gomez-Parra, A., 1998. Fate and distribution of linear alkylbenzene sulfonates in the littoral environment. Environ. Sci. Technol. 32, 1636–1641.

Gonzalez-Mazo, E., Honing, M., Barcelo, D., Gomez-Parra, A., 1997. Monitoring long-chain intermediate products item the degradation of linear alkylbenzene sulfonates in the marine environment by solid-phase extraction followed by liquid chromatography/ionspray mass spectrometry. Environ. Sci. Technol. 31, 504–510.

HERA, 2007. Human & Environmental Risk Assessment on ingredients of European household cleaning products: LAS.

Ingerslev, F., Torang, L., Nyholm, N., 2000. Importance of the test volume on the lag phase in biodegradation studies. Environ. Toxicol. Chem. 19, 2443–2447.

Lara-Martín, P.A., Gómez-Parra, A., González-Mazo, E., 2008. Sources, transport and reactivity of anionic and non-ionic surfactants in several aquatic ecosystems in SW Spain: a comparative study. Environ. Pollut. 156, 36–45.

Larson, R.J., Payne, A.G., 1981. Fate of the benzene ring of linear alkylbenzene sulfonate in natural waters. Appl. Environ. Microbiol. 41, 621–627.

León, V.M., Gomez-Parra, A., Gonzalez-Mazo, E., 2004. Biodegradation of linear alkylbenzene sulfonates and their degradation intermediates in seawater. Environ. Sci. Technol. 38, 2359–2367.

León, V.M., Lopez, C., Lara-Martin, P.A., Prats, D., Varo, P., Gonzalez-Mazo, E., 2006. Removal of linear alkylbenzene sulfonates and their degradation intermediates at low temperatures during activated sludge treatment. Chemosphere 64, 1157–1166.

Marcomini, A., Pojana, G., Sfriso, A., Quiroga Alonso, J.M., 2000. Behavior of anionic and nonionic surfactants and their persistent metabolites in the Venice Lagoon, Italy. Environ. Toxicol. Chem. 19, 2000–2007.

Matthijs, E., Stalmans, M., 1993. Monitoring of LAS in the North Sea. Tenside Surf. Det. 24, 193–198.

Mauffret, A., Rottiers, A., Federle, T.W., Gillan, D., Hampel, M., Blasco, J., Temara, A., 2009. Colonized beads as inoculum for marine biodegradability assessment: application to linear alkylbenzene sulfonate. Environ. Int. 35, 885–892.

McAvoy, D.C., Masscheleyn, P., Peng, C., Morrall, S.W., Casilla, A.B., Lim, J.M.U., Gregorio, E.G., 2003. Risk assessment approach for untreated wastewater using the QUAL2E water quality model. Chemosphere, 52.

Modler, R., Blagoev, M., Inoguchi, Y., 2009. CEH report linear alkylate sulfonates. SRI consulting.

Nyholm, N., Kristensen, P., 1987. Screening test methods for assessment of biodegradability of chemical substances in sea water. Water Quality Institute Report, Danish Academy of Technical Sciences.

OECD306, 1992. Biodegradability in seawater. Organisation for Economic Co-operation and Development.

Painter, H.A., 1995. Detailed review paper on biodegradability testing. OECD Environ. Monogr. 98, 1–162.

Sales, D., Quiroga, J.M., Gomez-Parra, A., 1987. Primary biodegradation kinetics of anionic surfactants in marine environment. Bull. Environ. Contam. Toxicol. 39, 385–392.

Shimp, R., 1989. LAS biodegradation in Estuaries. Tenside Surf. Det. 26, 390–393.

Swisher, R.D., 1987. Surfactant Biodegradation. Marcel Dekker Inc., New York.

Takada, H., Mutoh, K., Tomita, N., Miyadzu, T., Ogura, N., 1994. Rapid removal of linear alkylbenzenesulfonates (LAS) by attached biofilm in an urban shallow stream. Water Res. 28, 1953–1960.

Takada, H., Ogura, N., 1992. Removal of linear alkylbenzenesulfonates (LAS) in the Tamagawa Estuary. Mar. Chem. 37, 257–273.

Temara, A., Carr, G., Webb, S., Versteeg, D., Feijtel, T., 2001. Marine risk assessment: linear alkylbenzenesulponates (LAS) in the North Sea. Mar. Pollut. Bull. 42, 635–642.

Terzic, S., Hrsak, D., Ahel, M., 1992. Primary biodegradation kinetics of linear alkylbenzene sulphonates in estuarine waters. Water Res. 26, 585–591.

Torang, L., Nyholm, N., 2005. Biodegradation rates in adapted surface water can be assessed following a preadaptation period with semi-continuous operation. Chemosphere 61, 1–10.

Vives-Rego, J., Lopez-Amoros, R., Guindulain, T., Garcia, M.T., Comas, J., Sanchez-Leal, J., 2000. Microbial aspects of linear alkylbenzene sulfonate degradation in coastal water. J. Surfactants Deterg. 3, 303–308.

Whelan, M.J., Van Egmond, R., Guymer, I., Lacoursiere, J.O., Vought, L.M.B., Finnegan, C., Fox, K.K., Sparham, C., O'Connor, S., Vaughan, M., Pearson, J.M., 2007. The behaviour of linear alkyl benzene sulphonate under direct discharge conditions in Vientiane, Lao PDR. Water Res. 41, 4730–4740.

Wiggins, B.A., Jones, S.H., Alexander, M., 1987. Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. Appl. Environ. Microbiol. 53, 791–796.