INTRODUCTION

Toxicity bioassays play a crucial role in assessing the potential or actual impact of anthropogenic substances on the natural environment. They may be used to predict the ecological impact of toxic agents before their release (predictive approach) and to probe the extent to which an ecosystem has been perturbed by pollutants (monitoring approach) [1,2]. The need for toxicity bioassays for wildlife protection that are more stringent than traditional ones using adults or juveniles has recently encouraged the development of toxicity tests evaluating pollution effects in the more critical stages of animal life cycles, such as reproduction and embryo development [3]. Toxicty bioassays combining rapidity of response with high sensitivity and reproducibility are increasingly preferred to chronic bioassays in monitoring programs [4].

For marine environments, the reliability of the critical early stages of sea urchin as bioindicators is recognized worldwide [4–6], and standard procedures are available in some countries [7,8]. In Italy, several studies using embryo and larval stages have been carried out to study the toxicity and teratogenic activity of pure substances [9–11] and sediments [12,13], but less importance has been given to the use of gametes as biomonitoritors. Concerning toxicity tests using egg fertilization success as endpoint, most procedures are based on the exposure of sperm to a toxicant (the so-called sperm cell test) and are a derivation of the method originally developed by Dinnel et al. [14] for U.S. species. Sea urchin sperm cell test performances are well documented by Chapman [15]. A sperm cell toxicity test procedure has been developed by our research group using the North Adriatic autochthonous sea urchin Paracentrotus lividus Lamarck (Echinodermata: Echinoidea), the dominant echinoid species in the Mediterranean. The procedure derives from the general one proposed by Dinnel et al. [14] and is in harmony with U.S. Environmental Protection Agency standard procedure [16].

In the present work, the sensitivity and discriminatory ability of the sperm cell toxicity test were investigated for surfactants of the anionic (linear alkylbenzene sulfonates [LAS]) and nonionic (alcohol polyethoxylate [AE] and nonylphenol polyethoxylate [NPE]) types. The toxicity of the main aerobic biotransformation products of LAS, NPE, and AE was also examined. Sulfophenylcarboxylates (SPC), nonylphenol (NP), carboxylated AE (AEC), polyethylene glycols (PEG), and carboxylated PEG (PEGC) were tested in order to compare their toxicities with those of their parent surfactants. The choice of surfactants and their aerobic biodegradation products was motivated by their widespread presence in coastal marine environments and by the scarce amount of information on their effects on fertilization in marine organisms. Because of their affinity for the plasma membrane, surfactants can impoverish the reproduction of marine species with external fertilization, such as sea urchin, by acting on free gametes.

Although sea urchins have been used to assess the toxicity of many xenobiotic compounds, to the best of our knowledge, only toxicity data for the anionic surfactant C12,LAS toward embryo development of P. lividus have been reported. Alterations in skeletal development at commercial LAS concentrations >0.45 mg/L were observed, indicating that sea urchin embryos are more sensitive to this surfactant than mussels or tubificid oligochaetes [11].

Commercial LASs are mixtures of 10 to 13 homologues, each containing different isomers, depending on the phenyl...
Commercial NPEs are mixtures of ethoxymers (with usually 1–20 ethoxy groups and an average ethoxy number, eu AV, in the range of 9–10) and isomers (depending on the different alkyl branching of the nonyl chain attached to the aromatic ring). The biodegradation of NPE may produce carboxylated NPE, containing a carboxyl group on the polyethoxyl chain, or shorter ethoxymers, as well as the completely deethoxylated product NP [19,20]. Nonylphenols have higher environmental persistence than NPE parent surfactants due to their lower biodegradability and higher hydrophobicity [21]. The toxicity of NPE generally increases with decreasing ethoxylic chain length because of the increased hydrophobicity of the molecule. A review of the literature data shows for NPE median lethal concentration (LC50)/median effective concentration (EC50) values differing by some orders of magnitude with changing chain length (eu AV = 1–50), whereas NP has acute toxicity values that are generally one to three orders of magnitude lower than those of the parent compounds [22].

Aliphatic alcohol polyethoxylates are the most extensively used nonionic surfactants. The AE commercial blends are complex mixtures of homologues with linear and monobranched alkyl chains with 11 to 18 carbon atoms. Multibranched AE, synthesized via ethoxylation of isostearic acid (C18, C34, C57 homologue mixtures), still account for approximately 10% of overall AE production. Each AE homologue has a Poisson-like ethoxymeric distribution, with typically 1 to 30 ethoxy units and an average ethoxylation number (eu AV) in the range of 5 to 15. Polyethylene glycols are the main biodegradation products of AE, formed through attack on the alkyl carbon atom linked to the polyethoxyl chain (central cleavage mechanism) or by hydrolytic, nonoxidative shortening of the AE alkyl chain. Other important aerobic metabolites of AE are carboxylated AE, arising from the oxidation of the alkyl and/or polyethoxy groups of the AE molecule [23,24]. Acute AE toxicity depends both on length and branching of the alkyl chain as well as on the length of the polyethoxylic chain; in fact, toxicity decreases with the increase of the ethoxylic units and branching of the alkyl chain, whereas it increases with the length of the alkyl chain [25].

Laboratory-derived acute (EC50 or LC50) toxicity data for all compounds investigated in this study are well documented in freshwater species. Fewer studies have examined the acute toxicity of LAS [18,26], NPE and NP [22], and AE [25,27,28] toward marine species, and no information about the toxicity of LAS and AE biodegradation products can be found in the available literature. The acute toxicities of LAS, NPE, and their biodegradation compounds, SPC and NP, respectively, have also been investigated by in vitro tests [29]. Laboratory-based chronic (no observed effect concentration) toxicity data for all tested compounds are available for freshwater species but are very scarce for marine organisms; only NPE and NP have been extensively studied [22]. Recently, toxicological interest in NP has increased because there is concern that it may interfere with endocrine functions in both vertebrates [30] and invertebrates [31].

Literature toxicity data often lack detailed structural information about studied compounds, particularly LAS and AE. Moreover, most of the quoted toxicological studies do not focus on molecular structure/toxicity relationships. Chemically and biologically exhaustive results on surfactants, such as LAS and AE, are available only toward standard freshwater species, e.g., *Daphnia magna* and *Pimephales promelas* [32,33].

The aims of the present work were to investigate the discriminatory ability of the sperm cell toxicity test procedure by studying the toxic effects of surfactants and their biodegradation products and to relate the EC50 value of each compound with its chemical structure in order to elucidate the structure/toxicity relationships. Another objective of this study was to obtain original toxicity data for sea urchins regarding surfactants; greater attention was applied to AE isoforms and their biodegradation compounds because of their current widespread use and the great scarcity of data in available literature for marine species. Data were discussed comparing the sensitivity of the test with available toxicity data for other marine biological systems and in vitro tests.

**MATERIALS AND METHODS**

**Chemicals**

Copper nitrate, potassium chloride, and ethanol were analytical-grade products purchased from Merck (Milan, Italy), Carlo Erba (Milan, Italy), and Prolabo (Paris, France), respectively. Inorganic salt used for preparing artificial seawater (Ocean Fish) for experiments was purchased from Prolabo International (Cittadella, Padua, Italy). Deionized water for toxicity bioassays was purified by a MilliR® system (Millipore, Bedford, MA, USA).

The tested commercial surfactants (95 to >99% active material) were kindly provided by the manufacturers. The single homologue anionic surfactant C12 LAS was from W. Kolb AG (Hedingen, Switzerland) and the standard C12–C13 LAS mixture (C12 LAS, analytical grade), with an average alkyl chain length (ac AV) of 11.6, was from Carlo Erba (Milan, Italy). Individual SPC isomer 2-sulphophenylbutyrate (C12 SPC) was synthesized according to the literature [34]. The compound 11-sulphonylundecanoate (C11 SPC) was technical grade from Eastman Kodak (Rochester, NY, USA). Imbentin 120/90 (L-C12 AE), from Kolb AG, is an oleochemical AE containing only the C12 AE homologue with an average number of ethoxy units (eu AV) of 9. Marlipal 28/100 (L-C13 AE), from Hüls AG (Marl, Germany), is an oleochemical AE containing C12, C14, C16, and C18 AE homologues (55, 20, 9, and 16%, respectively, by weight, as reported by the manufacturer), with an eu AV of 10 and an ac AV of 13.7. Lialtet 125/7 (O-C16 AE), from Condea Augusta (Milan, Italy), is a mixture of linear and monobranched C12–C15 AE with an eu AV of 7 and an ac AV of 13.6. The composition (w/w) of the parent unethoxylated alcohol mixture of Lialtet 125/7, provided by the manufacturer, indicated that 46% of the oxo-AE mixture is linear AE and 54% is 2-alkyl substituted AE isomers. Novel 12/5 (B-C12 AE), from Condea Augusta, is a fully monobranched C12 AE, namely 2-buty1, octyl alcohol polyethyloxylate (2Bu-O-C12 AE), with a 2-alkyl side chain with four carbon atoms and an eu AV of 5. Isotridecanol EO8 (M-C13 AE), from Condea Augusta, is a multibranched AE mixture with an eu AV of 8 and an ac AV of 13. Lialtet 123/6, 5-COOH (AEC-5.5) and Lialtet 123/4, 5-COOH (AEC-3.5), from Condea Augusta, are two C12–C13 oxo-AE mixtures containing a carboxylic group at the terminal carbon of the polyethyloxyl chain, eu AV of 5.5 and 3.5 respectively, and an ac AV of 12.4. The nonylphenol polyethoxylate NPE10 (NPE), from Carlo
Erba, is a standard mixture (analytical grade) with an eu₄₀ of 10. The compounds PEG200, PEG400, and PEG3000 (purity > 98%) are polyethylene glycol mixtures from Fluka (Buchs, Switzerland) with average eu₄₀ of 4.5, 9, and 68, respectively. The dicarboxylated PEG oligomer 3,6,9-trioxadacanedioic acid (E₄DC) and 4-nonylphenol (NP) were both technical grade from Fluka. All organic solvents employed were high performance liquid chromatography grade from Baker (Deventer, Netherlands). Water for chromatographic purposes was purified by a MilliQ system (Millipore).

**Sperm cell toxicity test: procedure and intralaboratory reproducibility**

The test procedure was developed according to the autoecological characteristics of *P. lividus*, following the pioneering paper of Dinnel et al. [14], in which the author listed 13 general steps that should be followed to maintain a standardized method using any echinoderm species. Some modifications were introduced in order to increase the reliability and reproducibility of results.

Sea urchins were induced to spawn by injecting 1 ml of 0.5 to 1 M KCl solution into the coelom through the peristome. The animals were allowed to spawn for about 30 min in 50 ml of artificial seawater (35%) and then the sperm obtained from a minimum of three males [16] was put together in a volume of seawater that depended on the abundance of emission. The density (number of sperm cells/L) of this prediluted sperm suspension was determined by adding a 0.1-ml subsample to 1 ml of glacial acetic acid in a 10-ml graduated cylinder, brought to volume with artificial seawater. Counts were performed using a Neubauer hemocytometer counting chamber (American Optical, Buffalo, NY, USA) under a dissecting microscope at ×40, applying the formula reported in step 4 of Dinnel’s procedure. The eggs obtained from a minimum of three females [16] were filtered through 400-µm nylon gauze and put together to obtain a prediluted egg suspension in a large beaker. Some previous experiments had shown that, using separately the eggs obtained from three females in a sperm cell toxicity test, their successful fertilization was closely linked to small differences in egg quality, less evident when the eggs were put together.

The sperm:egg ratio was fixed at 20,000:1, the optimal value for *P. lividus*. In order to keep this ratio constant, sperm density had to be adjusted to a suitable test density (4 × 10⁶), applying the formula reported in step 6 of Dinnel et al.’s procedure [14]. The results of a set of experiments had shown that, at a sperm:egg ratio of 5,000:1, fertilization success was low (39 ± 3.5%), while at larger ratios (10,000:1, 20,000:1, and 30,000:1), a larger percentage of fertilized eggs resulted (88 ± 4.24%, 92 ± 3.46%, 95 ± 1.15%, respectively). Consequently, a sperm:egg ratio of 20,000:1 was chosen as the most suitable to ensure higher constancy in the percentage of fertilization in controls.

A volume of 0.1 ml of adjusted sperm suspension (prethermostated at 18°C) was exposed to 10-ml aliquots of test solution (prepared using 35% artificial seawater) and left to incubate in a thermostatic bath at 18°C for 60 min. During sperm exposure, egg suspension density was determined by adding 0.1 ml of the prediluted suspension of eggs in a 10-ml graduated cylinder and counting 1 ml of this diluted suspension using a plankton counting camera under a dissecting microscope at ×10. In order to keep the sperm:egg ratio constant, egg density had to be standardized to 2,000/ml, applying the formula reported in step 8 of Dinnel’s procedure. After 60 min of exposure, 1 ml of standardized egg solution (prethermostated at 18°C) was added to the sperm suspension, and a period of 20 min was allowed to pass to ensure that fertilization had occurred. Samples were preserved by 1 ml of concentrated buffered formalin.

The percentage of fertilization in each treatment was determined by counting (with the plankton counting camera) 200 eggs instead of 100, as reported by U.S. Environmental Protection Agency procedure [16], in order to increase the reliability of the assessment. Repeated counts of 50, 100, 200, and 300 eggs showed that determination of fertilization percentage was more reliable when the number of counted eggs was at least 200. Counts of 50 and 100 eggs provided coefficients of variation, respectively, of 11.6 and 14%, whereas when the number of counted eggs was increased, the coefficient of variation decreased to 4% and 3% for 200 and 300 eggs, respectively.

The intralaboratory reproducibility of the method was evaluated by generating a reference toxicant chart (copper as reference toxicant) in four years of experiments (from 1995 to 1999) by different operators (4) using adult organisms from different batches (13) collected in different sampling sites (5) along the Northern Adriatic coast. This coast is heavily anthropized; eastward (Gulf of Trieste, Dalmatian coast) it presents rocky shores, whereas westward (Gulf of Venice) it is mainly sandy, formed by deposition of fine sediment and sand by rivers. In this environment, hard substrata suitable for sea urchin colonization are mainly artificial (i.e., jetties, rocky break waters, rocky embankments), and this framework makes possible the presence of different habitats in a few miles. The sampling sites are named and located as follows: site A, on a rocky embankment of the island of Pellestrina (Gulf of Venice); site B, on a rocky break water of the island of Lido (Gulf of Venice, about 7 km from site A); site C, on a rocky break water inside the Lagoon of Venice; site D, in the Gulf of Trieste (about 115 km from site A); site E, on a jetty of the island of Lido (about 15 km from site B). The recent artificial construction of new beaches along the Venetian coastline has progressively caused a loss of hard substrata, probably forcing sea urchins to move. This was one of the main reasons for looking for new sampling sites.

All experiments were performed during the reproductive period of the species. A maximum of 100 animals for sampling was stored in one 250-L glass aquarium containing filtered and aerated natural seawater kept in a conditioned room at 18 ± 0.5°C with a natural photoperiod. Animals used for experiments were kept in an identical aquarium and then taken back to the sea. Sea urchins were fed on macroalgae (*Ulva* sp.) and mollusks (*Mytilus galloprovincialis*) collected at the same sampling sites and were kept at mean salinity and a pH of 35 ± 1.6% and 7.78 ± 0.38, respectively. Sea urchins were used for tests after at least one week of acclimatization.

Sperm cell toxicity tests were carried out from 1995 to 1996 after the setting-up of the method for an initial evaluation of test precision. These experiments were conducted by one operator using different animal batches from sampling site A. The method showed good precision, with a mean EC₅₀ ± standard deviation (SD) (as Cu²⁺) of 0.058 ± 0.0042 mg/L (coefficient of variation [CV] = 7.2%, n = 9) and high quality of data with very narrow 95% confidence ranges (confidence range percentage showed a mean ± SD of 7.28 ± 2.63%) due to the high replicability of the test (95% confidence ranges of
Surfactant's toxicity using sea urchin sperm cell test

Courtney E. Steenbergen

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Results obtained for copper by this procedure with the Mediterranean species are in good agreement with those found in the literature for other North American echinoid species in comparable procedures. In fact, the literature reports very close data for Strongylocentrotus droebachiensis (EC50 = 0.059 mg/L) [35] and similarly for Arbacia punctulata (EC50 = 0.028 mg/L) [4], Strongylocentrotus purpuratus (EC50 = 0.031 mg/L) [4], and Dendraster excentricus (EC50 = 0.026 mg/L) [35].

Toxicity tests using surfactants

Each toxicant solution was prepared by dissolving a concentrated solution in artificial seawater (pH 8 ± 0.2) and stirring it at test temperature (18 ± 0.1°C). All experiments were performed at a constant temperature 18 ± 0.1°C using the same 35% seawater for control tests and diluting solutions in 50-ml glass beakers, used as test chambers. Static experiments (sperm exposure = 60 min) were performed using the above-reported procedure maintaining test chambers with gentle stirring using a thermostatic oscillating water bath in order to ensure better homogeneity of the suspension.

The compounds NP, B-C12AE, and M-C13AE needed ethanol as organic cosolvent because of their low water solubility. A maximum allowable concentration of 0.1%, i.e., the highest concentration below the detectable toxicity level of the sperm cells, was previously determined for ethanol. Artificial seawater of 35% salinity with 0.1% ethanol concentration (v/v) was used in all dilutions for the above-mentioned three toxicants. Preliminary tests, at concentration levels distant by one order of magnitude, were performed for each toxicant in order to determine the 0 to 100% range of fertilized eggs. In definitive tests, male gametes were exposed to six toxicant concentrations chosen (at a logarithmic distance) in the range predetermined by preliminary tests plus a control test in which gametes were exposed only to artificial seawater. Three replicates were used for each concentration level and for control tests. The reliability of the toxicity test was verified throughout the experiments using the reference toxicant.

The EC50 values with 95% confidence intervals were calculated by two statistical methods, the trimmed Spearman–Karber [36] and the probit. The test responses for each treatment (percent of fertilized eggs) were corrected applying Abbott's formula [37] in order to take into account effects in control tests.

RESULTS AND DISCUSSION

The chemical structures of the tested compounds together with the mean EC50 values with 95% confidence interval are listed in Tables 1 and 2. The EC50 values obtained using the two statistical methods were always very similar, so only EC50 calculated by the trimmed Spearman–Karber method are reported here.

In all experiments, control tests showed 93.98 ± 1.98% of fertilized eggs, higher than the 70% minimum level recommended by U.S. Environmental Protection Agency [16]. The high reliability of the data, highlighted by the very narrow 95% confidence interval (<5%) for all tested compounds is due to factors such as the high biological homogeneity (because the same pool of gametes was used for all replicates), the huge number of male gametes employed in each test, and the high number of counted eggs, providing low coefficients of variation.

The average EC50 value measured with the reference toxicant during the experiments was 0.056 ± 0.012 (n = 3). As reported in the Materials and Methods, this value was very close to previously recorded values (0.058 ± 0.0042, n = 9) and was perfectly included in the acceptability range.

The EC50 values are distributed over a wide range, about four orders of magnitude, with a maximum of >200 mg/L for PEG, C8-SPC, and EO, and a minimum of 0.062 mg/L for L-C13,7AE. An upper limit of 200 mg/L, at which no effect was observed, was chosen since higher concentrations are not realistic in the aquatic environment. The toxicity of the tested compounds depended strongly on their chemical structure, in particular on the lipophilic portion of the molecule. This portion has high affinity to biological structures and allows these compounds to pass easily through the cell membrane and consequently to induce toxic effects.

Linear alkylbenzene sulfonates and SPC

The toxicity of linear alkylbenzene sulfonates (LAS) increased with increasing alkyl chain length, thus increasing hydrophobicity (Table 1). The pure compound C13LAS, with its relatively short alkyl chain, was less toxic (EC50 = 2.71 mg/L) than the C11,6LAS standard mixture (EC50 = 1.12 mg/L); an increase in the average alkyl chain length of 2.6 carbon atoms led to an increase in toxicity of about 2.5 times. The toxicity increase with increasing alkyl chain length is in good agreement with studies carried out on D. magna [18] and P. promelas [38].

The literature reports LAS acute toxicity data toward marine species over a wide range (0.1–>100 mg/L), with higher LC50-EC50 values for adult bivalves and crustaceans and lower ones for several fish species [18,26]. Most data on LAS toxicity come from very old papers lacking information on the chemical composition of the tested LAS, so no real comparison is possible with other marine species. The P. lividus embryos may be more sensitive to C11,6LAS than male gametes; a high percentage of alterations in skeletal development at concentrations >0.45 mg/L has been reported [11] but cannot really be compared with our data because the two experiments were carried out at different temperatures (25 vs. 18°C). Comparable sensitivity with SMP (EC50 = 0.6 mg/L) is found for C11,6LAS [29].

The LASs are aerobically biotransformed to SPC through oxidation of the alkyl chain, thus increasing hydrophilicity and reducing toxicity [18]. The measured toxicity for C11,6SPC and C13SPC provided EC50 values of 21.3 and >200 mg/L, respectively (Table 1). The toxicity value of C11,6SPC was about one order of magnitude lower than that of C11,6LAS, despite the similar number of carbon atoms in the alkyl chain. The different structure, a terminal carboxyl group in C11,6SPC in-
Table 1. Chemical structures, acronyms, and toxicity values (median effective concentration [EC50] and 95% confidence limits [CL]) for Paracentrotus lividus for linear alkylbenzenesulfonates (LAS), nonylphenol polyethoxylates (NPE), and their biodegradation products; for C4-SPC, no observable effect until 200 mg/L was found.

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Acronym</th>
<th>Compound</th>
<th>EC50 (95% CL)</th>
</tr>
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<tbody>
<tr>
<td>( R - C_{11} - SO_3^- )</td>
<td>C_{11}-LAS</td>
<td>Nonylbenzene sulfonate mixture</td>
<td>1.12 (1.10–1.14)</td>
</tr>
<tr>
<td>( R - C_{12} - SO_3^- )</td>
<td>C_{12}-LAS</td>
<td>2-Sulfophenyl butirate</td>
<td>&gt;200</td>
</tr>
<tr>
<td>( R - (CH_2)<em>{10} - C</em>{11} - SO_3^- )</td>
<td>C_{11}-SPC</td>
<td>11-Sulfophenyl undecanoate</td>
<td>21.3 (21.1–21.5)</td>
</tr>
<tr>
<td>( R - HO - C - CH_2 - CH_2 - OH )</td>
<td>NPE</td>
<td>Nonylphenol polyethoxylate with eu_{AV} = 10</td>
<td>1.94 (1.91–1.97)</td>
</tr>
<tr>
<td>( R = C_9 ) multbranched</td>
<td></td>
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<tr>
<td>( R = C_9 ) multbranched</td>
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\( ^1 \text{eu}_{AV} = \text{average number of ethoxy units.} \)

stead of a methyl group in C_{11}-LAS, is responsible for this significant toxicity decrease and confirms data from the literature on the structure–toxicity relationships of SPC, i.e., the toxicity of SPC increases with increasing length of the carboxylated alkyl chain. In fact, for C_{4}-SPC and C_{11}-SPC, acute toxicity for standard freshwater species *D. magna* and *P. promelas* gives EC50 values of >6,000 and 77 to 200 mg/L, respectively [18]. To the best of our knowledge, no data of effects of SPC on marine species, nor chronic toxicity information, are available for comparison.

**Nonylphenol polyethoxylates and nonylphenol**

The toxicity of NPE (eu_{AV} = 10) and NP was assessed through the sperm cell toxicity test with *P. lividus*, yielding EC50 values of 1.94 and 0.27 mg/L, respectively (Table 1). The presence of an average number of 10 ethoxylic groups in the NPE commercial mixture, increasing its hydrophilicity, makes this molecule about one order of magnitude less toxic than NP, which contains no ethoxylic groups. The increasing toxicity of the biodegradation compound, NP, is in agreement with literature data [22].

Most common commercial compounds (eu_{AV} = 9–10) show most acute data in the 2 to 14 mg/L range, with higher values for some adult crustacean species (>100 mg/L) and for *Photobacterium phosphoreum* (60 mg/L). The EC50 value yielded by the sperm cell toxicity test for NPE (eu_{AV} = 10) is in agreement both with available acute toxicity values for the marine fish *Gadus morrhua* and *Pleuronectes flesus* (96-h LC50 = 2 and 3 mg/L, respectively) [22] and for SMP (EC50 = 1.3 mg/L) [29] and is also close to that obtained for the similar compound NPE (eu_{AV} = 9) for the mysid *Mysisopsis bahia* (48-h LC50 = 2 mg/L) [22].

Most literature EC50-LC50 values for NP are in the range 0.027 to 0.37 mg/L [22], including the EC50 value obtained for *P. lividus* (0.27 mg/L). Also for this compound, the sensitivity of the sperm cell test is very close to that of fish species (96-h LC50s were 0.31 and 0.37 mg/L for *Cyprinodon variegatus* and *Gasterosteus aculeatus*, respectively), while algae (*Skeletonema costatum*, 96-h EC50 = 0.027) and crustacea (*M. bahia*, 96-h LC50 = 0.043) show higher sensitivity [22]. Available NP chronic toxicity data on reproduction for crustacea are some orders of magnitude lower (*M. bahia*, no observed effect concentration = 0.007) than the EC50 value for *P. lividus*, indicating that the sperm cell toxicity test cannot absolutely mime the chronic effects of NP on reproduction. Recent studies evidenced that NP disturbs steroid-metabolic processes, with serious implications for reproductive functions in crustacea; exposure of *Daphnia* sp. to NP decreased fecundity and affected environmental sex determination (female offspring) and normal development [31,39].

**Alcohol polyethoxylates and biodegradation compounds**

The five tested AE mixtures are very representative, in terms of molecular structure, of the marketed blends, which contain linear, monobranched, and multibranched AE homologues (Table 2). The compound L-C_{13,7}AE, a commercial mixture of fully linear AE homologues with an ac_{AV} of 13.7, was the most toxic AE, with an EC50 value of 0.062 mg/L. By comparison, the tested L-C_{12}AE blend, containing only the C_{12} homologue, showed a much higher EC50 value of 0.94 mg/L,
Table 2. Chemical structures, acronyms, and toxicity values (EC50 [median effective concentration] and 95% confidence limits [CL]) for *Paracentrotus lividus* for alcohol polyethoxylates (AE) and their biodegradation products; for polyethoxylates PEG200, PEG400, PEG3000, and E₄DC, no observable effect until 200 mg/L was found.

| Chemical structure | Acronym | Compound | EC50 (95% CL) (
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<tbody>
<tr>
<td>R-(O-CH₂-CH₃)-OH</td>
<td>L-C₁₂AE</td>
<td>Dodecanol polyethoxylate with acₑᵥ₅ = 9</td>
<td>0.94</td>
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<tr>
<td>R = C₁₂ linear</td>
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<tr>
<td>R-(O-CH₂-CH₃)$_n$-OH</td>
<td>L-C₁₃.₇AE</td>
<td>Linear C₁₂,₁₄,₁₆,₁₈ AE blend with acₑᵥ₅ = 13.7 and euₑᵥ₅ = 10</td>
<td>0.062</td>
</tr>
<tr>
<td>R = C₁₂,₁₄,₁₆,₁₈ linear; n = 10</td>
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<tr>
<td>R-(CH₂-CH₃)₂-O-(CH₂-OCH₂)$_n$-OH</td>
<td>O-C₁₃.₅AE</td>
<td>Oxo-C₁₃-C₁₅ (46% linear, 54% monobranched) blend with acₑᵥ₅ = 13.6 and euₑᵥ₅ = 7</td>
<td>0.12</td>
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<tr>
<td>R' = CH₃</td>
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<tr>
<td>R' = H, Methyl, Ethyl, Propyl</td>
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<tr>
<td>n = 7</td>
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<tr>
<td>R-(CH₂-CH₃)$_n$-OH</td>
<td>B-C₁₂AE</td>
<td>Monobranched C₁₂AE with euₑᵥ₅ = 5</td>
<td>4.03</td>
</tr>
<tr>
<td>R = C₁₂ linear</td>
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<td></td>
</tr>
<tr>
<td>R' = Buetyl</td>
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<td></td>
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<tr>
<td>n = 5</td>
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<td></td>
</tr>
<tr>
<td>R-(O-CH₂-CH₃)$_n$-OH</td>
<td>M-C₁₂AE</td>
<td>Multibranched AE blend, with acₑᵥ₅ = 13 and euₑᵥ₅ = 8</td>
<td>0.92</td>
</tr>
<tr>
<td>R = C₁₃ multibranched; n = 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-O(CH₂-OCH₂)$_n$-OH</td>
<td>PEGs 200, 400, 3000</td>
<td>PEG blend with euₑᵥ₅ = 4.5, 9, 68</td>
<td>&gt;200</td>
</tr>
<tr>
<td>n = 4.₅, 9, 6₈</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOOC-CH₂-O(CH₂-CH₃)$_n$O-CH₂OOH</td>
<td>E₄DC</td>
<td>Carboxylated PEG with euₑᵥ₅ = 4</td>
<td>&gt;200</td>
</tr>
<tr>
<td>n = 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-(O-CH₂-CH₃)$_n$CH₂-COOH</td>
<td>AEC-3.₅</td>
<td>Carboxylated AE blend with euₑᵥ₅ = 3.₅, 5.₅</td>
<td>2.₉₈</td>
</tr>
<tr>
<td>R = C₁₃ partially branched; n = 3.₅, 5.₅</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC-₅.₅</td>
<td>4.₄₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2.₈₇–3.₁₀)</td>
<td>(4.₃₃–₅.₅₀)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* acₑᵥ₅ = average number of ethoxy units.
* acₑᵥ₅ = average length of alkyl chain.

About one order of magnitude greater. An increase in alkyl chain length equivalent to 1.7 carbon atoms produced a toxicity increase of about 15 times. This remarkable influence of alkyl chain length is similar to that reported for LAS homologues [40].

Toxicity of AE toward *P. lividus* also depended on alkyl chain branching. The fully linear individual AE homologue L-C₁₂AE had an EC50 value of 0.94 mg/L, whereas the fully monobranched AE B-C₁₂AE, with the same number of carbon atoms and an alkyl side chain of four carbon atoms, had an EC50 value of 4.03 mg/L. The presence of an alkyl side chain induced a decrease in toxicity of about four times. The influence of branching on AE toxicity was confirmed by data from the other tested AE blends. O-C₁₃.₅AE, a commercial mixture of linear (46%) and monobranched (54%) C₁₂-₁₅AE homologues with an acₑᵥ₅ of 13.6, had an EC50 value of 0.062 mg/L, about two times higher than that exhibited by the fully linear L-C₁₃.₇AE (0.062 mg/L). The lower toxicity of O-C₁₃.₅AE with respect to L-C₁₃.₇AE may be ascribed to the monobranched isomers (with an average length of two carbon atoms) contained in the tested blend, which appear to mitigate the toxicity of the overall mixture. The potential toxicity of the monobranched portion of the O-C₁₃.₅AE mixture toward *P. lividus* may be estimated by taking into account the amount of monobranched isomers (54%, by weight) in the tested blend and the measured EC50 values of O-C₁₃.₅AE (0.12 mg/L) with respect to the fully linear L-C₁₃.₇AE (0.062 mg/L). The estimated value of the monobranched AE component, 0.18 mg/L, is about three times lower than that shown by L-C₁₃.₇AE and shows good agreement with the experimental values obtained for L-C₁₂AE and B-C₁₂AE. The length of the side chain does not seem to induce any detectable effects; the observed toxicity decrease of O-C₁₃.₅AE with respect to L-C₁₃.₇AE (obtained by comparing the estimated value for the monobranched portion of O-C₁₃.₅AE with the experimental values of L-C₁₃.₇AE, 0.18 and 0.062 mg/L, respectively) and of B-C₁₂AE with respect to L-C₁₂AE (4.03 and 0.94 mg/L, respectively) was similar, despite the different lengths of the alkyl side chain (two and four carbon atoms, respectively). A decrease in AE toxicity toward *P. lividus* of three to four times may be expected when a monobranched AE instead of a linear AE with the same alkyl chain length is tested. The compound M-C₁₃AE, a commercial multibranched AE blend with several methyl groups bound to the alkyl chain, further elucidated the influence of branching on AE toxicity and yielded a toxicity value of 0.92 mg/L. The presence of several methyl groups along
the alkyl chain dramatically lowers the toxicity of M-C_{12}AE with respect to O-C_{13.6}AE, by about one order of magnitude. Experimental results from tests on the main biointermediates of AE, i.e., PEG (arising from central scission of the AE molecule), carboxylated PEG (produced by oxidative shortening of the AE alkyl chain), and carboxylated AE (forming as a result of hydrolytic-oxidative attack on the AE chain), indicate that their biodegradation products are far less toxic than their parent surfactants. The EC50 values of PEG and carboxylated PEG for *P. l l i v i d a s* were >200 mg/L for all tested oligomers, regardless of their average ethoxy unit number (eu_{AV} = 4.5, 9, 68, and 4, respectively). The toxicity of carboxylated AE was also tested; AEC-3.5 and AEC-5.5 are two AE blends with average ethoxy numbers of 3.5 and 5.5, respectively, containing the same alkyl chain structure and a carboxyl group on the polyethoxyl chain. An increase in the toxicity of carboxylated AE with decreasing ethoxy unit number was observed, AEC-3.5 and AEC-5.5 yielding EC50s of 2.98 and 4.41 mg/L, respectively. These values are about one order of magnitude lower than those of the parent AE blends, such as O-C_{13.6}AE (0.12 mg/L).

The relationships between toxicity and structural characteristics of the molecule demonstrated for AE parent compounds confirm the statement of Dorn et al. [25], showing clearly that acute toxicity depends on both length and branching of the alkyl chain. Literature toxicity data on AE often lack detailed structural information about the chemical composition of the tested blends. No comparisons can be made with other literature data because no data for marine organisms are available, to the best of our knowledge, for the tested compounds. No information about the toxicity of AE biodegradation products has been found in the available literature.

**CONCLUSIONS**

The sperm cell toxicity test showed good discriminatory ability among all the tested compounds; EC50 values differ by about four orders of magnitude, ranging from 0.06 to >200 mg/L. This feature allows the test to record significantly different toxicity responses even when differences in molecular structure are slight.

Experimental results show that LAS toxicity depends mainly on the length of the alkyl chain and AE toxicity is due to both alkyl chain length and branching, i.e., the presence of alkyl side chains. The biodegradation products of both LAS and AE have lower toxicity than their parent surfactants, whereas the biodegradation product NP has greater toxicity than NPE. These structure–toxicity relationships are in good agreement with data from other studies.

The obtained EC50s were comparable with available literature data except for NP. They constitute new toxicity data regarding surfactants for sea urchins. Moreover, original information was obtained on toxicity of AE and their biodegradation compounds in view of their current widespread use and of the great scarcity of data for marine species from the available literature.

The sperm cell toxicity test toward *P. l l i v i d a s* yielded reliable results for surfactants and their biointermediates and demonstrated its predictive value in determining the toxicity of xenobiogenic compounds with similar molecular structures. The test also combines good sensitivity and discriminatory ability with rapidity, reproducibility, and ecological relevance. These characteristics make it a good instrument for monitoring purposes in coastal and marine environments.

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Surfactant’s toxicity using sea urchin sperm cell test