Marine Pollution of Chemicals Detergents Contamination Induced Apoptosis and Necrosis in Fish Liver (*Sardine aurita*) by Flow Cytometry DNA Measurements

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Abstract

Background: Presently, there are wide uses of detergents, which discharged to marine environment (Mediterrean sea). Detergents are organic chemical compounds, consisting of fatty acids, and different components of Alkyl Benzene, Sulphonates, Polyphosphates, Cellulose, Sucrinic acid, and Sodium Lauryl Sulfates. Further, even if at low concentrations, the synthetic detergents are considered to be toxic to marine biodiversity. Since, these surfactants are not completely degraded by waste water treatment. It has ability to damage the DNA and accumulated on fish and other marine organisms. Methods: Flow cytometry technique was applied, because it's accuracy for analysis of DNA contents. Hence, the present study evaluated the impact of sodium lauryl sulfate as synthetic detergent on the DNA of sardine fish as a marine biological indicator. *Sardinella aurita* was collected from clean and SLS-contaminated sites. The flow cytometry technique was employed to assess levels of active (apoptosis) and passive (necrosis) cell death in both exposed and unexposed fishes to SLS. Result: Chemical detergent contains 120 ml (*i.e.,* 20mg/L) of sodium lauryl sulfates. The result shows more distinct and significant differences of the early and late apoptosis/ necrosis of Sardine fish liver, compared with unexposed fish samples from cleaned reference site. Conclusions: The present data indicate that SLS was able to cause toxicity to fish. Data recorded revealed that toxicity as a significant accumulation of hepatic tissue cellular DNA during the G0-G1 in a significant way (P<0.05), compared with that of unexposed liver tissue. In the same way, there was an insignificant accumulation of hepatic cell's DNA in the S phase but not during the G2/M phase, finally there was a significant arrest / DNA accumulation during the PreG1- phase (P<0.05). In the meantime, accompanies of apoptotic profile of cells exposed to SLS showed a significant total apoptotic form significantly elevated compared with reference site.
Keywords: Detergents; Apoptosis; Necrosis; Flow cytometry; DNA content; Histopathology.

1. Introduction

Biological indicators are widely used in an aquatic environmental monitoring, because it allows examining specific target organs, such as gills, kidney and liver, that are responsible for main functions, for instance respiration, excretion, accumulation, and biotransformation of xenobiotic in the fish [1]. Since, this alteration found in those organs are normally easier to quantified than functional ones [2], which serves as warning singe of biomarkers health damage [3].

The Mediterranean Sea is characterized by huge varied quantities of biodiversity, including fish. It has been estimated that more than 8500 species of fish live in the Mediterranean Sea, which is considered to be between 4% and 18% of the world marine species. Compared to the world oceans, the Mediterranean Sea is only 0.82% in surface area and 0.32% in volume [4]. In addition, about 84% of the marine species are animals, of which 77% invertebrates and 7% vertebrates, while the 16% left are algae and sea grasses [4]. Since, the habitat and biological diversity preferring clear saline water with minimum temperature below 24° C, (United Nation Food and Agricultural Organization). But, any anthropogenic activities near the coastal region will resulted in an increase of pollution levels, which released into aquatic ecosystem [5]. The Mediterranean Sea is becoming worse, due to human activities like urbanizations, and industrializations, emerging an Ecological Disaster. The wide array of pollutants discharged into the aquatic environment, which is may have physic, chemical, biological, toxic and pathogenic effects [6].

According to the United Nation Environmental Program reports [7], the Mediterranean Sea is receiving hug quantity of untreated waste water, beside the effluents of industrial factories, and oil refinery. These contaminants include metals, metalloids, solvents, pesticides, and organic compounds of pharmaceutical and detergents.

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Further, chemical detergents are more likely is a mixture of synthetic chemicals and additives cooked up in a huge chemical plant and, unlike traditional soap; they're generally liquids rather than solids. Detergents are used in everything from hair shampoo and clothes washing powder to shaving foam and stain removers.

Detergents are not fully degraded by sewage, treatment and also they again discharged to the water bodies, which may cause very harmful effect on the biodiversity, health and environment.

These surfactants (detergents) have high biological activity even at low concentrations, and also have the power to accumulate in the organisms. Several types of surfactants such as soaps (anionic), Alkyl benzene sulphonate (ABS), SLS (anionic) and Ethoxylated fatty alcohols (non-ionic) are used to make detergents. Sodium Lauryl Sulfate (SLS) is an anionic surfactant detergent derived from Alcohol Sulfates and alternatively, it is also known as SDS [8]. While it's chemical formula is C12H25NaO4S, or CH3-(CH2)11-O-SO3-Na+, [9].

SLS is widely used in household products such as, toothpastes, shampoos, shaving foams, bubble baths, and cosmetics. It is used as leather softening agent, wool cleaning agent, in paper industry as penetrate and flocculating agent, in construction as additive of concrete, and as ingredient of floor cleaners and car wash soaps.

The presence of SLS in environment arises mainly from its presence in complex domestic and industrial effluents as well as its release directly from some applications (e.g., oil dispersants and pesticides) as shown on figure 1. It has been reported that SLS is toxic and affects survival of aquatic animals such as fishes, microbes like yeasts and bacteria [10]. It is also toxic to mammals like mice and humans but to a lesser extent [11].

Figure-1. Illustrates the sources of an ionic surfactants in an aquatic environment
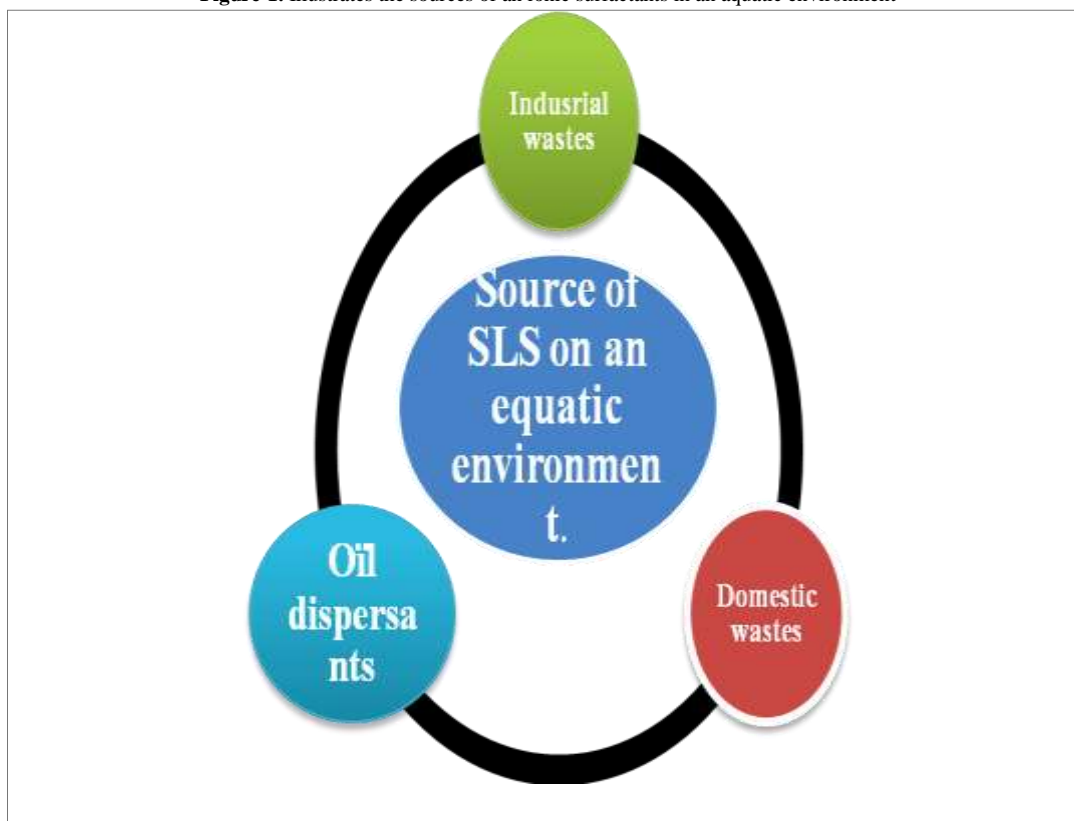
Surfactants toxicity has motivated a worldwide warning persuaded by distinct ruling. There are yet worries concerning the biodegradability and eco friendliness of surfactants [12].

As mentioned, fish in aquatic ecology is responded to variation of environmental toxicity, because its functions of metabolism with different pollutants [13]. Whereas, the sentinel fish species was widely used to evaluate the degree of toxicants accumulations and their health impacts [14]. Fish was preferred in toxicological studies because of their well-developed osmoregulatory, endocrine, nervous, and immune system compared to invertebrates [13]. Further, fish was capable to adsorb toxicant directly from the surrounding water and sediments, or ingesting them through contaminated food via food chain [15].

Due to widespread use of fish there have been increasing concerns about its environmental behavior in aquatic ecology. The use of biota as an indicator of pollution have an advantageous over chemical analysis as they are ecologically realistic [16]. Fauna1 and chemical monitoring has frequently been used to assess environmental quality [17].

The analysis of DNA damage in aquatic organisms has been shown to be a suitable method for evaluating the genotoxic contamination of environments [18, 19], with the advantage of detecting and quantifying the genotoxic impact without requiring a detailed knowledge of the identity and the physical/ chemical properties of the contaminants present [20]. Some of the methodologies commonly adopted in the assessment of genotoxic impact include the detection of structural damage into DNA such as DNA adducts [21, 22] and DNA strand breaks [23].

The structural perturbations occurring into the DNA molecule that do persist and do not result in the death of cell may interfere with the fidelity of DNA replication resulting in abnormal DNA. This abnormal DNA has been associated with clastogenic events such as chromosomal aberrations, micronuclei formation and aneuploidy, which have also been widely used as biomarkers of genotoxicity in aquatic organisms [21, 24, 25]. Flow cytometry (FCM) is one of the techniques used to evaluate chromosomal damage induced by genotoxic agents, by detecting variations in nuclear DNA content. This technique allows a rapid analysis of a large number of samples, applicable to any organism or tissue from which cellular or nuclear suspensions can be obtained [26]. FCM has been used successfully to evaluate the genotoxic effects of environmental pollutants in fish from laboratory exposures and in field experiments [27-29].

Sublethal exposure to environmental chemicals may result in changes in the structure of cells and the occurrence of pathologies which can significantly modify the function of tissues and organs. Histopathological changes are thus mediumterm responses to sublethal stressors that have been used as valuable tools to evaluate the toxic effects of contaminant exposure. Several studies have used histopathological biomarkers in fish to identify and evaluate the impact of aquatic pollutants [30-34]. Fish liver, being the primary organ for biotransformation of xenobiotics and where many contaminants tend to accumulate, has been the target of many of these studies. A relation between exposure to pollutants and the development of hepatic lesions has been demonstrated in fish [32, 33], making toxicopathic liver lesions effective biological markers of chemical exposures.

An advantage of histopathology as a biomarker lies in its intermediate location in the hierarchy of biological organization. However, as reported for other biomarkers, histological alterations may be influenced by factors other than chemical exposure such as age, diet, environmental factors, seasonal variation, and reproductive cycle [35]. These factors may induce several structural states which may represent normality and could act as potentially confounding issues when attempting to use histological criteria as biomarkers of aquatic pollution.

In the aquatic environment, fish are usually regarded as organisms of choice for assessing the biological effects of environmental pollution on aquatic ecosystems. Fish can be found virtually everywhere in the aquatic environment, they occupy the top of the aquatic food chain, are a highly visible resource, and are known to accumulate toxicants [1, 36]. In addition, they are in contact with pollutants either via the direct uptake from water by their gills and their body surface, or via ingestion of contaminated sediments. For these reasons fish are frequently used as bioindicator species, by giving information about the environmental conditions of its habitat.

The global aim of the present work was the study of the biological effects of SLS in fish, particularly the molecular events imposed by contaminants and that might be involved in the toxic response. Because the molecular changes constitute the earliest signals of response to the toxicant exposure and also because their detection may allow the intervention before irreversible damaging effects become inevitable, and could be potentially employed as molecular biomarkers. These molecular responses also contributed for a better understanding of the molecular events involved in fish response to aquatic contamination.

Consequently, in this study, the effect of exposure to SLS on S.aurita fish liver from the contaminated sites, where this detergent contains approximately 120ml of anionic surfactants (i.e., 20mg/L of SLS).

2. Materials and Methods

In this study we use a traditional detergent (SLS) which is frequent use as surfactants. It contains 20.15% of an ionic surfactant of 600 mg. and S. aurita fish as bioindicators.

Test Animal:

Sardinella aurita (Sardine or Round Sardinella) as shown on Figure 2, was chosen as the test animal for the study as they are most commonly and abundantly found in many parts of the world like Africa, Europe, North and South America. However, its distributions occur on two regions, (i.e., Mediterranean and the western Atlantic) [37]. Its characterizations:

- Family: Clupeidae (Sardines and Herrings).
- Order: Clupeiformes (Sardines, Herrings and Anchovies).
- Class: Actinopterygii (Ray-finned Fish).

2.1. Morphology

Sardinella aurita has a sub cylindrical elongated body, flattened side way sand with a rounded belly (Fig. 2). The length of sardines was generally 12-15cm at maturity, but it may reach up to 28cm in some cases, it is one of the largest Sardinella species, and weigh mostly 230g. The reflective scales that cover their body are silver in color, and smooth. At the top of the head was a golden, soft faint mid side line, besides, on the gill cover was a black distinguished spot. A typical clupeid mouth shape was visible in these fish, with a short but deep lower jaw. Sardines also hold pointedly ramified tails.

2.2. Flow Cytometry

A flow cytometer is used, which is an instrument that illuminates cells (or other particles) as they flow individually in front of a light source and then detects and correlates the signals from those cells that result from the illumination.

Liver and gill cell suspensions were also frozen in liquid nitrogen and stored at -70 ℃. Frozen samples of liver fish suspensions were thawed and suspended in a nuclear isolation buffer (LB01, Dolezel, et al. [38]. Isolated nuclei were treated with 50 mg/ ml RNase (Fluka, Buchs, Switzerland) and stained with 50 mg/ml propidium iodide (PI,

Fluka). Samples were incubated for 10 min on ice before the analysis. The fluorescence intensity of, at least, 5000 nuclei was measured per sample with a Becton-Dickinson, flow cytometry (San Jose, CA, USA). The instrument was equipped with an air-cooled argon ion laser (15 mW, operating at 488 nm) and PI fluorescence was collected through 645 nm dichroic long-pass and 620 nm band-pass filters.

In each sample, the half-peak coefficient of variation (HPCV) of the G0/G1 peak of isolated nuclei was registered (SYSTEM II software v. 3.0). This dispersion measurement of nuclear fluorescence has been previously shown to be positively correlated with chromosomal damage, as a result of cell-to-cell variation in nuclear DNA content [39].

For statistical analyses, all data were tested for normality and homogeneity of variances. Since an invalidation of normality and variances homogeneity was observed, data were analysed using a non-parametric Kruskal-Wallis ANOVA on ranks, followed by a Mann–Whitney U pairwise comparison test to detect which groups were significantly different (SigmaStat 3.11 Software).

3. Results and Discussion

The present data suggest that the liver tissue of fish may be useful marker for evaluation to fish toxicity damage. These findings agree with previous studies, which indicate the fish may respond to surfactants contamination.

There were an indication of SLS concentration ranged from (0-15 mg/L) motivated the morphological variation in kidney and spleen of gilthead (Sparus aurita L.) with an important inhibitory impacts on success fertilization [40].

While exposed of different concentrations of SLS (3, 5, 7 and 10mg/L.) to twenty juvenile turbots (Scophthalmus maximus L.), revealed that 50% mortality at 384, 190, 12, and 4h respectively. The results indicate that, the sub lethal chronic impacts of SLS on the survival, metabolism, and growth of juveniles Centropomus parallelus at three different salinities [41]. There were another study showed that SLS affects metabolism and swimming capacity of Cyprinus Carpio L. [42]. The acute toxicity of Daphnia magna increased with growing alkyl chain length of Alcohol Sulfates [43].

Since the primary target impacts of SLS on bioindicators are the cell membrane, due to its ability to physical and biochemical effects. This might cause epidermal cell proliferation and differentiations in vitro [44]. There was an indication that the frequent revelation of SLS it could be the source of skin irritations and hyperplasia in guinea pigs and is more sensitive to Rabbit skin cultures than human skin [45].

On the other hand, it has been reported that, the SLS may be unsafe by the oral route in mammals (LD50 1200 mg/kg bw), by the dermal route in rabbit (LD50 = ~600 mg/kg bw) and guinea pig (>1200 mg/kg bw) with skin and eye irritation in all respectively [46].

While the treatment of rats with SLS (100-1000 mg/kg bw/day) was showed the augmented level of cholesterol esters and phospholipids but, simultaneously reduced the levels of triglycerides, irritation of the gastro-intestinal tract, systemic toxicity on epididymal sperm and slight to moderate maternal toxicity [47].

The SDS might cause damage to human health, if individual consumed ≤150 g [48]. The direct contact to SLS (≤20%) may produce moderate inflammations, irritations of the skin, in addition, the continuous exposures of SLS is able to induce dermatitis like redness, swelling and blistering [49]. Hence in some people SLS may cause respiratory irritations, breathing difficulties, and lung damage [50]. The hyperactivity of a body against an antigen (non-allergic condition) was known as reactive airways dysfunction syndrome (RADS).

Further, the SLS was toxic on gram negative bacteria, [51]. Since, the increased amount of SLS in the cytoplasm contributes to mis-folding of denatured protein which could be toxic to the cell with other toxic effects [52] . Whereas, it has an effect on various cell organelles and expressed up regulated and down regulated genes in Saccharomyces cerevisiae [53].

Even though, the surfactant detergent as synthetic are reported to be acutely toxic to fish in concentrations ranged between 0.4 and 40 mg/L, [54], so in this study we exposed chemical detergents to S.aurita fish liver for more than 12 hours, where this detergent contains approximately 120ml of anionic surfactants(i.e., 20mg/L). The result as shown on the flowing graphs indicates that the detergents (i.e., SLS) are able to cause toxicity to fish. Data recorded revealed that toxicity of Na–lauryl sulfate (Table 1), demonstrated that there is a significant accumulation of hepatic tissue cellular DNA during the G0-G1 in a significant way (P<0.05) compared with that of un-treated liver tissue. In the same way there was an insignificant accumulation of hepatic cell's DNA in the S phase but not during the G2/M phase, finally there was a significant arrest / DNA accumulation during the PreG1- phase (P<0.05) .

Table-1. The difference in cell cycle phases between SLS-exposed and unexposed fish sample.

No	Sample data	Cell cycle Phases			
	Code	G0-G1%	S%	G2/M%	Pre-G1%
1	Unexposed	88.62	4.39	6.99	9.41
2	SLS-exposed	93.49	5.07	1.44	15.32

Figure-2. Variation in DNA content detected by flow cytometry and expressed as HPCV (%) in liver tissue isolated from two Sardine fish (S. auriata) population, exposed to SLS contamination and unexposed (clean) one. Data are expressed as means ± SEM (n = 4 animals/ month). Asterisks denote a statistically significant difference from the unexposed population
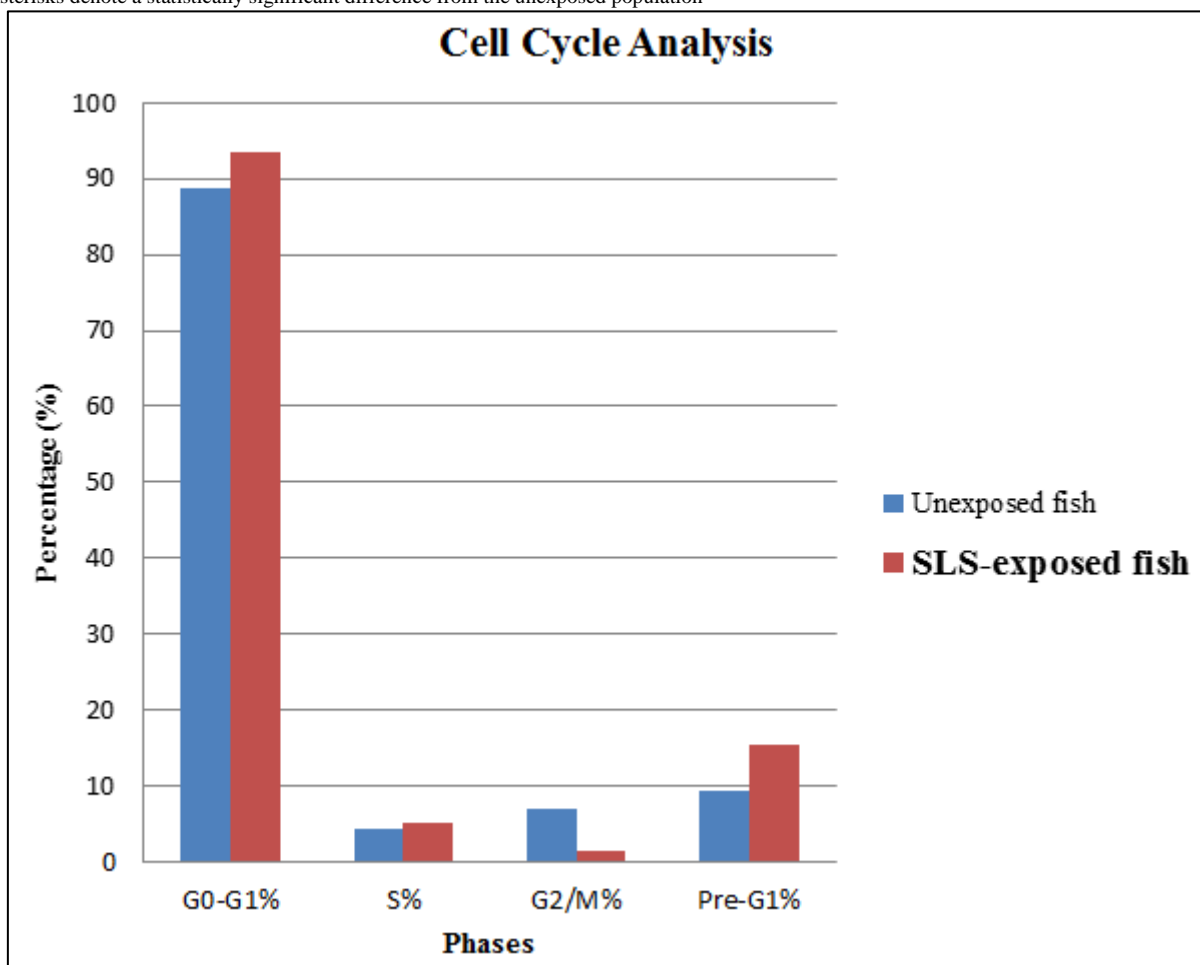
* P < 0.05; ** P < 0.01

The SLS-exposure induced a major changes in ploidy level (e.g., polyploidy) in the tested fish liver tissues. For putative chromosomal damage evaluation, mean HPCV values of the G0/G1 peak were calculated varying between 88.62% in liver of unexposed fish population, and 93.49% in liver of SLS-exposed fish population. A significant difference in HPCV values (P > 0.05) were found in liver cells of SLS-exposed Sardine fish collected from contaminated Tripoli site, compared to unexposed (clean) Sabratha (Table 1; Fig. 3). A great difference showed a significant increase (P < 0.01) in HPCV values of G2/M at unexposed fish population (6.99%) of controlled cell division, while, such values was decrease to about 1/3 (1.44%) in SLS-exposed population. Finally, a distinct duplication in Pre-G1 phase was detected (15.32%) in SLS-exposed population, while, in normal value (9.41%) during the cell cycle arrest in the cleaned fish population.

Table-2. The percentage (%) of apoptosis and necrosis of liver fish tissue

No	Code	Apoptosis			Necrosis
		Total	Early	Late	
1	Unexposed fish	9.41	0.42	2.82	6.17
2	SLS-exposed fish	15.32	2.52	4.19	8.61

Table 2, illustrated different degrees of apoptotic (early, late) cells pathway, compared with un-reversible necrotic pathway. Thus, a cell became necrotic when the plasma membrane integrity is compromised sufficiently to let viability dyes such as PI, into the cell. Late apoptosis or necrosis can be identified by SubG1/ DNA analysis. Early necrosis can now be identified in SLS-exposed fish population with a value of (8.61), which was corresponding with higher apoptotic cell accumulations (15.32), compared to a normal pattern value (9.41%) in non-contaminated fish population. Dead or necrotic cells display a differential signal of SLS contamination. DNA analysis of high and low intensity SLS events show that low intensity events have a greater level of DNA in the SubG1 area compared to the high intensity events, as in table 3 and figure 4. Whereas, dead cells can now be identified as early or late necrosis by a combination of a viability dye, SLS, quantification of DNA and side scatter.

Table-3. The difference in cell cycle phases between SLS-exposed and unexposed fish samples

Sample data	Cell cycle Phases			
Code	G0-G1%	S%	G2/M%	Pre-G1%
Unexposed	88.62	4.39	6.99	9.41
SLS-exposed	93.49	5.07	1.44	15.32

Figure-3. A histogram shows the different degrees of apoptosis and necrosis processes of unexposed fish population (purple colour) in comparison with the SLS-contaminated fish population (red colour)
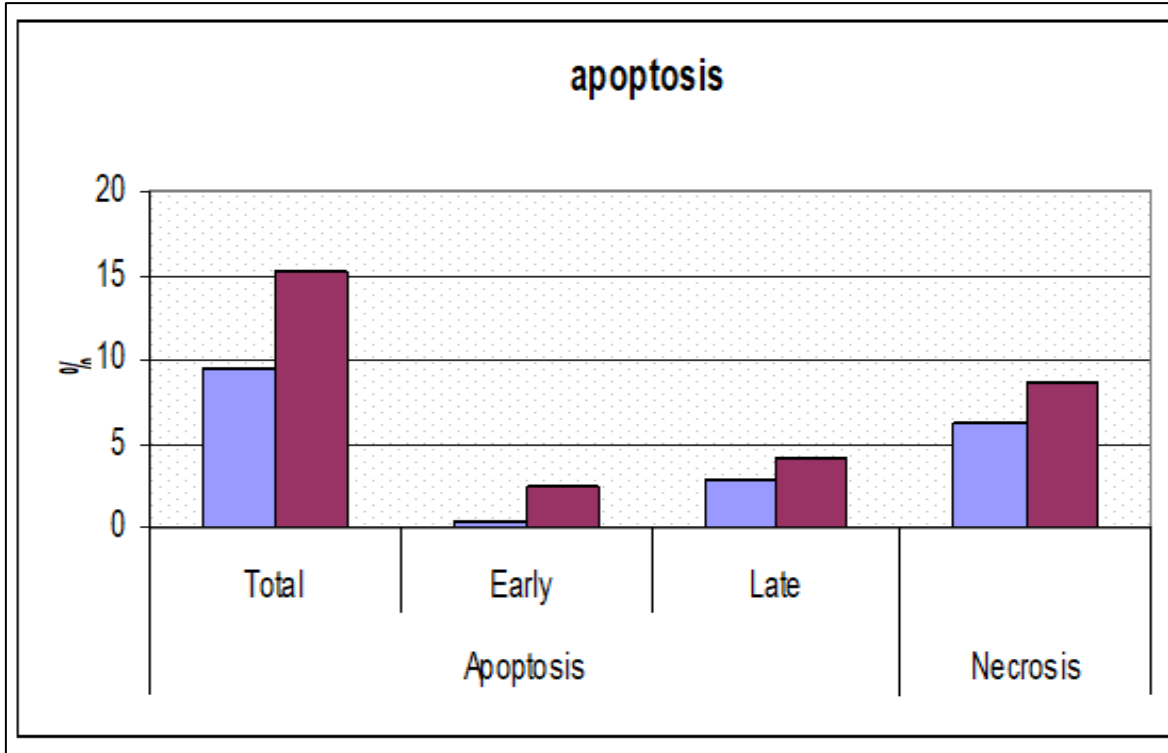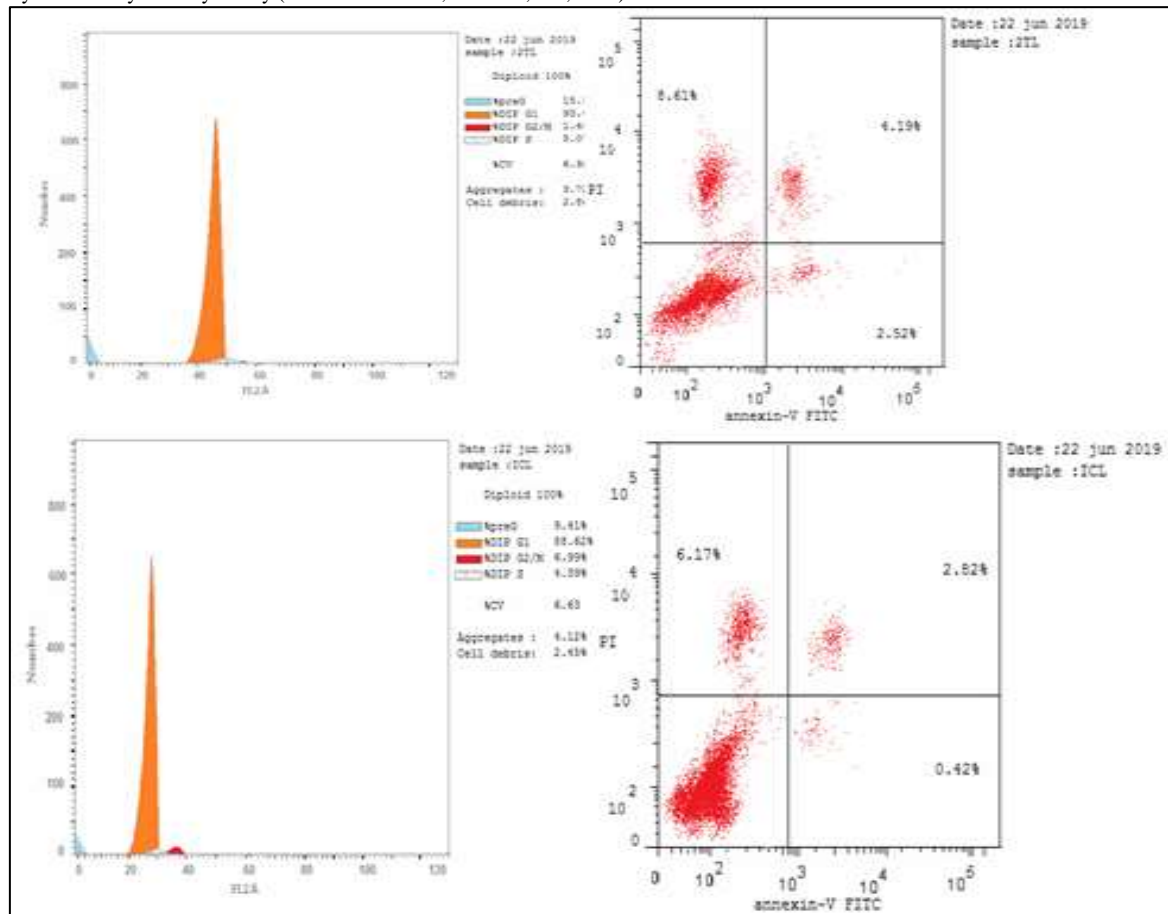

Figure-4. A charts and histogram show a diploid DNA and single-peak aneuploid DNA histograms with minimal debris contamination. Data chart by obtained by Flow cyomtery (Becton-Dickinson, San Jose, CA, USA)

4. Histopathology

The Light micrograph of liver of unexposed fish population showing the normal hepatic cells with a polyhedral and vacuolated tissue mass, and the pancreatic cells (Pc) of pancreatic part contain large amount of deeply stained granules.

Figure- 5. A photomicrograph showing liver tissue from unexposed (clean) fish population with normal hepatic lobule with central vein and hepatic cells arranged in cards. V= vacuolated hepatic cells; Pc= Pancreatic cells. H&E 10□
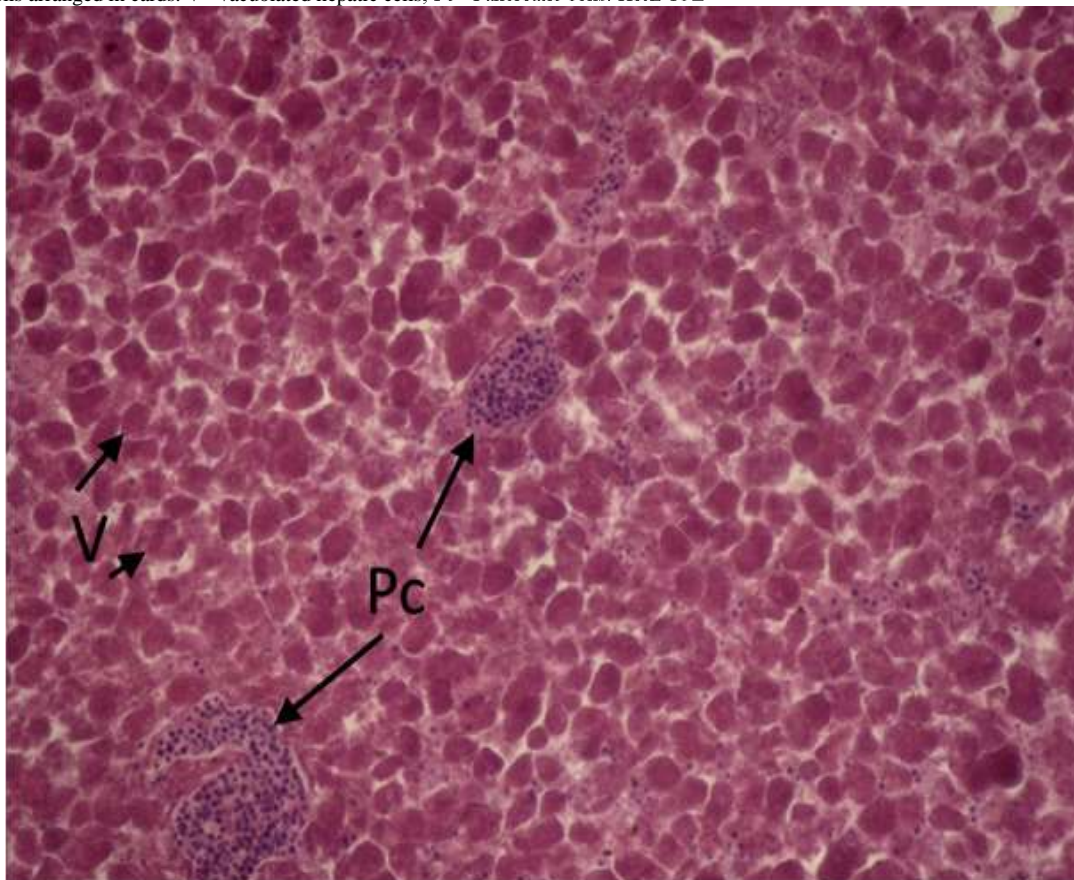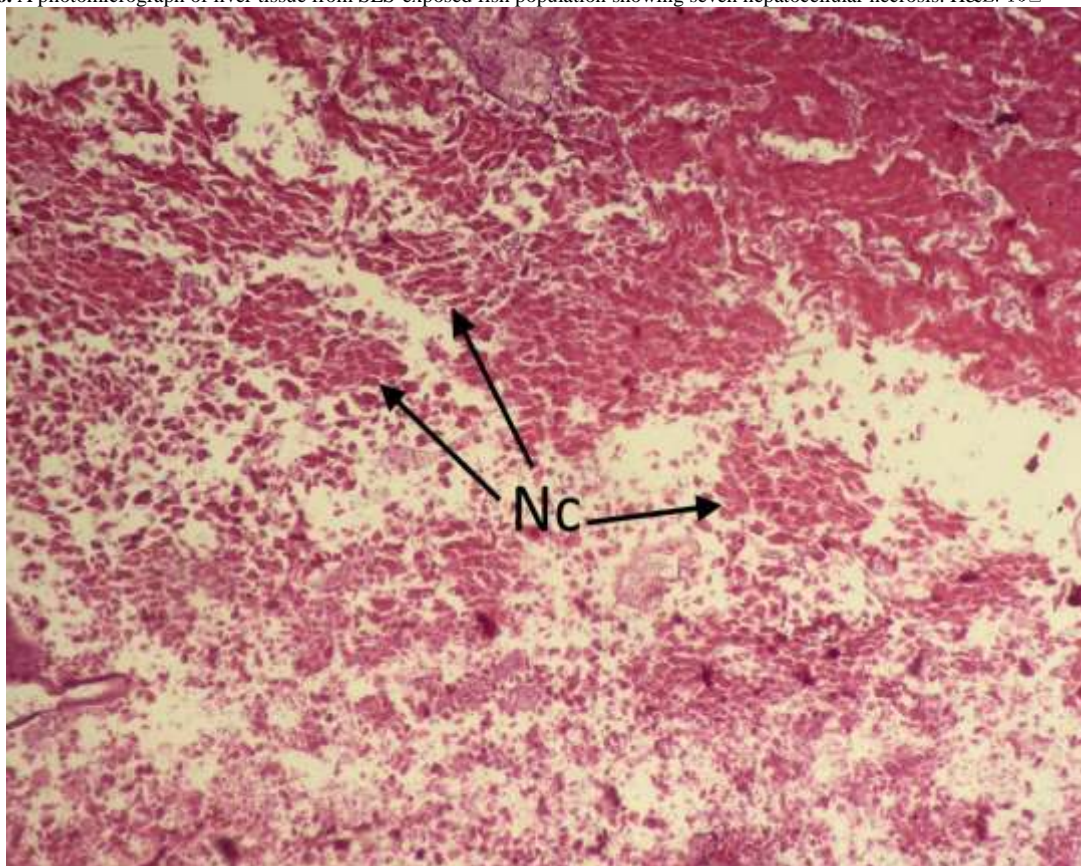

Figure- 6. A photomicrograph of liver tissue from SLS-exposed fish population showing seven hepatocellular necrosis. H&E. 10□

5. Conclusion

The present work can be mentioned that, the presence of DNA damage suggests putative disruptions of the main functions of liver tissue, which can lead to detrimental effects at the organism level.

The present data indicate that the detergents (SLS) are able to cause toxicity to fish. Data recorded revealed that toxicity of Na –lauryl sulfate indicated that there as a significant accumulation of hepatic tissue cellular DNA during the G0-G1 in a significant way (P<0.05) compared with that of un-treated liver tissue In the same way there was an insignificant accumulation of hepatic cell's DNA in the S phase but not during the G2/M phase, finally there was a significant arrest / DNA accumulation during the PreG1- phase (P<0.05). In the mean time the accompanies apoptotic profile of cells exposed to SLS showed a significant total apoptotic form significantly elevated compared with control.

Acknowledgment

References

[1]   Gernhofer, M., Pawet, M., Schramm, M., Müller, E., and R., T., 2001. "Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams." *Journal of Aquatic Ecossystem, Stress and Recovery,* vol. 8, pp. 241-260.

[2]   Fanta, E., Rios, F. S., Romão, S., Vianna, A. C., and S., F., 2003. "Histopathology of the fish Corydoras paleatus contaminated with sublethal levels of organophosphorus in water and food." *Ecotoxicology and Environmental Safety,* vol. 54, pp. 119-130.

[3]   Hinton, D. E. and Laurén, D. J., 1990. *Liver structural alterations accompanying chronic toxicity in fishes: potentioal biomarkers of exposure. Pp. 51-65. In: McCarthy, J.F. & L.R. Shugart (Eds.). Biomarkers of Environmental Contamination*. Boca Raton: Lewis Publishers.

[4]   Bianchi, C. and Morri, C., 2000. "Marine biodiversity of the mediterranean sea: Situation, problems and prospects for future research." *Marine Pollution Bulletin - MAR POLLUT BULL,* vol. 40, pp. 367-376.

[5]   Bhattacharya, S. B., 2007. "Induction of oxidative stress by arsenic in Clarias batrachus: involvement of peroxisomes." *Ecotoxicol. Environ. Saf.,* vol. 66, pp. 178-187.

[6]   Goel, P. K., 2000. *Water Pollution: causes, effects and control*. New Delhi: New age international publishers, Ltd.

[7]   United Nation Environmental Programm. "UNEP."

[8]   El-Bakatoushi, R., 2010. "Genetic diversity of winter wheat (Triticum aestivum L.) growing near a high voltage transmission line." *Romanian Journal Biology-Plant Biology,* vol. 55, pp. 71-87.

[9]   Technical Evaluation Report, 2006. "Sodium lauryl sulfate crops." In *compiled by ICF consulting for the USDA national organic program.* pp. 1-10.

[10]   Jeen, J. P., Midhu, K., and Johnson, P. C. A., 2018. "Histological study on gills and liver of freshwater fish rasbora dandia exposed to sodium lauryl sulfate (sls)." *International Journal for Research in Applied Science and Engineering Technology,* vol. 6, pp. 995-1002.

[11]   Fendinger, N. J., Versteg, D. J., Weeg, E., S., D., and Rapaport, R. A., 1994. *Environmental behavior and fate of anionic surfactants. In: Environmental chemistry of lakes and reservoirs, (ed. L. A baker)*. Washington Dc, USA: American Chemical Society. pp. 527-557.

[12]   Sharrel, R., Aju, A., Sathish, M., and Jisha, S., 2014. "Surfactants: Toxicity, remediation and green surfactants." *Environmental Chmistry Letters,* vol. 12, pp. 275-287.

[13]   Yancheva, V., Stoyanova, S., and Georgieva, E., 2015. "Histological biomarkers in fish as a tool in ecological risk assessment and monitoring programs: A review." *Applied Ecology and Environmental Research,* vol. 14, pp. 47-75.

[14]   Torre de, L., Ferrari, F. R., and Salibián, L. A., 2005. "Biomarkers of a native fsh species (Cnesterodon decemmaculatus) application to the water toxicity assessment of a periurban polluted river of Argentina." *Chemosphere,* vol. 59, pp. 577-583.

[15]   Moiseenko, T. I., Kudryavtseva, L. P., and Gashkina, N. A., 2005. "Assessment of the geochemical background and anthropogenic load by bioaccumulation of microelements in Fish." *Water Resources,* vol. 32, pp. 640-652.

[16]   Pocklington, P. and Wells, P. G., 1992. "Polychaetes: Key Taxa for marine environmental quality monitoring." *Marine Pollution Bull.,* vol. 24, pp. 593-598.

[17]   Martin, M. and Richardson, B. J., 1995. "A paradigm for integrated marine toxicity research: Further views from the Pacific Rim." *Marine Pollution Bulletin,* vol. 30, pp. 8-13.

[18]   Jha, A. N., 2004. "Genotoxicological studies in aquatic organisms: an overview." *Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 552, pp. 1-17.

[19]   Shugart, L. R., 2000. "DNA damage as a biomarker of exposure." *Ecotoxicology,* vol. 9, pp. 329-340.

[20]   Frenzilli, G., Nigro, M., and Lyons, B. P., 2009. "The Comet assay for the evaluation of genotoxic impact in aquatic environments." *Mutation Research, Reviews in Mutation Research,* vol. 681, pp. 80-92.

[21]   Akcha, F., Leday, G., and Pfohl-Leszkowicz, A., 2004. "Measurement of DNA adducts and strand breaks in dab (limanda limanda) collected in the field: Effects of biotic (age, sex) and abiotic (sampling site and

period) factors on the extent of DNA damage." *Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 552, pp. 197-207.

[22] Lyons, B. P., Stewart, C., and Kirby, M. F., 1999. "The detection of biomarkers of genotoxin exposure in the european flounder (platichthys flesus) collected from the river tyne estuary." *Mutation Research, Genetic Toxicology and Environmental Mutagenesis,* vol. 446, pp. 111-119.

[23] Mitchelmore, C. and Chipman, J., 1998. "DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring." *Mutation Research,* vol. 399, pp. 135-147.

[24] Custer, T., Custer, C., Hines, R., Sparks, D., Melancon, M., Hoffman, D., Bickam, J., and Wickliffe, J., 2000. "Mixed-function oygenases, oxidative stress, and chromosomal damage measured in lesser scaup wintering on the Indiana Harbor Canal." *Archives of Environmental Contamination and Toxicology,* vol. 38, pp. 522-529.

[25] Frenzilli, G., Falleni, A., Scarcelli, V., Del Barga, I., Pellegrini, S., Savarino, G., Mariotti, V., Benedetti, M., Fattorini, D.*, et al.*, 2008. "Cellular responses in the cyprinid Leuciscus cephalus from a contaminated freshwater cosystem." *Aquatic Toxicology,* vol. 89, pp. 188-196.

[26] Bickham, J., Mazet, J., Blake, J., Smolen, M., Lou, Y., and Ballachey, B., 1998. "Flow cytometric determination of genotoxic effects of exposure to petroleum in mink and sea otters." *Ecotoxicology,* vol. 7, pp. 191-199.

[27] Easton, M. D. L., Kruzynski, G. M., Solar, I. I., and M., D. H., 1997. "Genetic toxicity of pulp mill effluent on juvenile chinook salmon (Oncorhynchus tshawytscha) using flow cytometry." *Water Science and Technology,* vol. 35, pp. 347-355.

[28] Goanvec, C., Theron, M., Lacoue-Labarthe, T., Poirier, E., Guyomarch, J., Le-Floch, S., Laroche, J., Nonnotte, L., and Nonnotte, G., 2008. "Flow cytometry for the evaluation of chromosomal damage in turbot Psetta maxima (L.) exposed to the dissolved fraction of heavy fuel oil in sea water: a comparison with classical biomarkers." *Journal of Fish Biology,* vol. 73, pp. 395-413.

[29] Marchand, J., Tanguy, A., Laroche, J., Quiniou, L., and Moraga, D., 2003. "Responses of european flounder platichthys flesus populations to contamination in different estuaries along the atlantic coast of France." *Marine Ecology Progress Series,* vol. 260, pp. 273-284.

[30] Bernet, D., Schmidt-Posthaus, H., Wahli, T., and Burkhardt-Holm, P., 2004. "Evaluation of two monitoring approaches to assess effects of waste water disposal on histological alterations in fish." *Hydrobiologia,* vol. 524, pp. 53-66.

[31] Handy, R. D., Runnalls, T., and Russell, P. M., 2002. "Histopathologic biomarkers in three spined sticklebacks, Gasterosteus aculeatus, from several rivers in Southern England that meet the freshwater fisheries directive." *Ecotoxicology,* vol. 11, pp. 467-479.

[32] Stehr, C. M., Myers, M. S., Johnson, L. L., Spencer, S., and Stein, J. E., 2004. "Toxicopathic liver lesions in English sole and chemical contaminant exposure in Vancouver Harbour, Canada." *Marine Environmental Research,* vol. 57, pp. 55-74.

[33] Stentiford, G. D., Longshaw, M., Lyons, B. P., Jones, G., Green, M., and Feist, S. W., 2003. "Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants." *Marine Environmental Research,* vol. 55, pp. 137-159.

[34] Teh, S. J., Adams, S. M., and Hinton, D. E., 1997. "Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress." *Aquatic Toxicology,* vol. 37, pp. 51-70.

[35] Au, D. W. T., 2004. "The application of histo-cytopathological biomarkers in marine pollution monitoring: a review." *Marine Pollution Bulletin,* vol. 48, pp. 817-834.

[36] van der Oost, R., Beyer, J., and Vermeulen, N. P. E., 2003. "Fish bioaccumulation and biomarkers in environmental risk assessment: A review." *Environmental Toxicology and Pharmacology,* vol. 13, pp. 57-149.

[37] Brehmer, P., François, G., Charline, L., Pascal, C., Alina, A., and Birane, S., 2007. *Schooling behaviour of small pelagic fish: Phenotypic expression of independent stimuli*. Ebook Germany: Otto KInne.

[38] Dolezel, J., Binarova, P., and Lucrett, S., 1989. "Analysis of Nuclear DNA content in plant cells by Flow cytometry." *Bolgaria Plantarum,* vol. 31, pp. 113-120.

[39] Matson, J. L., Luke, M. A., and Mayville, S. B., 2004. "The effects of antiepileptic medication on the social skills of individuals with mental retardation." *Research in Developmental Disabilities,* vol. 25, pp. 219-228.

[40] Rosety, M., Ordonez, F. J., Rosety, R. M., Rosety, J. M., Rosety, I., Carrasco, C., and Ribelles, A., 2001. *Histology andHistopathology,* vol. 4, p. 1091.

[41] Rocha, A. J., Gomes, V., Ngan, P. V., Passos, M. J., and Furia, R. R., 2007. "Effects of anionic surfactant and salinity on the bioenergetics of juveniles of Centropomus parallelus (Poey)." *Ecotoxicology and Environmental Safety,* vol. 3, pp. 397-404.

[42] Bantseev, V., McCanna, D., Banh, A., Wong, W. W., Moran, K. L., Dixon, D. G., Trevithick, J. R., and Sivak, J. G., 2003. "Mechanisms of ocular toxicity using the in vitro bovine lens and sodium dodecyl sulfate as a chemical model." *Toxicological Science,* vol. 73, pp. 1-98.

[43] Ahlers, J., Riedhammer, C., Vogliano, M., Ebert, R.-U., Kühne, R., and Schüürmann, G., 2006. *Environmental toxicology and chemistry,* vol. 25, p. 2937.

[44] Wong, D. C. L., Toy, R. J., and Dorn, P. B., 2004. "A stream mesocosm study on the ecological effects of a C12-15 linear alcohol ethoxylate surfactant." *Ecotoxicology and Environmental Safety,* vol. 58, pp. 173–186.

[45]   Dunphy, J. C., Pessler, D. G., Morrall, S. W., Evans, K. A., Robaugh, D. A., Fujimoto, G., and Negahban, A., 2001. "Derivitization LC/MS for the simultaneous determination of fatty alcohol and alcohol ethoxylate surfactants in water and wastewater samples." *Environmental Science and Technology,* vol. 35, pp. 1223–1230.

[46]   Dyer, S. D., Stanton, D. T., Lauth, J. R., and Cherry, D. S., 2000. "Structure-activity relationships for acute and chronic toxicity of alcohol ether sulfates." *Environmental Toxicology Chemistry,* vol. 19, pp. 608–616.

[47]   Boeije, G. M., Cano, M. L., Marshall, S. J., Belanger, S. E., Van Compernolle, R., Dorn, P. B., Gümbel, H., Toy, R., and Wind, T., 2006. "Ecotoxicity quantitative structure-activity relationships for alcohol ethoxylate mixtures based on substance-specific toxicity predictions." *Ecotoxicology and Environmental Safety,* vol. 64, pp. 75-84.

[48]   Eadsforth, C. V., Sherren, A. J., Selby, M. A., Toy, R., Eckhoff, W. S., McAvoy, D. C., and Matthijs, E., 2006. *International Biodetermination Biodegradation,* vol. 63, pp. 981-987.

[49]   Basketter, D. A., York, M., McFadden, J. P., and Robinson, M. K., 2004. "Determination of skin irritation potential in the human 4-h patch test, ." *Contact dermatis,* vol. 51, pp. 1-4.

[50]   Federle, T. W. and Itrich, N. R., 2006. "Fate of free and linear alcohol-ethoxylate-derived fatty alcohols in activated sludge." *Ecotoxicology and Environmental Safety,* vol. 64, pp. 30–41.

[51]   Belanger, S. E., Dorn, P. B., Toy, R., Boeije, G., Marshall, S. J., Wind, T., Van Compernolle, R., and Zeller, D., 2006. "Aquatic risk assessment of alcohol ethoxylates in North America and Europe." *Ecotoxicology and Environmental Safety,* vol. 64, pp. 85-99.

[52]   Rajagopal, S., Sudarsan, N., and Nickerson, K. W., 2002. "Sodium dodecyl sulfate hypersensitivity of clpP and clpB mutants of Escherichia coli." *Applied and Environmental Microbiology,* vol. 68, pp. 4117-4121.

[53]   Sirisattha, S., Momose, Y., Kitagawa, E., and Iwahashi, H., 2004. "Water Research, 1, 61. S-phase as prognostic factors in node-positive breast cancer: results from patients enrolled in two prospective randomized trials." *J. Clin. Oncol.,* vol. 11, pp. 351–359.

[54]   Abel, P. D., 1974. "Toxicity of synthetic detergents to fish and aquatic invertebrates." *Journal of Fish Biology,* vol. 6, pp. 279-298.