Science of the Total Environment 557–558 (2016) 612–619

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Parallel pathways of ethoxylated alcohol biodegradation under aerobic conditions

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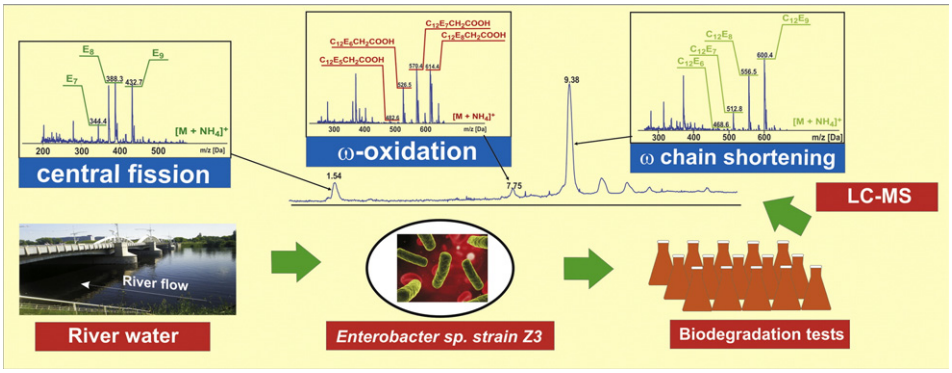
HIGHLIGHTS

- Two parallel biodegradation pathways of alcohol ethoxylates have been discovered.
- Apart from central fission, oxyethylene chain shortening has been evidenced.
- Both a single *Enterobacter* strain and raw river water cause the same two pathways.
- Ω-carboxylated alcohol ethoxylates are intermediate metabolites for chain shortening.
- Homogeneous alcohol ethoxylate $C_{12}E_9$ was the model used in this study.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 December 2015
Received in revised form 15 March 2016
Accepted 16 March 2016
Available online 31 March 2016

Editor: D. Barcelo

Keywords:
LC-MS
LC-MS/MS
Nonionic surfactants
Oxyethylene (5–9) dodecyl ethers
Metabolites
Enterobacter sp

ABSTRACT

Non-ionic surfactants (NS) are a major component of the surfactant flux discharged into surface water, and alcohol ethoxylates (AE) are the major component of this flux. Therefore, biodegradation pathways of AE deserve more thorough investigation.

The aim of this work was to investigate the stages of biodegradation of homogeneous oxyethylated dodecanol $C_{12}E_9$ having 9 oxyethylene subunits, under aerobic conditions. *Enterobacter* strain Z3 bacteria were chosen as biodegrading organisms under conditions with $C_{12}E_9$ as the sole source of organic carbon. Bacterial consortia of river water were used in a parallel test as an inoculum for comparison. The LC-MS technique was used to identify the products of biodegradation. Liquid-liquid extraction with ethyl acetate was selected for the isolation of $C_{12}E_9$ and metabolites from the biodegradation broth. The LC-MS/MS technique operating in the multiple reaction monitoring (MRM) mode was used for quantitative determination of $C_{12}E_9$, $C_{12}E_8$, $C_{12}E_7$ and $C_{12}E_6$. Apart from the substrate, the homologues $C_{12}E_8$, $C_{12}E_7$ and $C_{12}E_6$, being metabolites of $C_{12}E_9$ biodegradation by shortening of the oxyethylene chain, as well as intermediate metabolites having a carboxyl end group in the oxyethylene chain ($C_{12}E_8COOH$, $C_{12}E_7COOH$, $C_{12}E_6COOH$ and $C_{12}E_5COOH$), were identified. Poly(ethylene glycols) (E) having 9, 8 and 7 oxyethylene subunits were also identified, indicating parallel central fission of $C_{12}E_9$ and its metabolites. Similar results were obtained with river water as inoculum.

It is concluded that AE, under aerobic conditions, are biodegraded via two parallel pathways: by central fission with the formation of PEG, and by Ω-oxidation of the oxyethylene chain with the formation of carboxylated AE and subsequent shortening of the oxyethylene chain by a single unit.

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http://dx.doi.org/10.1016/j.scitotenv.2016.03.112
0048-9697/

1. Introduction

Non-ionic surfactants (NS), due to their amphiphile structure, have acquired a huge number of household and industrial applications, often typified by down-the-drain disposal. They are a major component of the surfactant stream discharged into surface water (Cassani et al., 2011; Fuerhacker et al., 2001; Kopiec et al., 2014; Nowicka et al., 2013a; Traczyk et al., 2006, Traverso-Soto et al., 2016; Zembrzuska et al., 2014, 2016; Zoller, 1994) and relatively high NS concentrations have been determined in river water (Krogh et al., 2002; Scott and Jones, 2000; Szymanski et al., 2001) and at sewage treatment plants (-Eadsforth et al., 2006; Evans et al., 1997). In the EU, 1.397 million tonnes of NS were manufactured in 2012 (CESIO, 2012), and alcohol ethoxylates (AE) were the major component of the NS flux (approximately 1 million tonnes). The ratio of AE manufactured from renewable sources and from petrochemicals was approximately 3:4. High AE participation in total NS was confirmed by an investigation of sewage composition, where approximately 100 individual AE but no other NS were found (Kopiec et al., 2015).

NS potentially include a very large number of individual substances (Arias et al., 2008). Several hundred substances belonging to the NS category may be present in surface water (Brunner et al., 1988). This is because of the polydispersity of the majority of NS, being a result of the technology used for their manufacture (Marcomini et al., 2000a; Wind et al., 2006). The use of AE is preferred due to their relatively easy biodegradation under aerobic conditions. Their 'central fission' with the formation of poly(ethylene glycols) (PEG) and free fatty alcohol is considered to be the dominant AE biodegradation pathway (Patterson et al., 1967; Swisher, 1987; Szymanski et al., 2002a, 2002b). PEG are also a common type of technological impurity of NS (Eubeler et al., 2010; Plata et al., 2011). Unlike AE, oxyethylated alkylphenols (APhE) (Hoenicke et al., 2007) undergo a gradual Ω-shortening of the oxyethylene chain (Ahel et al., 1994). However, 'central fission' of APhE has also been observed (Franska et al., 2003a; Marcomini et al., 2000b). Partly hypothetical pathways, including Ω-oxidation of both oxyethylene and alkyl moieties, were proposed by Schröder (Schröder, 2001). The formation of carboxylated AE due to Ω-oxidation of the oxyethylene chain has been reported (Nowicka et al., 2013b; Scott and Jones, 2000). The presence of metabolites which have been oxidized in the alkyl chain is reported in (Di Corcia et al., 1998). It can be presumed that the formation of Ω-carboxylated AE should lead to shortening of both oxyethylene and alkyl moieties, like in the case of oxyethylated alkylphenols. Such a process is hardly observable with a polydisperse substrate, because the product is already present as a component of the polydisperse mixture. However, the shortening should be easily observed by investigation of homogeneous AE having defined lengths of both oxyethylene and alkyl chains. In spite of the limited availability of such AE, several homogeneous AE are commercially available. The homogeneous surfactant $C_{12}E_9$ matches these requirements well.

The aim of this work was to investigate the stages of biodegradation of homogeneous oxyethylated dodecanol $C_{12}E_9$ having 9 oxyethylene subunits, under aerobic conditions. 'Central fission' with the formation of PEG consisting of 9 oxyethylene subunits, and the parallel formation of Ω-carboxylated $C_{12}E_9$, were expected. The main purpose of the experiment was to observe further steps of biodegradation, i.e. whether shortening of the oxyethylene or alkyl chain would be observed. Generally, the task of identification of biodegradation products of $C_{12}E_9$ can be classified as suspected screening (Schymanski et al., 2014; Gago-Ferrero et al., 2015), i.e. the products are suspected, but standards are generally unavailable. The polydisperse surfactant $C_{12}E_{10}$ was selected as a standard for metabolite identification. The $C_{12}E_{10}$ mass spectrum contains identified $C_{12}E_x$ homologues having 3–22 oxyethylene subunits (Nowicka et al., 2013b). Additionally, the mass spectra of $C_{12}E_{10}$ biodegradation products contain mass peaks of carboxylated $C_{12}E_x$ homologues having 7–15 oxyethylene subunits. The polydispersity of ethoxylates facilitates identification of particular homologues, because the mass spectra contain series of peaks which differ by $m/z = 44$ (Franska et al., 2003b), i.e. a single oxyethylene subunit. *Enterobacter* strain Z3 bacteria were chosen as biodegrading organisms under conditions with $C_{12}E_9$ as the sole source of organic carbon. The bacteria were isolated from river water and previously investigated for the ability to split oxyethylated alcohols with PEG formation. The LC-MS technique was selected as a tool for identification of the biodegradation products, and the LC-MS/MS technique operating in the MRM mode was used for their quantitative determination. MRM mode was also used for confirmation of the identity of shorter $C_{12}E_x$ homologues. Liquid-liquid extraction with ethyl acetate was selected for the isolation of $C_{12}E_9$ and metabolites from the biodegradation broth.

2. Materials and methods

2.1. Reagents

Homogeneous surfactants $C_{12}E_9$, $C_{12}E_8$, $C_{12}E_7$ and $C_{12}E_6$ of high purity grade (>98%) and polydisperse surfactant $C_{12}E_{10}$ were purchased from Sigma-Aldrich (St. Louis, MO, USA), as were MS-grade acetonitrile and methanol, and ammonium acetate used as a mobile phase additive. Water was prepared by reverse osmosis in a Demiwa system from double distillation from a quartz apparatus. Only freshly distilled water was used.

The reagents used for liquid-liquid (LLE) sequential extraction, chloroform and ethyl acetate of analytical grade, were from POCh (Gliwice, Poland), as were sodium chloride and sodium hydrogen carbonate. A stock standard solution of $C_{12}E_9$ was prepared in acetonitrile. The solution was stored at 4 °C.

All reagents used for the preparation of inoculums – urea, $NaHCO_3$, $K_2HPO_4 \cdot 2H_2O$, NaCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$ – and mineral salt media – $Na_2HPO_4 \cdot 2H_2O$, K_2HPO_4, KH_2PO_4, NH_4Cl, $FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, H_3BO_3, $ZnSO_4 \cdot 7H_2O$, $(NH4)_6Mo_7O_{24}$ – were from POCh, Poland, with the exception of meat extract and peptone, which were from BTL, Poland.

2.2. Enterobacter strain Z3

The bacterium *Enterobacter* strain Z3 is a microorganism isolated from river water. Pure cultures of the bacteria were maintained on an agar medium.

An inoculum was prepared by suspending a loopful from a 3-day culture in 5 mL of a sterile enrichment broth. The solution was incubated at 30 °C for 24 h. The obtained solution was introduced into 45 mL of the sterile enrichment broth, and the mixture was incubated at 30 °C for 24 h; it was then introduced into 450 mL of the sterile enrichment broth ('artificial sewage') (Zgola-Grzeskowiak et al., 2005; Zgola-Grzeskowiak et al., 2008) containing (mg L^{-1}): 85 of meat extract, 15 of urea, 10 of peptone, 98 of $NaHCO_3$, 14 of $K_2HPO_4 \cdot 2H_2O$, 3.5 of NaCl, 2 of $CaCl_2 \cdot 2H_2O$ and 1 of $MgSO_4 \cdot 7H_2O$, and incubated at 30 °C for 24 h. Thermally sterilized river water was used as the enrichment broth.

2.3. Biodegradation test

The tests were performed in bottles filled with 200 mL of medium, consisting of a mineral salt medium spiked with $C_{12}E_9$ surfactant (10 mg L^{-1}) and an inoculating suspension of the investigated bacterial strain (5 mL) or raw river water (10 mL). Apart from the tested surfactant, the solution contained (mg L^{-1}): 33.5 of $Na_2HPO_4 \cdot 2H_2O$, 21.75 of K_2HPO_4, 8.5 of KH_2PO_4, 27.5 of $CaCl_2 \cdot 2H_2O$, 22.5 of $MgSO_4 \cdot 7H_2O$, 20 of NH_4Cl, 0.25 of $FeCl_3 \cdot 6H_2O$, 0.40 of $MnSO_4 \cdot H_2O$, 0.06 of H_3BO_3, 0.04 of $ZnSO_4 \cdot 7H_2O$ and 0.035 of $(NH4)_6Mo_7O_{24}$. The samples were left in open bottles, protected against dust and light, at room temperature. All bottles were periodically shaken on a rotary shaker to provide