**Biodegradability and aquatic toxicity of quaternary ammonium-based
gemini surfactants: effect of the spacer on their ecological properties**

M. Teresa Garcia^{a*}, Olga Kaczerewska^b, Isabel Ribosa^a, Bogumił Brycki^b,
Paulina Materna^b, Małgorzata Drgas^b

^aDepartment of Chemical and Surfactant Technology, Institute of Advanced Chemistry of
Catalonia, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona Spain.

M. Teresa Garcia e-mail: teresa.garcia@iqac.csic.es

Isabel Ribosa e-mail: isabel.ribosa@iqac.csic.es

^bLaboratory of Microbiocides Chemistry, Faculty of Chemistry, Adam Mickiewicz
University, Umultowska 89b, 61-614 Poznań, Poland.

Olga Kaczerewska e-mail: olga.kaczerewska@wp.pl

Bogumił Brycki e-mail: brycki@amu.edu.pl

Paulina Materna e-mail: p_materna@wp.pl

Małgorzata Drgas e-mail: madrgas@wp.pl

* corresponding author. Phone: +34 93 400 61 00; fax: +34 93 204 59 04.

e-mail address: teresa.garcia@iqac.csic.es

20 Graphical Abstract
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ABSTRACT

Aerobic biodegradability and aquatic toxicity of five types of quaternary ammonium-based gemini surfactants have been examined. The effect of the spacer structure and the head group polarity on the ecological properties of a series of dimeric dodecyl ammonium surfactants has been investigated. Standard tests for ready biodegradability assessment (OECD 310) were conducted for C₁₂ alkyl chain gemini surfactants containing oxygen, nitrogen or a benzene ring in the spacer linkage and/or a hydroxyethyl group attached to the nitrogen atom of the head groups. According to the results obtained, the gemini surfactants examined cannot be considered as readily biodegradable compounds. The negligible biotransformation of the gemini surfactants under the standard biodegradation test conditions was found to be due to their toxic effects on the microbial population responsible for aerobic biodegradation. Aquatic toxicity of gemini surfactants was evaluated against *Daphnia magna*. The acute toxicity values to *Daphnia magna*, IC₅₀ at 48 h exposure, ranged from 0.6 to 1 mg/L. On the basis of these values, the gemini surfactants tested should be classified as toxic or very toxic to the aquatic environment. However, the dimeric quaternary ammonium-based surfactants examined result to be less toxic than their corresponding monomeric analogs. Nevertheless the aquatic toxicity of these gemini surfactants can be reduced by increasing the molecule hydrophilicity by adding an heteroatom to the spacer or a hydroxyethyl group to the polar head groups.

KEYWORDS

Gemini surfactants, spacer, aerobic biodegradation, mineralization, aquatic toxicity, *Daphnia magna*

ABBREVIATIONS

ISO	International Organization for Standardization
SDS	sodium n-dodecyl sulfate
IC ₅₀	estimated concentration to immobilize 50% of the daphnias after 48 h exposure
12-6-12	1,6-hexamethylene-bis(<i>N</i> -dodecyl- <i>N,N</i> -dimethylammonium) dibromide
12-O-12	3-oxa-1,5-pentamethylene-bis(<i>N</i> -dodecyl- <i>N,N</i> -dimethylammonium) dichloride
3N-12	3-azamethyl-1,5-bis(<i>N</i> -dodecyl- <i>N,N</i> -dimethylammonium) dibromide
QSB2-12	1,4-bis-[<i>N</i> -(1-dodecyl)- <i>N,N</i> -dimethylammoniummethyl]benzene dibromide
G6-MOH-12	1,6-hexamethylene-bis(<i>N</i> -dodecyl- <i>N</i> -hydroxyethyl- <i>N</i> -methylammonium) dibromide
TOC	total organic carbon
DOC	dissolved organic carbon
BOD	biochemical oxygen demand
OECD	Organization for Economic Co-operation and Development
CI	confidence interval
DT	dodecyl trimethyl ammonium bromide
BDD	dodecyl benzyl dimethyl ammonium bromide

1. INTRODUCTION

Dimeric alkylammonium salts are a class of gemini surfactants made up of two amphiphilic moieties having the structure of monomeric quaternary alkylammonium salts connected by a spacer linkage (Menger and Keiper, 2000; Tawfik, 2015). These cationic gemini surfactants possess at least two hydrophobic hydrocarbon chains and two polar ammonium head groups linked by a spacer group. The spacer can be either hydrophobic (polymethylene chain) or hydrophilic (polymethylene chain with ether, hydroxyl or aza groups), and from a structural point of view the spacer can be flexible (polymethylene chain) or rigid (aromatic or unsaturated hydrocarbons) (Brycki et al., 2014). Gemini alkylammonium surfactants show

biological and surface activities much higher than monomeric analogs (Negm et al., 2010; Tehrani-Bagha et al., 2015). It means that the same biocidal or surface effect can be reached using much smaller amounts of gemini surfactants, what has a fundamental importance from a toxicological and ecological point of view (Schram et al., 2003).

Owing to their unique properties cationic surfactants are widely used in household (Gerba, 2015; Lara-Martin et al., 2010), medicine, biotechnology, pharmacy (Grabińska-Sota, 2011; Kumar and Tyagi, 2014; Mishra et al., 2009) and other industries (Mori et al., 2015; Simoncic and Tomsic, 2010) as emulsifiers (Kumar and Tyagi, 2014), antimicrobial agents or corrosion inhibitors (Xue et al., 2015; Zhang et al., 2011). Because of the extensive consumption of these compounds, some part of them can get into the environment, i.e. accumulate in sediments and soil and leak into groundwater and wastewater (Grabińska-Sota, 2011; Ivanković and Hrenović, 2010; Li et al., 2014; Tezel and Pavlostathis, 2015). Moreover, concentration of surfactants and their degradation products in the environment will be increasing due to the constant development of industry that use large amount of these chemicals (Ivanković and Hrenović, 2010). Typical sewage treatment plants remove major readily biodegradable compounds, however, some minor contaminants, including quaternary ammonium salts, goes through the treatment plant and leak into the environment (Tezel and Pavlostathis, 2015). The presence of the hydrophobic chain and the positively charged nitrogen atoms facilitates the adsorption of these compounds on sediments and sludge (Li et al., 2014). In addition, quaternary ammonium salts are harmful for organisms that live in water (Chen et al., 2014; Jing et al., 2012; Liang et al., 2013; Qv and Jiang, 2013; Van de Voorde, 2012). These compounds are ubiquitous in the environment; therefore it is important to study their biodegradability and aquatic toxicity (Khan et al., 2015; Li and Brownawell, 2010). Quaternary ammonium-based monomeric cationic surfactants have been extensively studied (Li and Brownawell, 2010; Van de Voorde et al., 2012; Zhang et al., 2015), but there

is little information on the ecological properties of quaternary ammonium-based dimeric surfactants (Brycki et al., 2014; Tehrani-Bagha et al., 2015). Because of the increasing use of gemini surfactants, detailed knowledge about their biodegradability and aquatic toxicity is necessary to prevent and reduce the risk to the aquatic environment.

In the present work, the ultimate biodegradation of a series of gemini cationic surfactants under aerobic conditions and their toxic effects on an aquatic organism, *Daphnia magna*, are studied. The dimeric alkylammonium surfactants investigated have the same alkyl chain length (C12) but they differ in the nature of the spacer and the substituents attached to the polar head groups. The results of this study will provide reference data about the environmental behavior of quaternary ammonium-based gemini surfactants and the relationship between the hydrophilicity of the dimeric surfactant molecule and its ecological properties.

2. MATERIALS AND METHODS

2.1. Materials

Gemini cationic surfactants have been prepared by different methods described in literature, i.e. 1,6-hexamethylene-bis(*N*-dodecyl-*N,N*-dimethylammonium) dibromide (12-6-12) (Brycki et al., 2011), 3-azamethyl-1,5-bis(*N*-dodecyl-*N,N*-dimethylammonium) dibromide (3N-12) (Wettig et al., 2007), 1,4-bis-[*N*-(1-dodecyl)-*N,N*-dimethylammoniummethyl]benzene dibromide (QSB2-12) (Mivehi et al., 2011), 1,6-hexamethylene-bis(*N*-dodecyl-*N*-hydroxyethyl-*N*-methylammonium) dibromide (G6-MOH-12) (Borse et al., 2004), 3-oxa-1,5-pentamethylene-bis(*N*-dodecyl-*N,N*-dimethylammonium) dichloride (12-O-12) (unpublished results). The structures of tested gemini surfactants are presented in Table 1.

Table 1. Structures of the dimeric alkylammonium surfactants investigated

Molecular structure	Surfactant abbreviation
12-6-12
12-O-12
3N-12
G6-MOH-12
QSB2-12

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126 2.2. Methods

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128 2.2.1. CO₂ headspace biodegradation test

129 The CO₂ headspace test (OECD, 2006; ISO, 1999) was applied to evaluate the
 130 biodegradability of the gemini surfactants under aerobic conditions. This method allows the
 131 evaluation of the ultimate aerobic biodegradation (mineralization to carbon dioxide) of an

organic compound in aqueous medium by measuring the net increase in total inorganic carbon over time with respect to unamended blanks. Gemini surfactants were tested at a concentration of 15 mg C/L instead of the usual 20 mg C/L in order to reduce the potential toxicity of quaternary-ammonium dimeric surfactants to the microbial population and also meet the requirement that the amount of dissolved organic carbon provided by the inoculum was less than 10% of initial carbon concentration introduced by the test surfactant. Samples were inoculated with activated sludge collected from a municipal wastewater treatment plant (Manresa, Barcelona) and then incubated in the dark at 20 ± 1 °C in sealed vessels (120 mL) with a headspace of air (headspace/liquid volume ratio, 1:2). Sodium n-dodecyl sulfate (SDS) (20 mg C/L) was used as reference substance. Two replicates of surfactants, blank and reference substance were performed for each sampling day except for the last day, when four replicates were performed. For each gemini surfactant inhibition tests were also carried out (SDS 20 mg C/L + tested compound 15 mg C/L in each bottle). The tests run for 28 days. Every sampling day, after injecting a sodium hydroxide solution to the vessels, shaking and allowing to settle, suitable volumes were withdrawn by syringe from the liquid phase of each vessel and kept in small beakers carefully filled to the brim and covered with a cap to prevent CO₂ exchange with the air. The concentration of inorganic carbon (IC) was determined in a carbon analyzer (Shimadzu TOC-5050). The extent of biodegradation was expressed as a percentage of the theoretical amount of inorganic carbon (ThIC) based on the initial amount of the tested compound.

2.2.2. *Daphnia magna* immobilization test

Daphnia magna, laboratory bred, not more than 24h old, were used in this test (OECD, 2004), where the swimming incapability is the end point. The pH of the medium was 8.0 and the total hardness was 250 mg/L (as CaCO₃), with a Ca/Mg ratio of 4/1. Tests were performed in the dark at 20 °C. Twenty daphnia, divided into four batches of five animals each, were used

at each test concentration. For each gemini surfactant, 10 concentrations in a geometric series were tested in the concentration range, 0.2-2 mg/L, first established in a preliminary test. The percentage immobility at 24 and 48 h was plotted against concentration on logarithmic-probability paper and a linear relationship was obtained. The Probit method was employed as statistical procedure to determine the IC₅₀ (the estimated concentration to immobilize 50% of the daphnia after 48 h exposure) and the corresponding 95% confidence interval (CI).

3. RESULTS AND DISCUSSION

3.1. Aerobic biodegradability

The ready biodegradability of the cationic gemini surfactants under aerobic conditions was determined in a standard biodegradation test, the OECD 310 - CO₂ Headspace Test (ISO, 1999; OECD, 2006). The OECD 310 method was selected for evaluating the aerobic biodegradability of the cationic dimeric surfactants investigated because is the reference test method adopted by the European Union for testing surfactant biodegradability (EC, 2004). In this test the ultimate biodegradation or mineralization of the surfactants, i.e., the microbial transformation of the parent molecule into inorganic final products of the degradation process, such as carbon dioxide and water, was determined. The surfactant tested was added to an aerobic aqueous medium inoculated with wastewater microorganisms and the formation of carbon dioxide was measured for a determined period of time and reported as a percentage of the theoretical maximum. The conditions of this biodegradation test are so stringent that a positive result, i.e., a biodegradation level exceeding 60% within 28 days, can be considered as reliable evidence of ready biodegradation. In addition, the OECD 310 test also allows

checking the inhibitory effects of the gemini surfactants on the microbial population responsible for aerobic biodegradation.

The biodegradation results are shown in Table 2. Gemini surfactants underwent less than 5% of biodegradation after a 28-day incubation period. No significant differences were found as a function of the spacer structure (flexible or rigid) nor between gemini surfactants containing a hydrophobic spacer (12-6-12) or a more hydrophilic one (12-O-12 and 3N-12). Likewise, the replacement of a methyl by a hydroxyethyl group did not improve the degradation extent in the test conditions.

Table 2. Biodegradation percentages of dimeric cationic surfactants and reference substance (SDS) as determined by the CO₂ headspace test. (95% CI of 28-day results calculated from 4 replicates)

Surfactant	Biodegradation (%)			
	7 d	14 d	21 d	28 d
SDS	74	85	93	88±5.5
12-6-12	0	0	0	0±1.5
12-O-12	0	0	0	0±0.6
3N-12	0	0	0	0±0.8
QSB2-12	0	0	0	0±2.3
G6-MOH-12	0	0	0	0±3.2

Regarding monomeric surfactants, alkyltrimethyl ammonium salts attain even 100% of biodegradation by dissolved organic carbon (DOC) removal (Garcia et al., 2000; Nishiyama et al., 1995) and around 50% by biochemical oxygen consumption (BOD) (Nishiyama et al., 1995), whereas benzyl dodecyldimethyl ammonium chloride attains 100% of degradation by DOC removal (Garcia et al., 2000) and around 90% of biodegradation by oxygen uptake in

the MITI (Ministry of International Trade and Industry, Japan) test (Karsa and Porter, 1995). However, the biodegradation percentages for dialkyl dimethyl ammonium chlorides (C₁₂-C₁₈) in the MITI and Sturm (CO₂ evolution) tests are lower than 5% (Karsa and Porter, 1995). The biodegradation of gemini surfactants has not been extensively studied, so far. However, the biodegradation results here obtained are consistent with the low degradation percentage (2%) reported for the gemini surfactant trimethylene-1,3-bis-(N,N-dimethyl-N-dodecylammonium salt by measuring the biochemical oxygen demand in the Modified MITI test (Banno et al., 2010).

The biodegradation data should be interpreted with caution taking into account the features of both the biodegradation test applied and the chemicals studied. Cationic surfactants with a high adsorption potential on surfaces and membranes can attain percentages as high as 100% when DOC removal is the parameter used to measure biodegradation. On the other hand, many quaternary ammonium salts are potential biocides and may inhibit growth of microorganisms capable of degrading quaternary ammonium salts. These effects have been already described for monomeric surfactants (Garcia et al. 1999; Hajaya and Pavlostathis, 2012). As gemini surfactants have been found to have biological activities superior to monomeric cationic surfactants (Brycki et al., 2014), similar or enhanced effects would be expected for dimeric surfactants. Thus, the high biological activity of cationic gemini surfactants might have a negative impact on their biodegradation.

To evaluate the toxicity of dimeric alkylammonium surfactants to the microorganisms responsible for aerobic biodegradation, solutions containing a mixture of the compound and a reference substance (SDS) were tested. Biodegradation percentages for the mixtures of each gemini surfactant and SDS were calculated based on the theoretical inorganic carbon (ThIC) yield anticipated from only the reference substance (Table 3).

Table 3. Inhibition of sodium n-dodecyl sulfate (SDS) degradation by cationic gemini surfactants

Compound	Concentration (mg C/L)	Inhibition (%)
SDS + 12-6-12	20+15	41
SDS + 12-O-12	20+15	37
SDS + 3N-12	20+15	71
SDS + QSB-12	20+15	52
SDS + G6MOH-12	20+15	56

All tested dimeric surfactants strongly inhibited the inoculum activity at the concentration used (15 mg C/L) in the biodegradation tests. The SDS biodegradation inhibition percentages (Table 3) were clearly higher than the limit commonly accepted to consider serious inhibitory effects ($\geq 25\%$). Dimeric surfactant 3N-12, containing an aza group in the flexible spacer, is the compound with the highest inhibitory effect since the SDS degradation percentage was only the 30% whereas for the rest of binary mixtures the SDS degradation attained about 50%.

From the results obtained, the failure in the biodegradation tests can be attributed to the high toxicity of quaternary ammonium-based gemini surfactants to the microorganisms responsible for aerobic biodegradation. These findings are consistent with the high antimicrobial activity reported for gemini alkylammonium compounds (Brycki et al., 2014; Negm et al., 2010). The action of microbial consortia, which are responsible for biodegradation processes, is inhibited by the gemini surfactants under the conditions used in the ready biodegradability test. It might be possible to have better results at a lower initial surfactant concentration but it is limited by the ready biodegradability test characteristics.

Quaternary ammonium-based gemini surfactants are not readily biodegradable but more work need to be done on their degradation properties because, as described for monomeric cationic

surfactants, adsorption on biomass can decrease inhibition on the inoculum activity and thus enhance surfactant biodegradation (Garcia et al., 2000). On the other hand, it should be noted that in some cases the ready biodegradability could be a non-desirable property. It occurs when gemini surfactants are used as preservatives or corrosion inhibitors since the permanency of the substance affects the effectiveness of its action.

3.2. Aquatic toxicity

Acute toxicity tests on freshwater crustacea (*Daphnia magna*) were carried out to assess the aquatic toxicity of dimeric cationic surfactants. *Daphnia magna* was selected as model organism because this planktonic crustacean is highly sensitive to pollution and has been widely used for evaluating toxicity of surfactants (Garcia et al., 2000; Garcia et al., 2008; Garcia et al., 2009; Hodges et al., 2006; Lavorgna et al., 2016) which allows the comparison of the results here obtained with those reported in literature for conventional monomeric cationic surfactants (Garcia et al., 1999). The results of *D. magna* 48 h immobilization test (IC₅₀) are given in Table 4 and the graph for one of the tested surfactants is presented as an example in Figure 1.

Table 4. Acute toxicity of gemini surfactants to *Daphnia magna*

Surfactant	IC ₅₀ (95% CI) (mg/L)
12-6-12	0.65 (0.59-0.72)
12-O-12	1.1 (0.99-1.2)
3N-12	1.0 (0.90-1.1)
QSB2-12	0.73 (0.68-0.80)
G6-MOH-12	1.1 (0.95-1.3)

Figure 1. Plots of percentage of immobilized daphnias at 24 and 48 h as a function of surfactant concentration for 12-O-12

The estimated concentration to immobilize 50% of the crustacea population after 48 h of exposure (IC_{50}) ranged from 0.6 to 1.1 mg/L for gemini cationic surfactants (Table 4). Based on the IC_{50} values and the Ecotoxicity Hazard classes proposed by the OECD (OECD, 2001), gemini surfactants 12-6-12, QSB-12 and 3N-12 belong to the class of chemicals considered as very toxic to aquatic life (Acute toxicity I) and surfactants 12-O-12 and G6-MOH-12 to the class of chemicals considered as toxic to aquatic life (Acute toxicity II).

Aquatic toxicity decreased when increasing the hydrophilicity of the surfactant molecule by adding an heteroatom to the spacer or replacing a methyl by a hydroxyethyl group in the ammonium polar head group. However, the structure of the spacer, rigid (benzene ring) or flexible (alkyl chain), has no a significant effect on the acute toxicity to *Daphnia magna*.

On the other hand, comparing the acute toxicity to *Daphnia magna* of gemini surfactants (Table 4) with that of monomeric surfactants, dodecyltrimethylammonium bromide (DT) and benzyldodecyldimethyl ammonium bromide (BDD), IC_{50} = 0.38 and 0.13 mg/L, respectively, (Garcia et al., 1999), dimeric surfactants result to be less toxic than monomeric surfactants

(Figure 2). In addition, since similar biocidal effect and surface activity can be attained by using smaller amounts of dimeric than monomeric surfactants (Negm et al., 2010; Tehrani-Bagha et al., 2015), the use of gemini surfactants seems to be less hazardous for the aquatic environment.

Figure 2. Comparison of acute toxicity to *Daphnia magna* of gemini (solid bars) and monomeric (dashed bars) quaternary ammonium-based surfactants

4. CONCLUSIONS

Quaternary ammonium-based gemini surfactants are not biodegradable by the CO₂ headspace test, a standard and reference method for ready biodegradability assessment. The failure in the biodegradation tests can be attributed to the toxic effects of these compounds towards the aerobic microorganisms responsible for biodegradation under the standard test conditions. The results of aquatic toxicity (*Daphnia magna* test) reveal that quaternary ammonium-based gemini surfactants are less toxic to the aquatic life than the monomeric ones. Furthermore, the

aquatic toxicity can be reduced increasing the hydrophilicity of the surfactant molecule by including a heteroatom in the spacer or replacing a methyl by a hydroxyethyl group in the ammonium polar head group.

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