




Communication

The Number of Ethylene Oxide Groups of Sulphate-Based Surfactants Influences the Cytotoxicity of Mixed Micelles to an Amphibian Cell Line

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Abstract: Sodium lauryl ether sulphate (SLE_nS) is an anionic surfactant that is widely used in many fields, such as cosmetics and detergents, among others. This study evaluated the influence of the number of ethylene oxide (EO) units, present in the head group of SLE_nS, on its cytotoxicity to the A6 cell line of *Xenopus laevis* using MTT and resazurin assays. The A6 cell line was exposed for 48 h to six SLE_nS variants: SLE₀S, SLE₁S, SLE₄S, SLE₁₁S, SLE₃₀S, and SLE₅₀S (subscript values correspond to the number of EO units). Overall, the six variants impaired the A6 cells' viability at low concentrations for the MTT assay, with the median lethal concentrations (LC_{50,48h}) ranging between 0.398 and 0.554 mg/L and for the resazurin assay between 0.557 and 0.969 mg/L. Further, the obtained results indicate SLE_nS variants with fewer EO units to be the most cytotoxic in the resazurin assay; although a similar cytotoxicity pattern was observed with the MTT assay, a significant association between the number of EO units and the values of LC₅₀ was not found. This result highlights the usefulness of in vitro assays with A6 cell lines as a first screening tool for assessing the structure–toxicity relationship of this type of surfactant, also providing a baseline for the development of environmentally friendlier chemical compounds while still maintaining their efficiency.

Keywords: in vitro assay; *Xenopus laevis*; cell viability; sodium lauryl ether sulphate; structure–toxicity assessment; A6 cell line

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1. Introduction

Sodium lauryl ether sulphates (SLE_nS) consist of anionic surfactants that are used worldwide in pharmaceutical and cosmetic products, cleaning products and detergents [1,2], pesticides [3], and land excavation processes [4], among others. Given the wide use of these surfactants, their occurrence in the environment is expected. Whereas information on the concentrations of SLE_{n>1}S in the aquatic environment is scarce, concentrations of sodium lauryl sulphate (SL₀S) ranging from 0.018 to 5.94 mg/L have been measured in urban effluents, agriculture, and industrial sewages [5,6], and concentrations from 40 to 500 mg/kg were measured in soil near excavation industries [4]. The presence of SLE_nS in these abiotic components raises environmental concerns since it can have adverse effects on the environment, with concentrations ranging from 0.004 to 3509 mg/L known to cause toxicity to biota, namely, aquatic species [7,8]. This reported toxicity has been proven to be dependent on many biological (e.g., species sensitivity) and physicochemical (e.g., water hardness, temperature) factors [9,10]. Specifically, the chemical structure of SLE_nS, as it regards the degree of ethyl oxidation (i.e., number of ethylene oxide groups on its headgroup), has been shown by Martins et al. [1] to change its toxicity, when present in

mixed micelles, to the marine bacterium *Vibrio fischeri*. The study showed that the bioluminescence of *V. fischeri* was less affected by micelles containing SLE_nS variants with a higher number of EO units. Also, Wong et al. [11] reported that a fewer number of EO units in nine alcohol ethoxylate surfactants led to increased acute toxicity to the zooplankton species *Daphnia magna* and the fish *Pimephales promelas*. Despite these two examples, few studies addressing the influence of the structure of SLE_nS on its toxicity to aquatic biota have been published. But understanding the structure–toxicity relationship of these types of polymers is of the most relevance to make predictions of their toxicity when conducting risk assessments using frameworks and supporting the design of more ecofriendly structures. With this type of study, when targeting the characterization of the effects on freshwater vertebrates, ethical issues arise related to the welfare and protection of animals used in scientific procedures, as regulated by the European Directive for animal protection (Directive 2010/63/EU). In this context, *in vitro* toxicity assays have been proposed as a good first screening alternative to the use of *in vivo* assays, thus minimizing the use of animal experimentation. Further, *in vitro* testing is considered a high-throughput, fast, and cheap methodology [12], allowing for the testing of a wider range of substances simultaneously at a higher number of concentrations, characteristics that are a major advantage when performing screenings of the influence of the structure–toxicity relationships of chemicals. The effect of surfactants on cells involves destabilizing the cell membrane through flip-flop movements, leading to phospholipid hydrolysis, protein denaturation, and membrane leakage [13,14]. This *in vitro* approach was used in the present work to predict the effects of SLE_nS variants on amphibian cell lines. Among the four classes of vertebrates, Amphibia is considered the most threatened, with 41% of extant species classified as threatened with extinction [15]. Chemical pollution has been pointed out as being amongst the most relevant factors contributing to this conservation status of amphibians, namely, because these organisms have highly permeable skin and a rudimentary immune system [16]. Nevertheless, amphibians typically have aquatic early life stages, which make them high-risk targets for exposure to surfactant contamination, since water is considered the ultimate fate of most surfactants [17]. Therefore, studying the potential risks that different surfactants pose to this group of organisms is highly relevant. The present study assessed the influence of the number of EO units present in the polar head group of SLE_nS on the cytotoxicity of mixed micelles, which is formed with alkylbenzene sulfonic acid, to the A6 cell line, which was derived from a male *Xenopus laevis*. This *in vitro* methodology was used as a preliminary and high-throughput approach to infer the possible impacts that these surfactants may have on amphibians, as recommended by several legislative guidelines regarding animal experimentation (e.g., EU Directive 63/2010). This cell line, A6, is one of the most used models for *in vitro* studies regarding amphibians and is responsive to several toxins (e.g., cadmium) [18,19] and mitogens (e.g., flavonoids) [20]. In addition, since the tissue of origin is the kidney, all processes concerning membrane dynamics are well studied [21,22], making this model suitable for this study.

2. Materials and Methods

2.1. Test Substance

The cytotoxicity of six variants of mixed micelles made of two anionic surfactants (sodium lauryl ether sulfate (SLE_nS) and alkyl benzene sulfonate (LAS)) was tested (Figure 1). A mixture of these two anionic surfactants was selected because it has been shown that together they self-assemble at a lower critical micelle concentration compared to when they are found alone, thus requiring a lower concentration of the surfactants, which leads to a higher viscosity of the final mixture, decreasing skin irritability (e.g., [23]). These micelles were obtained according to Martins et al. [1]. In brief, SLE_nS and LAS were mixed in distilled water using a ratio of 1:2 with 3300 mg/L of SLE_nS and 6600 mg/L of LAS. While LAS was kept constant in each micelle variant, SLE_nS varied in the number of ethylene oxide units. Micelles with six types of SLE_nS were produced: SLE₀S, SLE₁S, SLE₄S, SLE₁₁S, SLE₃₀S, and SLE₅₀S, where the subscript number corresponds to the number of EO units.

Since all of these types of SLE_nS were added to the exact same concentration of LAS, the differences in the observed cytotoxicity can be assumed to be a function of SLE_nS, and all of tested concentrations are presented in mg/L of SLE_nS. Therefore, SLE_nS notation is used to refer to the six variants of mixed micelles tested in this work. The company Greendet Lda. (Coimbra, Portugal) provided the surfactant LAS, while SLE_nS were acquired from BASF SE (BASF GmbH, Dusseldorf, Germany).

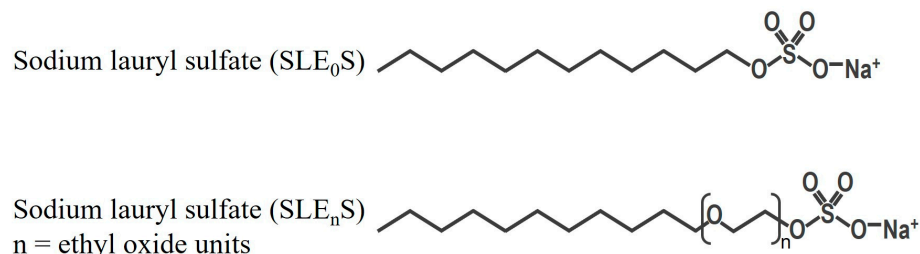


Figure 1. Chemical structure of sodium lauryl ether sulphate (SLE_nS) with and without ethylene oxide units.

2.2. Biological Model

The cell line used in this study was the A6 epithelial cell line (ECACC 89072613), which was isolated from the kidney of an adult male *Xenopus laevis* [24]. This cell type was selected to perform this study because it is an easy-to-culture cell line, and it is extensively used to study the impact of chemicals on amphibians (e.g., [18]). Furthermore, some of the processes involved in membrane dynamics (which may be altered by the chemicals tested in this study) with this cell line, especially concerning the opening of sodium and calcium channels, are already known. This cell line was kindly provided by Professor Peter Lorenz from the University of Rostock (Rostock, Germany). Cells were grown in 55% Leibovitz (L-15) culture medium (Biowest, Nuaille, France), diluted with 35% ultrapure water, and supplemented with 10% fetal bovine serum (Capricorn, Düsseldorf, Germany), 100 U/mL penicillin G, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B (Biowest, France) [25]. Cells were subcultured twice a week using Tryple Express (ThermoFisher Scientific, Porto Salvo, Portugal) as a dissociation agent and 70% phosphate-buffered saline (PBS) as washing solution [26], and they were routinely maintained in T75 flasks at 25 °C under atmospheric air.

2.3. Cytotoxicity Assays

To assess the cytotoxicity of the six SLE_nS variants to A6 cell lines, two different viability assays were performed, namely, the thiazolyl blue tetrazolium bromide (MTT) and resazurin assays, according to the National Institute of Health's (NIH) guidelines [27], which were adapted to the A6 cell line. Briefly, cells were plated on 96-well plates at a density of 1×10^4 cells per well (Countess[®] II FL Automated Cell Counter (ThermoFisher Scientific[®])) and allowed to adhere overnight at 25 °C. On the following day, test solutions of each SLE_nS variant were made, immediately before exposure, through the dilution of the initially prepared stock solution (3300 mg/L of SLE_nS and 6600 mg/L of LAS) in complete cell culture medium. Then, the cell lines were exposed to the following SLE_nS concentrations, which were selected based on prior range finding tests: 0.132, 0.264, 0.396, 0.528, 0.66, 0.792, 0.924, 1.056, 1.188, and 1.32 mg/L of SLE_nS. The duration of the tests was 48 h, with observations of the viability of the cells made after 24 h and 48 h of exposure. Three independent assays were performed, each containing four replicates per concentration and type of SLE_nS. For the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (TCI, Brussels, Belgium) was diluted in PBS (pH 7.36) at a concentration of 5 mg/mL, while for the resazurin assay, 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (Serva, Heidelberg, Germany) was diluted in PBS of pH 7.36 at a concentration of 0.15 mg/mL, and both reagents were sterilized by filtration (pore: 0.22 µm), stored at −20 °C, and protected from light. After the exposure period, the cells

were washed with PBS, 100 μ L of 10% MTT solution was added to each well, and the plates were incubated for 4 h at 25 $^{\circ}$ C. At this stage, the cells with an active metabolism transform the MTT to purple-colored formazan. Then, the MTT solution was removed and DMSO was added to the wells. The amount of formazan produced was measured through absorbance (at 570 nm and 690 nm using MultiSkan Spectrum ThermoFisher Scientific[®]). The cell viability was calculated as a percentage of the control. After the 24 and 48 h exposure periods, 10 μ L of resazurin was added to each well, and the cells were incubated for 4 h at 25 $^{\circ}$ C. After this period, the media were transferred to black 96-well plates. Viable cells will reduce resazurin to resorufin. The fluorescence was measured (Ex. 530.0—Em. 590.0 nm) with a fluorescence spectrophotometer (Hitachi F-7000; ©Hitachi, Ltd.; WVR, Lisbon, Portugal). The cell viability was calculated as a percentage of the control.

2.4. Data Analysis

Lethal concentrations causing a 10, 25, 50, and 90% effect on the cell viability (LC_{10} , LC_{25} , LC_{50} , and LC_{90} , respectively) were estimated by fitting the data to a sigmoidal nonlinear regression model with four parameters and are expressed in the text in mg/L of SLE_nS . A two-way analysis of variance (ANOVA) was conducted to analyze the cytotoxic interaction between the exposure time and SLE_nS concentrations. The assumptions to conducted the ANOVA were checked with the Shapiro–Wilk test (for normality) and Bartlett’s test (for homogeneity of variance). The coefficient of correlations (r) for linear regressions were determined to identify associations between the values of computed LC_{50} (with MTT and RES assays) and the number of EO groups in the SLE_nS variants. To check for associations between the LC_{50} computed using the MTT and the resazurin assays, coefficient correlations were computed after running Pearson correlations. The significance level for the ANOVA, linear regressions, and Pearson correlations was set at $p < 0.05$. All statistical analyses was performed using GraphPad Prism v9.1.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. MTT Assay

The cell viability showed a significant difference between the 24 h and 48 h exposure periods for SLE_1S and $SLE_{11}S$ ($p < 0.05$), while there were no significant differences in the exposure periods for SLE_0S , SLE_4S , $SLE_{30}S$, and $SLE_{50}S$ ($p > 0.05$; Figure 2; Table S2).

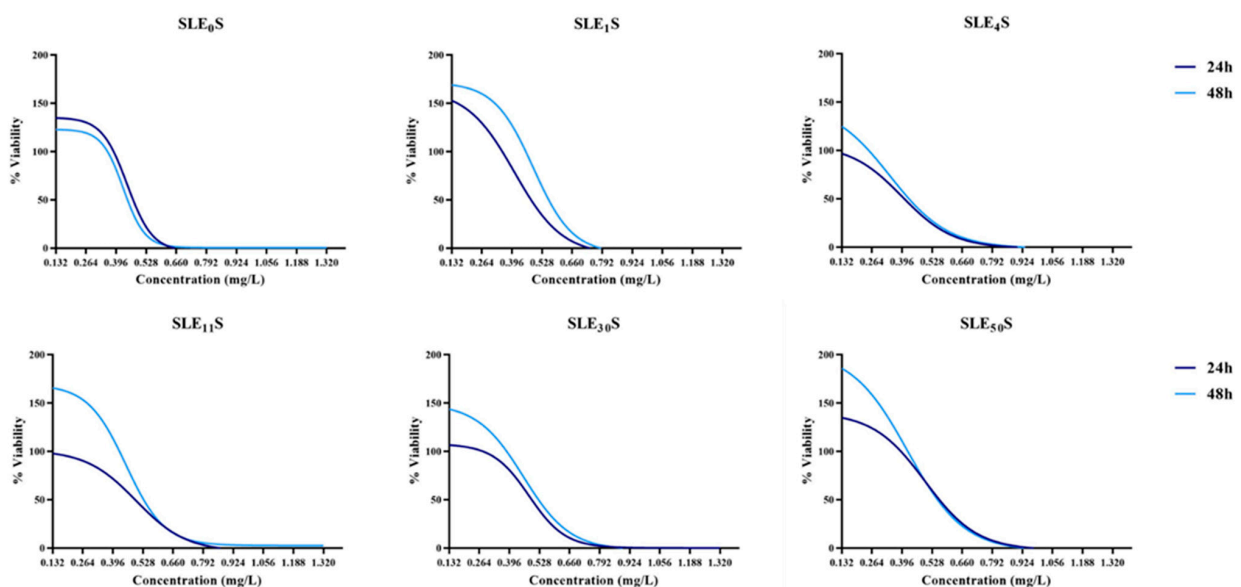


Figure 2. Fitted curves of the cell viability of the A6 cell line to the six variants of SLE_nS after 24 and 48 h of exposure based on the MTT cell viability assay.

Based on the estimated LC_{50} values at both the 24 h and 48 h exposure times, the variants with lower EO units, SLE_0S , SLE_1S , and SLE_4S (the values ranged between 0.398 and 0.475 and 0.417 and 0.552 mg/L, respectively), exhibited slightly higher cytotoxicity than $SLE_{11}S$, $SLE_{30}S$, and $SLE_{50}S$ (the values ranged between 0.488 and 0.554 and 0.517 and 0.548 mg/L, respectively) (Figure 3; Table S1). Despite this tendency of the SLE_nS variants with higher EO units to exhibit a lower cytotoxicity, a significant positive association between the number of EO units and the values of LC_{50} after 24 and 48 h of exposure was not observed ($p \geq 0.07$; $r \leq 0.55$; Table S3).

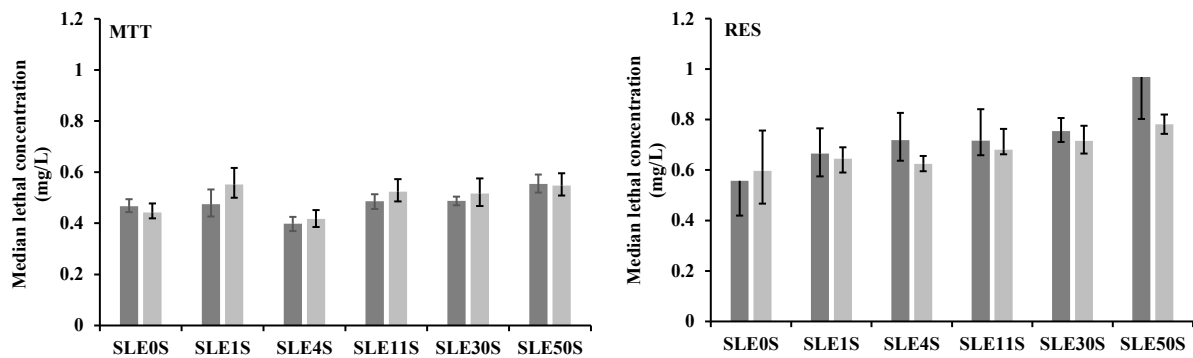


Figure 3. Median lethal concentrations calculated for the six SLE_nS variants after 24 h (dark grey bars) and 48 h (light grey bars) of exposure based on the MTT and resazurin (RES) assays. Error bars represent the 95% confidence limits, and missing upper bars mean that the upper 95% confidence limit could not be computed.

3.2. Resazurin Assay

The cell viability showed no significant differences between 24 h and 48 h of exposure for all six variants of SLE_nS ($p > 0.05$; Figure 4; Table S2).

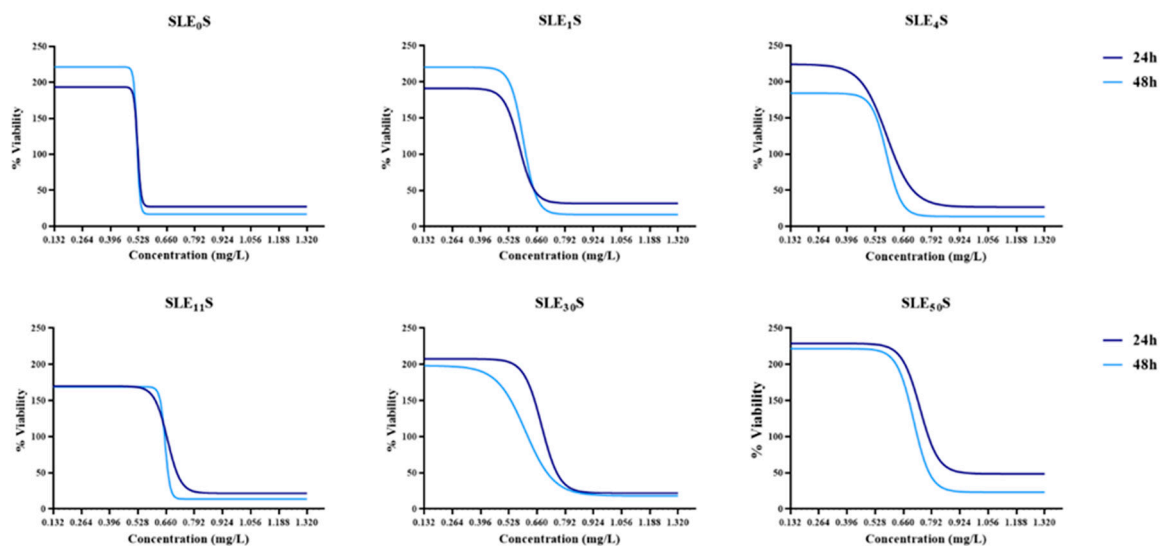


Figure 4. Fitted curves of the cell viability of the A6 cell line to the six variants of SLE_nS after 24 and 48 h of exposure based on the resazurin cell viability assay.

The estimated LC_{50} values increased with the number of EO units of the SLE_nS variants, ranging from 0.557 to 0.969 mg/L, at 24 h exposure, and from 0.597 to 0.782 mg/L, at 48 h of exposure, respectively, for SLE_0S and $SLE_{50}S$ (Figure 3; Table S1). A significant positive association was found at 24 h and 48 h of exposure between the number of EO units of the SLE_nS variants and the computed values of the LC_{50} ($p \leq 0.011$; $r \geq 0.91$; Table S3).

When comparing the computed LC_x for the six SLE_nS variants from the MTT and resazurin assays, no significant correlations were observed between the two assays at 24 and 48 h of exposure ($r \leq 0.688$, $p \geq 0.131$; Table S3). But, the MTT assay generally resulted in lower values of LC_x , suggesting its higher sensitivity to these surfactants (Table S1). Namely, the LC_{50} values obtained from the resazurin assay were 1.2- to 1.8-fold higher than those measured using the MTT assay (Figure 3).

4. Discussion

The ethoxylation of sodium lauryl sulphate (i.e., the addition of ethylene oxide (EO) groups) has been proven to reduce its hazards to humans regarding the skin irritation effects of this surfactant while maintaining its functionalities [28], although a parallel assessment of the influence of such ethoxylation on the toxicity of SLE_nS to the biota has rarely been conducted, even though this chemical is commonly discharged in freshwater ecosystems. Thus, the present work aimed to elucidate the effects of different degrees of ethoxylation (number of EO groups) of SLE_nS on the viability of the *X. laevis* kidney cell line A6 through MTT and resazurin assays. Although both assays estimate cellular viability due to the reduction of the active ingredient by oxidoreductases present in the cell, there is a preference for the enzymes that catalyze this reaction towards MTT or resazurin [29]. While MTT is mainly reduced by the oxidoreductases, dehydrogenases, oxidases, and peroxidases using NADH, NADPH, succinate, or pyruvate as electron donors that are present in the mitochondria and plasma membrane, resazurin is mainly reduced by reductases present in the cytosol, mitochondrial, or microsomal enzymes; by enzymes in the respiratory chain; or by electron transfer agents, preferably N-methylphenazinium methosulfate [27,30]. The results obtained varied according to the assay used. The resazurin assay indicated that SLE_nS variants with a fewer number of EO units were the most toxic to A6 cell lines, exhibiting lower values of LC_{50} . Though the results from the MTT assay showed a similar pattern of fewer EO units associated with higher toxicity, no significant association was observed between the computed LC_{50} values and the number of EO groups. This pattern of toxicity is in line with previously published works. Martins et al. [1] assessed the effects of SLE_nS on the bioluminescence of the marine bacterium *Vibrio fischeri* and reported that SLE_nS with a greater number of EO units were comparatively less toxic to the bacterium than those with a fewer number of EO units. These authors reported acute toxicity ranges (LC_{50}) from 0.56 to 2.81 mg/L⁻¹ for SLE_0S , SLE_1S , SLE_2S , and SLE_4S , while $SLE_{11}S$, $SLE_{30}S$, and $SLE_{50}S$ showed an acute toxicity from 8.09 to 8.60 mg/L⁻¹ [1]. Possible mechanisms for SLE_nS with a fewer number of EO units being more toxic were suggested by Martins et al. [1]. First, a higher number of EO units is linked with a high stabilization of micelles due to the screening of headgroup repulsion, which contributes to the presence of a lower quantity of monomers in the suspension. Therefore, this mechanism lowers the amount of surfactant (i.e., monomers) able to penetrate the cell membrane, resulting in lower toxicity [1]. The second mechanism is represented by the fact that surfactants with a fewer number of EO units are more hydrophobic and, consequently, more efficient at associating with cellular membranes, thus being more bioavailable [1]. Similarly, Wong et al. [11] determined the acute toxicity of nine variants of alcohol ethoxylate surfactants with different alkyl chain lengths and different numbers of EO groups on *P. promelas* and on *D. magna*. The authors reported increasing toxicity with decreasing number of EO units; for example, for *D. magna* the $EC_{50,48hs}$ ranged from 0.46 mg/L (for the variant with 5 EOs) to 1.2 mg/L (for the variant with 13 EOs) [11]. The exposure duration had no significant effect on the cell viability for any variant of SLE_nS in the resazurin assay, but a significant influence was registered for SLE_1S and $SLE_{11}S$ when the MTT assay was performed. This latter assay also showed a higher sensitivity to the variants of SLE_nS compared to the resazurin assay. One of the possibilities for the lower values of LC_{50} computed with the data obtained from the MTT assay may be due to the mode of action of the surfactants, since membrane destabilization and hydrolysis of cell membrane proteins and enzymes might impair the conversion of MTT to formazan by cellular membrane enzymes. The work from Magalhães

et al. [31] demonstrates that SLE₀S promotes denaturation of lipase with the consequent loss of function, but the increase in the number of EO units of the polar head of SLE₂S stabilizes the enzyme, maintaining its function. Surfactants that are prone to forming micelles, including SLE₀S, tend to induce curvature stress, causing membrane disordering and culminating in cellular lysis [32]; but recent developments have demonstrated that this effect is diminished with the incorporation of EO, with SLE₁S having a critical micelle concentration of 0.7 mM in comparison to SLE₀S with 8.7 mM and a membrane stiffness that increases instead of decreasing, diminishing membrane disordering [33].

Despite the differential sensitivity between the MTT and resazurin assays, the micelles composed of each of the six variants of SLE_nS and LAS were able to impair the viability of the A6 cells at low concentrations: for MTT the LC_{50,48h} ranged from 0.398 to 0.554 mg/L and for resazurin from 0.557 to 0.969 mg/L. These values are within the range of toxicity reported by Martins et al. [1] for the bacterium *V. fisherii*; as mentioned above, EC_{50,15min.} values were between 0.56 and 8.60 mg/L. Though, comparing the toxicity of SLE₀S here reported with that of other studies, a higher toxicity was observed in the present work. Song et al. [34] tested SLE₀S on the mouse fibroblast-like cells L-929 (ATCC, CCL-1) and reported an LC₅₀ of 173.6 mg/L in 72 h of exposure (converted from the original result of 602 µM reported in their article), which is considerably higher than the values estimated for the A6 cells in the present study. Bondi et al. [7] report LC₅₀ values ranging between 1 mg/L and 13.9 mg/L for aquatic organisms (invertebrates and fish) exposed for 96 h to sodium lauryl sulphate (SLE₀S). Nevertheless, this toxicity was influenced, apart from the target species, by several environmental factors such as water temperature and hardness [10,35]. Vater et al. [36] showed that human keratinocytes and fibroblasts cell lines, when exposed to 1.65 mg/L of SLE₀S (converted from the original result of 5% concentration reported in their article), have no active metabolism and, therefore, no viability after 24 h of exposure, with our work demonstrating far lower concentrations for LC₉₀, but this higher cytotoxicity may be due to the presence of LAS. Several studies focusing on the effect of SLE_nS and LAS in keratinocytes and other cellular models demonstrate that the exerted cytotoxic effects are not exclusive to membrane damage but also involve the formation of reactive oxygen species (ROS) together with altered calcium metabolism [37–39]. Bjerregaard et al. [37] using the same cellular model, A6 cell line, demonstrated that LAS was able to activate calcium channels in the apical membrane and destabilize ion homeostasis, contributing to a higher chloride secretion due to the activation of calcium-dependent chloride channels from the apical membrane [37]. Alterations to calcium metabolism induced by sodium lauryl sulphate (SLS) at concentrations higher than 7.21 mg/L (converted from the original result of 25 µM reported in the article) were also found in keratinocytes and in a reconstructed human epidermal skin model [38]. Mizutani et al. [39] demonstrated that membrane damage due to SLE₀S activates calcium channels, inducing an increase in intracellular calcium promoting the formation of ROS and the activation of IL-1, leading to skin roughness. Responses to surfactants are also dependent on the cellular membrane constitution, namely, the presence of cholesterol that induces rigidity to the membrane and, therefore, interferes with the penetration capability of surfactants [40–42]. This is also important for evaluating the effect of surfactants that are able to transverse the plasma membrane and interfere with organellar membranes, which have different compositions in comparison to the plasma membrane [43,44]. Such an example was found regarding ammonium lauryl sulphate (ALS), which promoted apoptosis through mitochondrial dysfunction, with alterations in mitochondrial membrane potential, reticulum, lysosomal shrinking, and LDH release [45].

5. Conclusions

Overall, this study showed that SLE_nS with a fewer number of EO units were more toxic, as previously shown by other studies at the organismal level. This result highlights the importance and usefulness of in vitro tests as first screenings for this type of study, which can be performed rapidly, are reliable, and do not require animal experimentation. For future works, experiments with other cell lines that exhibit different active metabolic

pathways (e.g., XTC-2), is recommended, targeting the establishment of better structure–toxicity relationships of these SLE_nS variants. Once established, this relationship between EO groups and toxicity may be explored using *in vivo* assays with amphibians with the variants identified as the least toxic to further assess their risks for this class of vertebrates and to confirm that the variants with a greater number of EO units are more ecofriendly.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app13158745/s1>, Table S1: Concentrations of the six variants of SLE_nS causing 10, 25, 50, and 90% effect (LC₁₀, LC₂₅, LC₅₀, and LC₉₀) and the respective 95% confidence limits within parenthesis after 24 and 48 h of exposure for the MTT and resazurin (RES) assays. Table S2. p-Values concerning two-way ANOVA followed by Tukey’s multiple comparisons test to distinguish differences between exposure times (significant differences are in bold, $p < 0.05$). Table S3. Parameters of the linear regressions performed between the median lethal concentrations and the number of EO units (significant differences are in bold, $p < 0.05$) and the Pearson correlations performed between the cell viabilities measured with the MTT and resazurin methods.

Author Contributions: S.T.: Investigation, Conceptualization, Methodology, Data curation, Formal analysis, Software, and Writing—original draft; M.A.: Methodology, Data curation, Formal analysis, Software, and Writing—Reviewing and Editing; M.O.: Funding acquisition, Conceptualization, Visualization, Investigation, and Writing—Reviewing and Editing; D.C.: Funding acquisition, Conceptualization, Validation, and Writing—Reviewing and editing; I.L.: Funding acquisition, Supervision, Investigation, Conceptualization, Methodology, Data curation, Formal analysis, Validation, and Writing—Reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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