BIOCHEMICAL EFFECTS OF ANIONIC, CATIONIC AND NON IONIC SURFACTANTS ON A TROPICAL TELEOST *OREOCHROMIS MOSSAMBICUS* (PETERS) AND A MARINE CYANOBACTERIUM *SYNECHOCYSTIS SALINA* WISLOUCH

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BY

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...to My Grand Mother

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Certificate

This is to certify that the thesis entitled **Biochemical Effects of Anionic, Cationic and Non ionic Surfactants on a Tropical Teleost Oreochromis mossambicus (Peters) and a Marine Cyanobacterium Synechocystis salina Wislouch** *is an authentic record of the research work carried out by* **Ms. Bindu P.C.** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of* **Doctor of Philosophy** *in Biochemistry of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

Kochi-16
January 2002

BABU PHILIP

Declaration

I hereby declare that the thesis entitled **Biochemical Effects of Anionic, Cationic and Non ionic Surfactants on a Tropical Teleost Oreochromis mossambicus (Peters) and a Marine Cyanobacterium Synechocystis salina Wislouch** *is a genuine record of research work done by me under the supervision and guidance of Prof. Dr. Babu Philip, Head, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.*

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List of Notations and Abbreviations

AE	-	alkyl ethoxylates
ALP	-	alkaline phosphatase
ALT	-	alanine transaminase
ANOVA	-	analysis of variance
AOS	-	alkyl oleiffin sulfate
APE	-	alkyl phenol ethoxylate
APHA	-	American Public Health Association
AS	-	alkyl sulphate
AST	-	aspartate transaminase
ATPase	-	adenosine triphosphatase
CAT	-	catalase
CD	-	conjugated dienes
CMC	-	critical micellar concentration
CTAB	-	cetyl trimethyl ammonium bromide
EO	-	ethylene oxide
GR	-	glutathione reductase
GSH	-	glutathione (reduced)
HPI	-	hypothalamic pituitary interrenal
IU	-	international unit
LAS/LABS	-	linear alkyl benzene sulphonate
LC_{50}	-	lethal concentration causing 50% mortality
LERA	-	lysosomal enzyme release assay
LSD	-	least significant difference
LSI	-	lysosomal stability index
MDA	-	malondialdehyde
NP	-	nonyl phenol
PUFA	-	poly unsaturated fatty acid
QAC	-	quaternary ammonium compound
SAS	-	sodium alkyl sulfate
SDS	-	sodium diodecyl sulfate
SOD	-	superoxide dismutase
TX-100	-	Triton X-100

Contents

CHAPTER

1

Introduction

1.1 Relevance of Surfactants as Aquatic Pollutants

Aquatic environment has been the dumping ground of several man-made pollutants. The detrimental effects of these anthropogenic compounds on aquatic flora and fauna have always been an interesting and relevant topic of research. Marine pollution research started 30 years ago with studies on radioactive wastes dumped into the sea. The first international congress on marine pollution took place in 1959. It was found that the major contaminants of the aquatic environment were petroleum hydrocarbons, heavy metals, oil, waste heat, radioactive wastes, pathogens etc. Based on their adverse effects and laws regarding their disposal these pollutants have been classified into black list and grey list compounds (Ruivo, 1972). Domestic wastes containing detergents and sewage effluents were a neglected group of pollutants as they were thought to be mild in their adverse effects.

India has a rich tradition of the use of natural products especially in the field of body adornment. This includes natural cleansing agents, herbal shampoos, pollen based powders, perfumes and the like. Ever since the evolution of *Homo sapiens*, next to the basic needs of food, shelter and clothing, cleanliness and sense of beauty have received priority. The traditional materials used for such purposes in earlier days gave way in course of time to many synthetic formulations.

Until 1918, soap (sodium/potassium salt of long chain fatty acids) was the main product used for cleaning. With the introduction of chemical industries, the production and use of detergents and cosmetics developed and the demand for

these products automatically increased with better standards of living. Moreover there has been an increased use of these synthetic detergents (syndets) since 1960. The syndets have an edge over soap as they are unaffected by the hardness of water and are superior to soaps in their efficacy. The manufacture of detergents in India is at present carried out by many units scattered in organised and small-scale sectors. There is no law so far pertaining to Indian Standard Specifications for the quality and quantity of the ingredients to be used in detergents and cosmetics. Ministry of Health and the Bureau of Indian Standards are now jointly working for setting up quality standards for detergents and cosmetics. Testing and analytical facilities for detergents are few and there is lack of stringent regulation or legislation (Mathur and Gupta, 1998). However, in spite of all these, the demand and the use of detergents have attained new dimensions in the fields of laundry industry, in pesticide formulations, pharmaceuticals, plastics, herbicides and many other products of day to day use.

Detergents are complex mixtures of surface-active compounds or surfactants (10-18%), builders and bleaches. Surfactants are mixtures of homologues of a material differing in chain length, degree of substitution etc. Usually the properties of these compounds are additive i.e., the total property is the sum of the properties of individual constituents. After use these are discharged as domestic waste and reach the environment via sewers and/or sewage treatment plants. Surfactant is an amphipathic molecule and may be anionic, cationic, non ionic or zwitter ionic based on the characteristic ionisable group present in it. The builder component used in earlier days was sodium tri polyphosphate (STPP). These phosphate containing detergents were found to cause accumulation of phosphate in rivers (40%) in early sixties which led to eutrophication and subsequent nitrogen imbalances (Patrick and Khalid, 1974; Salas and Martino,

1991). Thereafter a strict regulation was imposed which reduced the permissible amounts of phosphates in detergents. It was replaced by the less toxic zeolites/silicates. The builder additive commonly used is polycarboxylate and perborate is added as the bleaching agent. The studies reveal that almost all the detergent components are toxic to the aquatic organisms, especially the surfactant and the builder. In addition, the detergents may also contain enzymes, perfumes, dyes etc. (de Oude, 1992).

Detergents were also found to have adverse effects on humans. They affect the skin by removing the stratum corneum and react with other skin proteins. They also aid in the penetration of other substances as well. Skin irritation due to detergents is not a problem where machine washing is the rule, but in India where washing is done largely by hand it is of great significance. Skin irritation potential of several anionics like sodium dodecyl sulfate and non ionics were investigated and these were found to cause allergic dermatitis (Manning *et al.*, 1998). Occupational dermatitis was noted among workers in detergent manufacturing plant. Children are the victims of poisoning by oral consumption of household detergents. Detergents and cleaners rank third in the number of reported cases of accidental ingestion every year (Mathur and Gupta, 1998).

Extensive research has been done recently in the evaluation of the toxicity of detergents. This group has now received top priority among other aquatic pollutants because of their ever increasing use and discharge into water bodies. Also they have synergistic effect on other pollutants like oil, metals, pesticides etc. (Dennis, 1997; Panigrahi and Konar, 1990). Approximately 15 million tons of soap and synthetic surfactants were used world wide in 1987 (Berth and Jeschke, 1989). Surfactants most commonly used in commercial detergents were linear alkyl benzene sulfonate (LABS/LAS), alkyl ethoxylates (AE), alkyl phenol

ethoxylates (APE) and quaternary ammonium compounds (QAC). Alkyl benzene sulfonate (ABS) was the commercially important laundry surfactant in earlier days, but was banned as it was non biodegradable and also was highly toxic to aquatic life. It was substituted by the linear alkyl benzene sulfonate (LAS), an anionic compound. LAS is a petroleum product and is treated with oleum or sulphur trioxide gas to obtain LAS. It is then neutralised with alkali and then other ingredients like fillers are added (Wagle, 1996). LAS was the most extensively studied surfactant and several references are available regarding quantification and toxicity of the chemical to a large number of invertebrates and vertebrates (Kikuchi *et al.*, 1986; Huber, 1989; Kimerle, 1989).

Though legislators prescribe surfactant biodegradability, they are not completely mineralised in biological waste treatment plants. Unfortunately India being a developing country has poor wastewater treatment facilities. Water pollution prevention law (revised in 1990) puts up a policy for proper treatment of domestic wastewater. Also it has been identified that the presence of detergents create significant cost increases in sewage treatment. In India, domestic sewage treatment is limited only to 15 class1 cities (out of total 142) where as full sewerage treatment is present only in 7 (out of 190) class 2 towns. 55 class 1 cities, 35 class 2 towns have partial sewerage. Also 27 class 1 cities and 12 class 2 towns have partial treatment facilities. 72 class 1 cities and 147 class 2 towns have neither sewerage nor treatment facilities. Thus in our country sewage or domestic waste pose a major cause of aquatic pollution and would undoubtedly be the major threat in years to come (Chittkkara, 1998).

Coastal areas are the most prone to pollutant effects as they receive domestic waste/sewage/industrial effluents directly and it has been reported by Mukherjee *et al.* (1992) that detergent inputs into rivers have reached a point of

rising concern in India. The quality of coastal waters is largely affected by sewage pollution from large human settlements and industries that dump the wastes into the sea. It has been observed that the coasts most stressed by pollution load from sewage are Maharashtra, Tamilnadu and Kerala. In Kerala the hot spot is the southern half of coastline including Cochin, Ernakulam and Trivandrum (Chitkkara, 1998).

Thus, surfactants and their metabolites form the biggest group of anthropogenic pollutants. The ability of these compounds for foam formation is a serious problem as organic contaminants and pathogenic micro organisms are concentrated in the foam and thus present an epidemiological threat. In addition foaming also reduces aeration and causes hypoxia. More over the quantification of surfactants in rivers, sewage, marine waters etc has been done mainly in European and U.S. water bodies (Painter, 1992; Holt *et al.*, 1992). The local conditions of temperature, humidity, water quality etc also influence the extent of toxicity. Hence the researches done in foreign countries may not apply to the Indian scene.

Historically, LAS has received utmost attention among surfactants whereas other anionics like alkyl sulfates which are now increasingly used were neglected. Also the non ionics like alkyl ethoxylates and the cationics excluding ditallow dimethyl ammonium chloride have not been properly studied for their chronic toxic effects on aquatic species. Hence there is need for a database investigating on the toxicities of the alkyl sulfates, non ionics and the cationic, all the more so because of the increased use of the non ionics in pesticide formulations, emulsion stability and pharmaceuticals. Also alkyl phenol ethoxylates (APE) are now the most widely used at the industrial level and rank third for all types of applications with an annual volume of production of 370,000 tonnes (Raymond, 1996). Also

the estrogenic potential of alkyl phenols is said to cause a detrimental effect on male reproductive health (Jobling and Sumpter,1993; White *et al.*, 1994). The cationics now find applications in fabric softeners, drilling mud, antiseptics, disinfectants, eye drops etc. Though the laboratory studies have their own limitations while extrapolating the results to complex natural environmental conditions, yet they are of unquestionable benefit to provide an insight into the sub lethal chronic effects of the test compounds.

The present work is a base line attempt to investigate and assess the toxicities of three surfactants viz. anionic sodium dodecyl sulfate (SDS), non ionic Triton X-100 (TX-100) and cationic cetyl trimethyl ammonium bromide (CTAB). These compounds represent simple members of the often neglected group of aquatic pollutants i.e. the anionic alkyl sulfates, non ionics and the cationics. These compounds are widely used in plastic industry, pesticide/herbicide formulations, detergents, oil spill dispersants, molluscicides etc. The test organisms selected for the present study are the cyanobacterium *Synechocystis salina* Wislouch representing a primary producer in the marine environment and a fresh water adapted euryhaline teleost *Oreochromis mossambicus* (peters) at the consumer level of the ecological pyramid. The fish species, though not indigenous to our country, is now found ubiquitously in fresh water systems and estuaries. Also it is highly resistant to pollutants and has been suggested as an indicator of pollution in tropical region (Ueng and Ueng, 1995).

1.2 Scope of the Work

The present work investigates the chronic biochemical changes induced by the sub lethal concentrations of the three surfactants viz. anionic sodium dodecyl

sulfate (SDS), non ionic Triton X-100 (TX-100) and cationic cetyl trimethyl ammonium bromide (CTAB).

SDS - $H_3C-(CH_2)_{11}-OSO_3^- Na^+$

TX-100 $H_3C-(CH_2)_7-C_6H_4-(OCH_2)(CH_2O)_{10}H$

CTAB - $H_3C-(CH_2)_{15}-N^+(CH_3)_2-CH_3\ Br^-$

The thesis is divided into two sections.

Section 1 discusses the effects of surfactants on the teleost fish *Oreochromis mossambicus* after an exposure to 1 ppm (1/10 of LC_{50}) of each of the three surfactants for a period of 30 days. This corresponds to 3.468µM SDS, 0.0015µM Tx-100 and 2.74µM CTAB. The parameters studied include

1. Membrane stability

The stability of the hepatic lysosomal membrane (*in vitro and in vivo*) and erythrocyte membrane *(in vitro)* was studied in presence of surfactants. The release of acid phosphatase and hemoglobin respectively were used as the criteria for assessment of membrane stability.

2. Effect on lipid peroxidation

Studies were done for evaluating the peroxidative effects of surfactants on biological membranes. The important markers in lipid peroxidation viz., catalase,

superoxide dismutase, glutathione reductase, glutathione, malondialdehyde and conjugated dienes were assayed in tissues like liver, kidney and heart.

3. Hepatic enzymes and other biochemical parameters

Enzymes like acid and alkaline phosphatases (ACP and ALP respectively), alanine transaminase (ALT) and aspartate transaminase (AST) were assayed to assess the impacts of the surfactants on metabolic functions of the cell. The levels of glycogen, protein and lipid were also estimated.

4. Osmoregulation and branchial ATPases

Fresh water fishes engage in active ion uptake to maintain ionic homeostasis. Gill Na^+-K^+ ATPase and Mg^{2+} ATPase play a significant role in this respect. The activity of these enzymes as influenced by the surfactants was studied.

Section 2 deals with the biochemical effects of surfactants on the marine cyanobacterium *Synechocystis salina* Wislouch. Here the parameters studied include

1. Growth - as determined by cell count/fluorescence measurement.
2. Estimation of Chlorophyll *a*
3. Estimation of protein
4. Estimation of carbohydrate
5. Estimation of lipid

CHAPTER

2

Review of Literature

The studies regarding detergent toxicity started as early as 1970. The toxicity was monitored at first at the level of behavioural, physiological and histological changes. Common experimental animals were *Daphnia*, various fish species like *Ayu, Tilapia, Pimephales,* etc. Also molluscs like *Mytilus, Crassostrea* and clams were used as test organisms. Studies were focused mainly on exposure to high concentrations of the detergents and acute studies were given more importance. Later, chronic toxicity studies using the test compounds gained more relevance and the interest was diverted to the biochemical changes on exposure to sub-lethal and environmentally relevant concentrations of detergents. The most commonly studied detergents were branched alkyl benzene sulfonate (ABS) and linear alkyl benzene sulfonate (LAS or LABS) which were used extensively in commercial products mainly cleaners (Abel,1974).

2.1 Physiological Studies on Surfactant/Detergent Toxicity

Many of the reports available are related to the effects of anionic surfactants during exposure periods of 15 minutes to 30 days. Effects on olfactory responses, respiration and gill physiology were the most frequently monitored. It was found that concentrations greater than 0.1 ppm were sufficient to elicit characteristic physiological responses.

The blocking effects of cationic and anionic (ABS) compounds were noted on the olfactory epithelium of Atlantic Salmon at 1ppm (Sutterlin *et al.*, 1971).

No effects were observed for non ionics. It was also found that in many of the cases these effects were reversible.

Effects on feeding pattern/feeding rate were studied in presence of surfactants. Probably due to olfactory disturbances, it was observed that feeding rate in presence of detergents was reduced. The effects of linear alkyl benzene sulphonate on fish *Tilapia mossambica*, plankton *Diaptomus forbesi* and the worm *Branchiura* were studied by Konar and Chattopadhyay (1985). The feeding rates were decreased at 0.25, 0.38 and 1.1 ppm. The fishes were found to move towards the feed, swallowed it, tried to chew for 3-4 sec but ejected the food matter out of the buccal cavity. Swallowing and regurgitation continued 3-4 times, whereas the control fishes rushed towards the food and swallowed it quickly.

Respiration was largely affected in presence of surfactants. The respiratory rate was increased in *Lepomis machrochirus* at concentrations above 1.56 ppm when exposed to alkyl ethoxylates (Maki, 1979).

The detergent exposure (oil spill dispersants) was found to induce conditions similar to hypoxia. There was an increase in heart rate and ventilation volume (Kiceniuk *et al.,* 1978) and concomitant bradycardia in fish-*Tautogolabrus adspersus* (cunner fish) exposed to non ionics viz. Triton X-100, Tween 20 and the anionic sodium lauryl sulfate. Bradycardia so induced was sustained during the exposure period and was reversible. All the surfactants tested produced the same response differing only in the threshold concentrations. This rapid reversibility showed that the observed effect was the result of a reversible action on a peripheral site /sensory receptor of the gill epithelium.

Developmental abnormalities were caused by surfactants to a large extent. Studies on fat head minnows, tilapia, *poecilia* etc. revealed that hatching, growth and larval survival were affected at linear alkyl benzene sulphonate (LAS)

concentrations of 0.25-1.1 ppm for 90 days. Non ionics like C_{12}-C_{13} alcohol ethoxylates also created disturbances in development by interfering with growth, hatching and larval development when the exposure period was 90 days. Cationics like ditallow dimethyl ammonium chloride and tri methyl ammonium chloride were found to affect the developmental stages of fat head minnows when exposed for 28 days. Similar data regarding surfactant toxicity are also available for invertebrates like sea urchins, sponge, star fish etc. (Swedmark *et al.*, 1971; Hidu, 1965; Vailati et al., 1975 and Moffet and Grosch,1967). Marchetti (1965) and Pickering (1966) stated that developmental stages of fishes were especially sensitive to surfactants.

Holman and Macek (1980) found that there was a reduction in the survival rate of fish larvae at 0.5 ppm of linear alkyl benzene sulphonate (LABS). The survival of the first generation of larvae was significantly reduced at 0.25 ppm of linear alkyl benzene sulphonate and also there was a reduction in spawning. Hidu (1966) also reported stunted growth and decreased survival rates of veliger larvae of clams and oysters in presence of low concentrations of surfactants. Swedmark *et al.* (1971) studied the developmental abnormalities induced by alkyl benzene sulphonate (ABS), linear alkyl benzene sulphonate (LABS), linear ethoxy sulphate (LES), nonyl phenol 10 ethoxylate (NP 10 EO), tallow alcohol ethoxylate (TAE) in fish (cod, flounder), mussels (*Mytilus*), *Mya arneria* (clams), cockle and pecten and crustaceans like balanus. The effects of surfactants were studied on the eggs and larvae of the fishes, larvae of *Hya*s and larvae and juveniles of *Balanus*. The survival rate and percentage of normal development was similar to control fishes in cod at 0.02 ppm linear alkyl benzene sulphonate and 0.2 ppm nonyl phenol 10 ethoxylate. The eggs and veliger of *Mytilus* were more sensitive and at 2 ppm nonyl phenol 10 ethoxylate embryos never developed beyond the blastula stage

and at 1 ppm never beyond the veliger larvae. Planktonic larvae of *Balanus* and *Hyas* were found to be more sensitive than adults to the surfactants. Crustaceans in the inter moult stages were generally resistant and the degree of resistance decreased during the 15 h period after moulting.

Olfactory membrane receptors of fish are directly exposed to pollutants in the aquatic environment and are not protected by barriers. Thus alterations in water quality would easily interfere with their functions, the consequence being a break down in the communication among the fish and between the fish and the environment. Hara and Thompson (1978) reported that sodium lauryl sulphate (SLS) at 0.1 ppm depressed the olfactory senses in the white fish *Coregonus clupeaformis*. SLS was also found to cause a decrease in shell weight of the snails (*Tarazona)* at 0.61 ppm and alkyl benzene sulfonate interfered with the uptake of calcium.(Misra *et al.*, 1984).

2.2 Behavioural Studies on Surfactant Toxicity

Acute toxicity studies in surfactants as well as exposure to sub lethal concentrations gave sufficient opportunity to monitor the behavioural changes. Avoidance reactions were the most commonly observed in fishes. Sprague and Drury (1969) observed avoidance reaction of salmonids at very low concentrations of alkyl benzene sulphonate. It was also observed that the avoidance reaction was more pronounced in case of exposure to anionic surfactants.

Swedmark *et al.* (1971) have studied the responses of fish, mussels, clams and crustaceans on exposure to various anionic and non ionic surfactants. The swimming activity was affected in case of fishes and it was observed that more active fish species were affected the most. The ability for valve closure was

affected in *Mytilus edulis* on exposure to the non ionic nonyl phenol 10 ethoxylate. The siphon retraction was adversely affected in *Mya arneria*. The burrowing activity of the cockle was inhibited by the surfactants. Crustaceans like prawns, *Leander sp*, exhibited violent movements of abdomen and extremities on surfactant exposure. In barnacle the beat of cirri and shell closure were affected, the cirri beat gradually decreased on exposure to increasing concentrations of nonyl phenol 10 ethoxylate. Swimming of the nauplius larvae of *Balanus* and zoea of *Hya* were also seriously affected on exposure to surfactants.

Avoidance reactions for anionic surfactants were studied on a large scale by many workers. Concentrations between 0.002-0.011 ppm of linear alkyl benzene sulfonate and alkyl sulfates elicited avoidance reactions (Tatuskawa and Hidaka,1978) in *Plecoglossus*. In case of medaka, the concentration required was 0.007-0.027 ppm (Hidaka *et al.*, 1978). For alkyl benzene sulfonate the concentration eliciting the avoidance reaction was 0.001 for *Salmo gairdneri* and 0.02ppm for *Gadus morrhua*. A higher concentration of non ionic surfactants was required for eliciting avoidance reactions. For C_9 alkyl phenol 10 ethoxylates it was 2-4 ppm (Hoglund, 1976). The responses to the surfactants were erratic in most of the cases. Swimming and feeding responses were affected at higher concentrations.

2.3 Histological Studies on Surfactant Toxicity

Numerous histological studies were also reported on acute surfactant toxicity in fishes. The tissues subjected to analysis included gill lamellae, olfactory epithelium, club cells, taste buds etc. It was inferred from these studies that the surfactants induced great damage at the sub cellular level.

The fish *Rita rita* exposed to sodium dodecyl benzene sulfonate at concentration of 6.9 ppm showed a gradual decrease in lipid moieties of the epithelial cells, club cells, goblet mucus cells lining the gill arch and the gill filament epithelium. A gradual decrease was also noted in the protein constituents of these cells when subjected to histochemical techniques (Roy, 1990).

Pathomorphological changes in the skin were noticed under the scanning electron microscope in the fingerlings of *Cirrhina mrigala* exposed to 0.005 ppm (25% LC_{50}) of linear alkyl benzene sulphonate (Misra *et al.*, 1987). The epithelial cells present in the epidermis of the dosed fish were found to secrete more mucus than the control group. The gill lamellae showed a distorted appearance indicating severe damage that led to dysfunctions in respiration and osmoregulation.

Effects of a cationic detergent (Zephiran) were studied on the gills of *Salmo gairdneri* (Byrne *et al.*, 1989). This chemical was being widely used for the treatment of bacterial gill diseases and is an alkyl dimethyl benzyl ammonium chloride. Scanning and transmission electron microscope studies revealed that at 3 ppm the gill tissue showed severe spongiotic lesions, necrotic lesions, lamellar fusion, membrane vesiculation, hydropic degeneration, exfoliation of lamellae and interlamellar epithelium.

Acute toxicity of 3 detergents viz. linear alkyl benzene sulfonate, Triton X100 and sodium dodecyl sulfate were studied on *Arenicola* (Emilio conti, 1987). The anionics were found to cause more damage. Linear alkyl benzene sulfonate affected the papillae and the caudal epithelium. Linear alkyl benzene sulfonate also affected the epidermal receptors and decrease in olfactory response. Triton X-100 affected both the morphology and physiology of the olfactory mucosa. Triton X-100 also caused the rupture of the intestinal wall and blood vessels. Gills

were the most damaged tissue and linear alkyl benzene sulfonate destroyed the epithelium and the blood vessels of the gills.

Linear alkyl benzene sulfonate toxicity was reported by other workers like Pohlagubo and Adam (1982). They observed that LAS at 1 ppm caused skin degeneration in rainbow trout. Linear alkyl benzene sulfonate at 1 ppm was also found to cause intestinal damage in *Pisidium casertanum* (Maciorowski *et al.*, 1977).

A commercial detergent "Ariel" at 5 ppm was found to induce moderate degenerative changes in the respiratory lamellae in *Oreochromis mossambicus* on 2 days exposure and then chronic exposure led to drastic changes like separation of the epithelium layer and atrophy (Raju *et al.*, 1994).

Response of the mucus cells of the epidermis of *Clarias* exposed to a sub lethal concentration of sodium dodecyl sulfate was studied by Garg and Mittal (1993). At 4, 8, 24, 48 and 72 h of treatment most of the cells attained voluminous dimensions and appeared closely approximating to or even overlapping the adjacent ones. Statistically however no significant change was observed in the total number of these cells. The mucus cells were enlarged towards the end of the experiment signifying enhanced mucus production which may be considered as an adaptation to the environmental change. A shift in the histochemical nature of the secretory contents of the middle and basal parts of the cells from acid to neutral glycoproteins during early stages of treatment suggest that acid moieties could not simultaneously be synthesised as an immediate response to enhanced mucus secretion. The apical parts of the mucus cell showed no histochemical change throughout the experiment.

Rosety *et al.* (1985) studied the sodium dodecyl sulfate induced histological changes in the kidney and spleen of *Sparus aurata* at 5, 8.5, 10 and

15 ppm. Kidney showed loss of normal structure with tubular and renal corpuscle retraction. Spleen showed damage of the reticular structure and a progressive increase in the infiltration of leucocytes and red cells.

2.4 Biochemical Studies on Surfactant Toxicity

2.4.1 Studies on Cell Membrane

Gill tissue has its own importance while assessing the toxicity of surfactants. The large surface area of the tissue coupled with its important role in respiration and osmoregulation made it ideal for examining the toxic effects.

Gill viability in presence of surfactants linear alkyl benzene sulfonate and nonyl phenol was studied in rainbow trout by Part *et al.* (1985). The viability of gills deteriorated rapidly during 60 min of exposure to 100 micromoles/litre of linear alkyl benzene sulfonate and to nonyl phenol. Linear alkyl benzene sulfonate was also found to decrease cadmium (Cd) transfer whereas nonyl phenol increased Cd retention.. When tested at environmentally relevant concentrations (0.05 ppm), linear alkyl benzene sulfonate doubled the Cd transfer whereas nonyl phenol had no effect.

The effects of surfactants on gill osmoregulatory function were studied by monitoring the changes in the activity of the gill Na^+-K^+ ATPase. It was reported by many workers that low concentrations of surfactants activated this membrane-bound enzyme while high concentrations had an inhibitory effect. The effects of syndets like Idet 5L and Swanic 6L (SLS) on ATPase activity was studied by Verma *et al.* (1979) in the fish *Channa punctatus*. They exposed the animals to sub lethal levels of these syndets for 25 and 50 days. The analysis of the enzyme activity revealed that enzyme inhibitions were highest in the gill and brain

homogenates for oligomycin-sensitive Mg^{2+} ATPase with pronounced effects (65%) after 50 days of exposure to 7.5ppm of Swanic. Fish exposed to lower concentrations showed an insignificant activation of Na^{+}-K^{+} ATPase and Mg^{2+} ATPase in the gills. A similar study on *in vivo* responses of ATPase was done in *Mystus vittatus* (Verma et al., 1979) exposed to Swascofix (alkyl benzene sulfonate). Here brain, gill, liver and kidney tissues were sampled. After a period of 60 days the highest inhibition was noted in the brain followed by gill, kidney and liver. It was observed that low concentrations in some cases enhanced the activity. Roufogallis (1973) also reported enhanced Mg^{2+}-Ca^{2+} ATPase activity in the microsomal fraction of the bovine brain cortex treated with sodium deoxycholate and Lubrol-WX. The ATPase is concerned with the active transport of sodium ions out of the cell and potassium ions into the cell. Hence it is fundamental to functions like regulation of cell volume and electrolyte balance. Thus an inhibition of the enzyme would result in alterations in membrane/nerve transmission and uncoupling of oxidative phosphorylation. The surfactant is supposed to exert its toxic effect probably at the active site of the enzyme.

Rosas *et al.* (1988) studied the effects of sub lethal concentrations of sodium alkyl aryl sulfonate on 21 day exposure in *Ctenopharyngodon idella* at 3, 5 and 8 ppm. It was noted that plasma sodium levels were decreased below the normal levels of 150 mmol significantly after 15 days. An increase in opercular movements was also noted.

Sub cellular studies using surfactants were done mainly at the level of cell membrane. Red cell membrane was the commonly used one because a release of hemoglobin could serve as a criteria of stability. The differential release of red cell membrane components was done by Kirkpatrick *et al.* (1974). The surfactants used were SDS, Triton X-100 and deoxycholate. It was found that SDS extracted

lipids and proteins separately whereas Triton was found to initially effect membrane labilisation by interacting with the membrane proteins. It was also found that SDS bound to all components of the RBC membrane and that the release of membrane components roughly paralleled their water solubility. It was difficult for them to interpret the Triton behaviour. Though the CMC of Triton was 1 millimol, micelles were not formed until 5 millimol.

The effects of a cationic compound Zephiran were studied in the gills. It induced creation of the red cell membrane (Byrne *et al.*, 1989).

Dielectric, haematological and biochemical investigations on detergent toxicity in fish blood were done by Bielinska (1987) in *Cyprinus carpio.* The sub lethal exposure resulted in decrease in the RBC count, hemoglobin and hematocrit. Also there was an increase uptake of sodium into the cells and intra cellular potassium was also elevated.

The differential release of proteins and lipids from the cell membrane was studied in trout gill epithelium by Partearroyo *et al.* (1991). These studies highlighted the significance of critical micellar concentration in the solubilisation of the membrane components which has great applications in the extraction and study of a large number of membrane bound enzymes.

Surfactants also acted as fusogens as reported by Attwood and Florence (1983). Span 80 was effective in fusing hen RBC.

Hrabak *et al.* (1982) studied the effects of detergents like nonyl phenol 40, sodium dodecyl sulfate, deoxy cholate, Triton X-100 on poly morpho nuclear and mono nuclear lymphocytes of the tonsils. The cells were exposed to the test compounds for 0, 60, 90, 120, 180min and 24 h at 37°C. There was considerable increase in glucose oxidation and respiration in cells exposed to deoxycholate

whereas Triton and sodium dodecyl sulfate had only slight effects on oxidative processes. The cationic cetyl pyridinium bromide had a drastic effect even at low concentrations of 0.001% due to the hydrophobic nature of the cetyl pyridinium ion.

Inhibition of the release of phospholipase A_2 was studied in the sponge *Geodia cydonium* exposed to sodium dodecyl sulfate and cetyl tri methyl ammonium bromide at 0.1 ppb to 10 ppm by Vgarkovic *et al.* (1991). Preincubation of cells in presence of detergents at low concentrations strongly inhibited the release of phospholipase A_2 about 65% and 55% inhibition was effected by 10^{-8}gm/litre of sodium dodecyl sulfate and 10^{-7} gm/litre of cetyl tri methyl ammonium bromide respectively. Also uptake of thymidine precursors was affected by sodium dodecyl sulfate at 10^{-2} gm/litre.

The release of liver acid phosphatase was studied from rat lysosomes in presence of sodium dodecyl sulfate, benzalkonium chloride and tween. The release rate was increased by all the chemicals. It was $>10^{-6}$M for cationics, $>10^{-5}$ M for anionics and tween (Tabata *et al.*, 1990).

Interaction of non ionics with the cell membrane has been studied in detail by Regen *et al.* (1989). It was deduced that non ionics interacted with the cell membrane phospholipids and this led to modification of membrane structure and permeability. This in turn caused leakage of ions, amino acids, enzymes etc from the cell and resulted in cell damage. Supramolecular surfactants like polyethyleneglycol (PEG) as well as Triton readily disrupted the cell membrane of egg yolk. Studies by Naka *et al.* (1993) have shown that Triton and other surfactants caused leakage from palmitoyloleoyl phosphatidyl choline/cholesterol large unilamellar vesicles. Several studies on model membranes revealed that the effect of synthetic surfactants depend upon the cholesterol concentration of the

lipid bilayer where as the effect of Triton was not affected by the same (Nagawa *et al.*, 1991).

The interaction of surfactants with the artificial membranes was found to modify many physico-chemical properties of the cell membrane phospholipids. A fluorescence depolarisation study indicated that alkanoyl-N-methyl glucamide surfactants decreased the fluidity of the di palmitoyl phosphatidyl choline membranes (Inou *et al.*, 1988). Also the non ionics were found to decrease the phase transition temperature of negatively charged dilauroyl phosphatidic acid membrane. Non ionics were also found to increase the permeability of sarcoplasmic reticulum vesicles (Teruel *et al.*, 1991). Pluronic L81, a hydrophobic surfactant greatly influenced the cholesterol homeostasis of the intestinal mucosa (Pool *et al.*, 1991).

The structure-activity relationship of the non ionics was studied in great detail by Gallova *et al.* (1993) and it was inferred that the strength of the interaction depended upon the length of the ethylene oxide chain. It was therefore concluded that non ionic interactions with the cell membrane involved insertion of the hydrophobic moiety of surfactants into the apolar fatty acid domain of phospholipids. Linear structures like fatty acid/long chain alcohols are well accommodated and do not disturb the membrane organisation. Bulky hydrophobic moieties like alkylated phenols caused severe disturbances between the apolar fatty acid chains resulting in increased permeability and leakage. The hydrophilic ethylene oxide chain has probably two functions—it regulates the insertion depth of the hydrophobic moiety (longer chain draws the hydrophobic moiety towards the aqueous phase) indirectly influencing the membrane damaging effect or it binds to the polar head group of the phospho lipids (Cserhati, 1994).

Lewis *et al.* (1993) reported that the anionics and the cationics were more toxic than the non ionics in causing ocular irritancy. Grant *et al.* (1992) also reported that the cytotoxicity of surfactants on rabbit corneal epithelial cells was cationic>anionic=amphoteric>non ionic, how ever Triton had a ranking similar to the anionics. It was reported by Roguet *et al.* (1992) from their studies on uptake of neutral red by rabbit corneal epithelial cells that Triton had a lower toxicity than the cationics and anionics.

Surfactants were also found to modify the arrangement of integral membrane proteins like P-glycoprotein and presumably the glutathione transporters (Board, 1993). Polyoxyethoxylated non ionics inhibited the transport of 2,4-dinitro phenyl glutathione out of human RBC.

2.4.2 Interaction of Surfactants with Enzymes (*in vitro* Studies)

Interaction of surfactants with proteins has been studied by many workers as this forms an important part of extraction and solubilisation of membrane proteins and enzymes (Higgins, 1987). Also interactions of surfactants with many enzymes have also been investigated by many workers.

Large amount of research has been done on surfactant–protein interactions. Charge transfer chromatographic methods indicated that nonyl phenol ethoxylates only interact with some amino acids, the relative strength of the interaction in the increasing order was tyrosine, glutamic acid, phenyl alanine, hydroxy proline, glutamine, cysteine and glycine. A significant relationship was found between the strength of interaction and the hydrophobicity of the amino acids (Forgacs, 1993).

The alkaline phosphatase plays an important role in phosphate hydrolysis, transport of sugars etc. There are few data concerning the effect of detergents on

this enzyme. Joao *et al.* (1987) investigated the effects of sodium dodecyl sulfate Triton X-100 and cationics like quaternary ammonium compounds on alkaline phosphatase. It was found that non ionics like Triton increased the K_m of the enzyme and also its velocity. The same effect was observed for sodium dodecyl sulfate whereas deoxy cholate decreased these parameters. It was found that the enzyme preserves its Michelis-Menten behaviour even in presence of surfactants. The highest velocity was obtained for Triton (265%). This could be due to activation or release of enzyme activity that remained latent within the membrane. Cationics were diverse in their effects. Octa decyl tetramethyl ammonium chloride increased the k_m and decreased the velocity where as trimethyl ammonium bromide increased both these parameters.

Octa ethylene glycol dodecyl ether induced the dissociation of the membrane–bound Na^+-K^+ ATPase purified from the dog kidney (Mimura *et al.*, 1993).

The activation of *Aspergillus niger* catalase by sodium dodecyl sulfate was observed by Jones *et al.* (1987). In marked contrast to other enzymes it was found that the fungal enzyme activity was increased on sodium dodecyl sulfate binding. There was 180% activation when 150 sodium dodecyl sulfate molecules were bound. It was thought that the binding at pH 6.4 resulted only in small conformational change facilitating the enzyme action. In case of bovine catalase it was noted that incubation for 25 h with sodium dodecyl sulfate at pH 6.4 resulted in loss of activity whereas under the same conditions bacterial catalase *(Micrococcus luteus)* retained 80% of its activity for several weeks.

Diane and Christensen (1982) conducted *in vitro* studies on urease inhibition using several organic chemicals like organo phosphates, phenols, quinols, hydrocarbons etc. Of the organic chemicals studied it was found that

SDS was next only to 2,4-D a herbicide in toxicity, 8 x 10^{-4}M being the concentration causing 50% inhibition.

Binding of cationics to DNA, protein and DNA-protein mixtures was studied by Gani *et al.* (1999). It was observed that cetyl tri methyl ammonium bromide at pH 5 and ionic strength 0.05 M bound in high amounts to negatively charged phosphate ion of each nucleotide. But bovine serum albumin (BSA) at pH 5 was bound only moderately by cetyl tri methyl ammonium bromide (CTAB). DNA-protein mixtures had more surfactant binding sites than those in saturated BSA-CTAB and DNA-CTAB complexes. It was concluded that the chain length of amines and conformation of macromolecules play important roles in interaction.

Triton X-100 activation of lecithin-cholesterol acyl transferase (LCAT) was reported by Bonelli and Jonas (1993). Dygas and Zborowski (1989) reported the stimulating effect of Triton on rat liver mitochondrial phosphatidyl serine decarboxylase. Wenzel *et al.* (1990) observed the synergistic action of Triton and other non ionics like Myrj 52 and 59, Tween 20, Tween 80 etc. on the human proteinase elastase and cathepsin G. The long polyoxyethylene chain of non ionics have been shown to effectively increase the activity of *Chromobacterium lipase* (Yamada *et al.,* 1993). Sandstrom and Cleland (1989) reported the activation of plasma membrane ATPase by Triton.

The effect of SDS (20 micro moles) on rat liver and kidney enzymes like acid phosphatase, alkaline phosphatase, succinate dehydrogenase (SDH), aminotransferases like aspartate transaminase, alanine transaminase and ATPase was studied by Gupta and Dhillon (1983). The increase in acid phosphatase was thought to be due to the interaction of sodium dodecyl sulfate with the lysosomes.

Succinate dehydrogenase inhibition was suggested to be the result of interaction of the surfactant with the sulphydryl group of the enzyme.

2.5 Biochemical Studies on Surfactant Toxicity *in vivo*

Surfactants were found to increase the absorption of xenobiotics in rat colon augmenting the adverse effects (Martinez *et al.,* 1993). Emulgen 913 (polyoxyethyleneglycol nonyl phenol ether) decreased the liver weight and cytochrome P450, cytochrome b5 and microsomal heme content in rats whereas heme oxygenase was greatly enhanced. Emulgen 813 also significantly reduced the concentration of metal binding proteins in the hepatopancreas and decreased the heme oxygenase activity in the kidney of red carp (Ariyoshi *et al.,* 1991).

Toxicity of Swascol IP (SLS) to *Channa punctatus* and *Cirrhina mrigala* was studied by Verma *et al.* (1979). The enzymes assayed included acid phosphatase, alkaline phosphatase, succinate dehydrogenase (SDH) in the liver and kidney for 15 and 30 days. 1/2,1/3 and 1/6 of LC_{50} was taken as the sub lethal dose. Acid phosphatase activity was found to alter moderately in the tissues, this could be due to the inhibition of synthesis or increased turn over in presence of the pollutant. At lower doses of 3.25 ppm there was activation whereas at higher concentrations of 6.5 and 9.75 ppm there was inhibition. Alkaline phosphatase activity was significantly decreased in the liver of *Cirrhina mrigala* after 30 days. SDH activity showed a decrease possibly due to impairment of aerobic metabolism. It was inferred that the depletion of phosphatases could be due to uncoupling of oxidative phosphorylation followed by intoxication.

Influence of Idet 20 on the biochemical composition and enzymes of *clarias batrachus* liver was studied by Verma *et al.* (1984). The exposure was for

10, 20 and 30 days. The enzymes assayed included acetyl choline esterase, glucose-6-phosphate dehydrogenase, 5-nucleotidase. Other parameters studied were glycogen, protein, cholesterol, calcium and RNA. An increase of cholesterol was noted in the liver. The inhibitions were all concentration-dependent. The inhibition of glucose-6-phosphate dehydrogenase was supposed to be the result of damage of endoplasmic reticular membranes by the detergent. Decrease in calcium, the regulator of cell membrane permeability and membrane potential, affected the nervous and muscular functions. It was also suggested that the hormones regulating calcium metabolism like calcitonin and parathyroid hormone were affected by the surfactants.

Emmelot and Bos (1965) studied the influence of sodium deoxycholate on 5-nucleotidase activity and found the activating effect of the surfactant. Similarly Konopka *et al.* (1972) found an increase in the activity of this enzyme in rat liver exposed to Triton and sodium deoxycholate.

Effect of Ariel, a commercial powder on the oxidative enzymes and histology of the teleost *Oreochromis mossambicus* was studied by Raju *et al.* (1994). The sub lethal concentration was 5 ppm and the dosing period was 1 week. The tissues viz., liver, kidney, muscle, brain and gill were sampled at 2, 4, 6 and 8 days. The enzymes assayed were Lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH). There was an increase in SDH and decrease in LDH at first indicating aerobic metabolism to combat stress. Then after from fourth day onwards there was a shift towards anaerobic metabolism and increase of LDH.

Effects of detergents in a pond on the biochemical changes of *Machrobrachium lammarei* were studied by Maruthanayagam *et al.* (1997). There was a decrease in the protein, carbohydrate and lipid in the hepatopancreas,

muscle and gill indicating increased mobilisation of reserve substances to adapt to stress conditions.

The toxicity of the detergent alkyl benzene sulfonate to the fingerlings and the yearlings of *Cirrhina* and *Punctius* at 0.005 ppm was studied by Misra *et al.* (1991). The parameters studied included glycogen, lactic acid, sialic acid in the gill, muscle, liver and kidney. There was a significant decrease in the glycogen content of *Cirrhina* in the gills, liver and kidney, but not in the muscle. Lactic acid increased in the gill, liver and kidney in both animals indicating an increase in anaerobic metabolism. A decrease in sialic acid indicated toxicity to the membranes.

In vivo effects of syndets Swanic (poly oxy ethylene ether) and Idet (alkyl aryl sulfonate) on the membrane Na^+- K^+ ATPase of *Channa punctatus* was studied by Verma *et al.* (1979). The tissues studied were the brain and gill. The lowest concentrations of the detergents activated the enzyme whereas the oligomycin-sensitive Mg^{2+} ATPase was insignificantly activated. Significant inhibitions of the oligomycin-insensitive Mg^{2+} ATPase was noted in the gill after 50 days at 7.5 ppm, the maximum in the brain. It was also noted that the inhibitions of the oligomycin-insensitive enzyme was more pronounced than Na^+-K^+ ATPase and oligomycin-sensitive Mg^{2+} ATPase.

The effects of Swascofix (alkyl benzene sulfonate) on Na^+-K^+ ATPase at low concentrations of 0.88, 0.586 and 0.352 ppm was studied in the brain, gill, kidney and liver of *Mystus vittatus* (Verma *et al.*, 1979). The highest inhibition was noted in the brain. The lowest concentrations had a stimulating effect on enzyme activity. In all cases inhibition was concentration–dependent.

2.6 Metabolism and Bioaccumulation of Surfactants

In vivo metabolism and organ distribution of nonyl phenol was studied in Atlantic salmon by Augustine *et al.* (2000). It was observed that the chemical was metabolised *in vivo* to the corresponding glucuronide and hydroxylate conjugates and the major routes of excretion were the bile and urine. The half life of residues in the muscle and carcass was between 48 and 24 h in both water-borne and dietary exposure.

Bioaccumulation and tissue distribution of a quaternary ammonium compound (quaternary ammonium compounds), cetyl pyridinium bromide was studied by Knezovich *et al.* (1989). The test organisms were clams, minnows and tadpoles. The dose was 10 µg/litre. Whole body concentration factor was 21± 7, 22± 3 and 13± 4 respectively for these animals. The gill accumulated the largest amounts indicating high risk. In tadpoles quaternary ammonium compounds sorbed to food particles in the gastro intestinal tract and was subsequently available for sorption to the mucus cells. The tissue burden was found to decrease with time only for minnows and tadpoles. Other than gills, tissues of toxicological interest like liver, kidney and lipid reserves showed only trace amounts of the compound. This indicated that cetyl pyridinium bromide transport was limited across cell membranes. Gills and gastro intestinal tract were found to accumulate the largest amounts as they secreted negatively charged polysaccharides.

It was reported by Lewis and Wee (1983) that ditallow di methyl ammonium chloride showed the maximum accumulation in the viscera of *Lepomis machrochirus*. Also the compound was sorbed to the sediment and dissolved organic matter which decreased its bio availability. Cary *et al.* (1987) were of the opinion that the sorbed compound would be still available to the gastro intestinal

tract. Cutler and Drobeck (1970) reported that in mammals an oral dose of quaternary ammonium compounds caused delayed death and tissue pathology.

Fate and effects of sodium dodecyl sulfate were studied by Singer and Tjeerdema (1993). It was deduced that the metabolism of this compound was similar in fish and mammals. It first underwent omega oxidation to give carboxylic acid and then beta oxidation occurs to give butyric acid-4-sulfate. It was then non-enzymatically desulfurated to give gamma butyro lactone and inorganic sulfate.

Metabolism of a complex mixture of oil spill dispersants was studied by Payne (1982) in *Salmo gairdnerii*. The surfactants tested included Corexit, Syrperonic BP1100 and Oilsperse. It was found that these were metabolised by the lipase enzyme to give fatty acids.

Bioconcentration of linear alkyl benzene sulfonate in blue gill, *Lepomis machrochirus* was studied by Kimerle *et al.* (1981). Exposure to 35 days to a concentration of 0.5 ± 0.05 ppm C^{14} labelled surfactant resulted in greatest accumulation in the gall bladder with a body concentration factor of approximately 5000. The body concentration factor for liver, gills and viscera, remaining carcass and blood ranged between 64 and 283. Clearance of radioactivity was rapid with half- lives of 2-5 days.

The uptake, distribution and elimination of 2 labelled surfactants viz. sodium dodecyl tri oxy ethylene sulfate and penta oxy ethylene sulfate were investigated in *Cyprinus carpio* by Kikuchi *et al.* (1980). It was found that radioactivity was rapidly absorbed by gills and skin and transferred to other organs and tissues. After 24 h exposure, there was a comparatively high accumulation of radioactivity in the gills, hepato pancreas, gall bladder, intestinal content and nasal and oral cavity.

2.7 Synergistic Studies on Surfactant Toxicity

Panigrahi and Konar (1986) studied the effects of a mixture of crude petroleum oil and an anionic detergent Parnol J for 90 days on tilapia. The sublethal exposure (1.01 ppm) resulted in abnormal behaviour of fishes and there was a significant reduction in growth rate, maturity index and fecundity of male and female fishes. Gastrosomatic index (GSI) was significantly increased.

Effects of metals and non ionics was investigated by Dennis *et al.* (1997) on a transgenic nematode *Caenorhabditis elegans* containing *E.coli* lac z gene under control of hsp (heat shock protein) sequence. Pluronic surfactant was found to induce low beta galactosidase activity. The surfactant also promoted growth by enhancing nutrient uptake via membrane pores created by its action. The stress response to cadmium, mercury, manganese and zinc markedly increased lac z activity between 1.5-4 fold when Pluronic was present. Also the effect of surfactant was more pronounced at higher metal concentrations. Also the metal-induced stress was more toxic when combined with the non ionic surfactant, itself being present at low sub toxic concentrations.

High cutaneous toxicity of nickel and SDS was reported by Mathur *et al.* (1992). This was associated with increased lipid peroxidation and higher tissue accumulation of nickel in presence of sodium dodecyl sulfate.

2.8 Studies on Algae

The criteria for the assessment of surfactant toxicity in algae include lethality (Cabridenc, 1978; Whiton, 1967) reduction of standing crop (Nyberg, 1988; Ukeles, 1965), inhibition of algal growth rate (Kutt and Martin, 1974; Payne

and Hall, 1977). Lewis (1991) reported that there were 3 to 4 orders of magnitude differences between the sensitivity of various algal species and surfactant toxicity.

Toxicity of C_{10-14} linear alkyl benzene sulfonate on algae was studied by Holt *et al.* (1992). The LC_{50} values for 72-96 h were 0.9-300 ppm for fresh water algae, for marine algae 0.025-10 ppm. For C_{12-14} alkyl sulfates the values were 4-30 ppm for fresh water and 1-2 ppm for the marine groups. For alcohol ethoxylates with 12 to 14 carbons the values were 4-50 ppm for fresh water algae and 0.01-0.05 ppm for marine algae. It was reported by Ventullo *et al.* (1989) that the bacteria showed an adaptive response on chronic exposure to linear alkyl ethoxylates. For the non ionics the LC_{50} values ranged between 0.3 and 100 ppm. Quaternary ammonium compounds (QAC) showed the highest toxicity to algae and there was remarkable differences between laboratory, *in situ* and river studies (mainly due to sorption of quaternary ammonium compounds to sediments). The first effect concentrations were 0.1 ppm for alkyl tri methyl ammonium chloride and 1 ppm for ditallow dimethyl ammonium chloride.

The effects of 5 types of anionics (linear alkyl benzene sulfonate, alkyl ethoxy sulfate, alkyl oleffin sulfate, alkyl sulfates and alkyl ethoxylates), 3 non ionics of poly oxy ethylene alkyl ether type having chain lengths of 4, 9 and 13 and soap was studied by Atsuko *et al.* (1984). The test algae were *Selanastrum capricornutum, Microcystis aeruginosa* and *Nitzschia fonticola.* Here the retardation of specific growth rate was the assay criterion and the exposure duration was for not more than 3 days. The growth of algae in surfactants were not always linear on normal logarithmic scales (time-algal concentrations) and biphasic growth was observed. There was a delay in the inhibition of growth rate which was attributed to the time required for penetration of surfactants into the cell wall (Ukeles, 1965) and inhibit biochemical reactions within the cell. EC_{50} for

linear alkyl benzene sulfonate was 50–100 ppm, for AS-60 ppm, alkyl ethoxy sulfate 65 ppm, alkyl oleffin sulfate 45 ppm, soap 10-50 ppm for *Selanastrum.* For non ionics EC_{50} varied between 2-4 for alkyl ethoxylates of chain length 4 EO, 4-8 ppm for EO: 9, 10 ppm for ethylene oxide of chain length 10 units and for alkyl phenol ethoxylate 20-50 ppm. The most sensitive was *Microcystis* for linear alkyl benzene sulfonate, followed by *Nitzchia* and then *Selanastrum.* For AE (EO9), *Selanastrum* was the most sensitive followed by *Nitzschia* and then *Microcystis.* For soap the sensitive species were in the decreasing order *Microcystis, Selanastrum and Nitzschia.*

The anionic surfactant had more deleterious effects on marine planktonic algae *Gymnodinium breve* as reported by Kutt and Martin (1974) with respect to lethality and growth rate as compared with the non ionics and the cationics. Matulova (1964) observed that cetyl pyridinium bromide was the most inhibitory to the fresh water algae.

Effects of detergents linear alkyl benzene sulfonate, Triton and the amphoteric betaine on the sterol composition of the red alga *Porphyridium* were assessed using lipid fractionation and gas liquid chromatography by Nyberg and Saranpaa (1989). The growth time monitored was one month at 25°C and 100 microE/m/s light. A decrease was noted in 22-dehydro cholesterol at 10 and 20 ppm, the major sterol in these species. Betaine was also powerful in decreasing algae growth and here too 22-dehydro cholesterol decreased progressively at higher concentrations. Triton was also found to induce similar effects and the maximum decrease in 22 dehydrocholesterol was observed at 10 ppm.

Acute (3 and 6 h) toxic responses towards linear alkyl benzene sulfonate of light saturated and unsaturated photosynthesis was investigated by Petersons and

Kokusk (2000) for *Rhodomonas salina* and *Skeletonema costatum.* Linear alkyl benzene sulfonate toxicity was not dependent on the light conditions. However, photosynthetic rate was inhibited by linear alkyl benzene sulfonate.

Influence of the dispersant corexit on bioavailability of naphthalene from the water accommodated fraction of crude oil to the golden brown algae *Isochrysis galbana* was done by Wolfe *et al.* (1998). The results indicated that the dispersant had significant influence on the uptake of naphthalene but not on the bioaccumulation factor or metabolic fate. Thus it was inferred that the surfactants have the potential to increase the organism exposure to certain hydrocarbons without increasing their aqueous concentrations.

Effects of non ionics on cyanobacteria was studied by Waterbury and Ostroumov (1994). Triton X-100 acted differently on *Synechococcus W H 7805 and W H 8103.* 5 ppm of the surfactant inhibited the strain 7805 and favoured the development of strain 8103. A lower concentration of 0.5 ppm stimulated the growth of both the strains. Triton however had no effect on the motility of the 8103 strain.

Laboratory simulation studies were conducted by Mukherjee *et al.* (1994) to assess the impacts of detergent input, resulting phosphate load and its effects on the chemical and biological properties of water bodies. There was a significant increase of both inorganic carbon and phosphate with an increase in detergent concentration. Also there were increases in total alkalinity and pH. The high levels of phosphate and suitable temperature promoted the growth of *Microcystis* and caused blooms in November. There was a concomitant decrease in oxygen which resulted due to oxygen demand of detergents and this interfered with the productivity.

Responses of aquatic communities to 25-6 alcohol ethoxylate in a model stream ecosystem was studied by Belanger *et al.* (2000). Algae, heterotrophic, microbial, protozoan and invertebrate communities were assessed over an 8 week exposure period. The concentrations exposed to were 12, 37, 111, 333 and 1000 μg/litre. During the first 2 weeks of exposure there was an enhanced growth of microbial communities but by 4 weeks adverse effects were indicated at 259-760 μg /litre. The key species affected were *Stenonema* the may fly, *Chimarra*, the caddis fly and *Corbicula* the clam.

2.9 Environmental Fate and Biodegradation of Surfactants

The environmental fate of surfactants has been discussed at various international seminars and symposia (Richtler and Knaut, 1988).

Metabolic pathways are known in detail for linear alkyl benzene sulfonate, alkyl sulfates, alkyl ethoxy sulfate, only speculated for alkyl oleffin sulfate and less established for sodium alkyl sulfates. In rivers and sediments linear alkyl benzene sulfonate was removed to a large extent (68-87%). Also linear alkyl benzene sulfonate mineralisation was found to be dependent on chain length and temperature (Palmisano *et al.*, 1991). It was shown that a substantial role in mineralisation was realised by the micro biota of submerged plant detritus (Federle and Ventullo, 1990). Linear alkyl benzene sulfonate biodegradability generally increased with increasing chain length. It was observed that benzene ring was completely mineralised. Alkyl sulfates showed near complete degradation in marine and estuarine waters and there was only little effect of chain length on biodegradation (Mann and Reid, 1971). Alkyl ethoxy sulphates in general exhibited high primary aerobic and anaerobic degradation and

mineralisation in estuarine and marine waters was high. Here the rate of biodegradation was not significantly influenced by chain length or number of ethylene oxide groups (Oba *et al.*, 1976). Sodium alkyl sulfates were not anaerobically degradable like other sulfonates. There was only a marginal effect of chain length on biodegradability, with higher chain length homologues having a longer lag phase. Alkyl oleiffin sulfonates were mineralised with sewage inoculum at rates slightly lower than alkyl sulfates and slightly faster than linear alkyl benzene sulfonate. Soaps showed 100% biodegradation in river water die-away and batch activated sludge tests (Swisher, 1987).

All widely used non ionics showed a primary biodegradability and removal of more than 80%. Alcohol ethoxylates in general were aerobically mineralised. It was observed that higher ethoxylate numbers lowered the biodegradability. The structure of hydrophobic moiety also influenced the rate of biodegradation, the linear hydrophobes undergoing most rapid degradation whereas secondary and highly branched hydrophobes retard ultimate biodegradation. Straight/branched chain alcohol phenol ethoxylates were neither rapidly degraded nor mineralised. They are converted in the process to poorly biodegradable phenols (Holt *et al.*, 1992; Brown *et al.*, 1986).

Cationics like quaternary ammonium compounds have a strong tendency to adsorb onto suspended solids. They can also form complexes with anionics like linear alkyl benzene sulfonate in particular ditallow di methyl ammonium chloride and alkyl tri methyl ammonium chloride were rapidly biodegraded in river waters with half time for degradation being several days. Ditallow di methyl ammonium chloride degradation was found to be much slower in sediment–free water (Hand *et al.*, 1990). It was inferred that sediment associated and periphytic microbial

communities adapted to the cationics within 5-10 days of exposure and effected degradation (Shimp and Schwab, 1991). It was also suggested that quaternary ammonium compounds have a potential for photodegradation and aerobic conditions were necessary for degradation.

2.10 Quantification of Surfactants in the Environment

The levels of surfactants have been measured in various lakes, rivers, drinking water, sewage sludge, sediments, estuarine, marine and coastal waters (Sivak *et al.*, 1982; Giger *et al.*, 1987; Ventura *et al.*, 1989; Painter *et al.*, 1992). Concentrations of linear alkyl benzene sulfonate and methylene blue active substances have been reported for European and U.S. rivers by Painter *et al.* (1992). According to the data sewage has 3-12 ppm methylene blue active substances and 0.5-12 ppm linear alkyl benzene sulfonate, rivers had 0.005-6.9 ppm methylene blue active substances and up to 1.6 ppm linear alkyl benzene sulfonate in contaminated sites. Estuarine and coastal waters had 0.008-0.09 ppm linear alkyl benzene sulfonate, 0.01-0.54 ppm methylene blue active substances, marine waters had <0.5-1.2 μg /L methylene blue active substances in North sea (salinity 28-35 ppt) and <0.5-9.4 μg /L linear alkyl benzene sulfonate in the estuary up to open sea (salinity 16-34 ppt).

Gilbert and Pettigrew (1984) reported the estimated concentrations of alkyl sulfates (AS), linear alkyl ethoxy sulfates (alkyl ethoxy sulfate) and sodium alkyl sulfates (SAS) in the environment. Raw sewage had 1 ppm of primary AS, 5 ppm of alkyl ethoxy sulfate and 2 ppm of sodium alkyl sulfates. For river water, the levels were 0.005,0.05 and 0.02 ppm respectively.

Holt *et al.* (1992) estimated that concentration of non ionics in U.S. and European environment were -0.03 ppm in the raw sewage, 0.01-2.6 ppm in river waters and 1-5 μg/L in the marine water sediment.

Versteeg *et al.* (1992) and Boethling and Lynch (1992) reported the concentrations of cationics as follows: raw sewage 30-1400 μg/L at German, U.K. and U.S. domestic sewage treatment plans, 5-50 μg/L in German, U.K, Germany and France river systems.

SECTION 1

Studies on

Oreochromis mossambicus (Peters)

CHAPTER

3

Effects of Surfactants on Biological Membranes

3.1 Introduction

Biological membranes are complex structures comprising of numerous lipids, proteins, ions etc. The interaction of detergents with the cell membranes has been studied in great details with reference to the isolation, purification and solubilisation of various membrane proteins and enzymes. There are many factors that affect the interaction between membrane components and the surfactants. These include surfactant concentration, asymmetrical distribution of lipids, presence of carbohydrate moieties on the cell membrane and electrostatic and hydrophobic interactions between the lipids and proteins (Lichtenberg, 1983; Higgins, 1987).

Surfactant concentration plays an important role in the damage caused to the cell membrane. At low concentrations there might only be subtle changes in the membrane permeability whereas at higher concentrations there might be drastic effects like lysis or fusion (Jones and Chapman, 1995).

In the present study, the effects of three surfactants-sodium dodecyl sulphate (SDS), Triton X-100 and cetyl trimethyl ammonium bromide (CTAB) at a sub lethal concentration of 1 ppm were studied on the hepatic lysosomal membranes and the erythrocyte membranes (*in vitro* and *in vivo*).

3.2 Studies on Hepatic Lysosomal Membrane

3.2.1 Introduction

Lysosomal damage is well-established as a bio-marker of stress in a wide range of vertebrates and invertebrates (Bayne, 1976; Moore, 1990; Tabata *et al.*, 1990). The damage to the lysosomal membrane may be conceptualised as an increase/activation or decrease/inhibition of the lysosomal hydrolases or labilising/stabilising effects due to changes in membrane permeability effected by the contaminants (Hawkins, 1980). Membrane labilisation is unlikely to occur other than as a stress phenomenon, whereas elevation of activity can occur as a consequence of an increased metabolic demand, for example during gametogenesis, rather than as a stress response (Lowe and Fosstovu, 2000).

Earlier works on surfactant toxicity in fishes have revealed that liver is an important site of accumulation or metabolism of the surfactant (Kimerle, 1981; Kikuchi *et al.*, 1980). Hence liver lysosomes serve as perfect biochemical indices in assessing the impacts of surfactants on the cell membrane. Lysosomes are single membrane-bound organelles that enclose a battery of hydrolytic enzymes like acid phosphatase, beta glucuronidase, cathepsin, aryl sulfatase etc. They have an acid environment which is maintained by a membrane-bound Mg^{2+} ATPase dependent proton pump (Ohkuma *et al.*, 1982). These organelles are best with numerous functions like sequestration of foreign compounds, immune response, protein and organelle turnover, embryonic development, apoptosis etc. If the lysosomal membrane is damaged or destabilised then these marker enzymes are released. Hence the assay of these enzymes can be used as an index of lysosomal membrane damage. The release of lysosomal enzymes is related to necrosis or death of the cell or pathological or stressful conditions (Hawkins, 1980).

3.2.2 Materials and Methods

The fish species were collected from Rice Research Institute, Vyttila, Kochi, Kerala. They were fed on a commercial diet *ad libitum* and were acclimated in aquarium tanks for a month before the experiment. The fish of the size range 15 ± 3 gm and 8.5 ± 0.5 cm were used for the experiments. Six fish each were exposed to a sub lethal surfactant concentration of 1 ppm (1/10 of 96 h LC_{50}) of each of the surfactants (anionic sodium dodecyl sulphate, cationic cetyl tri methyl ammonium bromide and non ionic Triton X-100) in aerated fibre glass tanks. The surfactants were dissolved in tap water and diluted to obtain the required concentration of 1 ppm (APHA). A control group of six fishes was also maintained without any surfactant.

The tap water used had dissolved oxygen content of 7-8 ppm, hardness-below detectable limits, pH 7, temperature 25 ± 3°C and salinity 0 ppt. During the experimental period of 30 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and anti oxidant status. The water in the experimental tanks was replaced every 48 h with water containing fresh surfactant so as to avoid any possible degradation of the surfactant. Fishes were deprived of food 24 h before assay. They were sacrificed by pithing (by damaging the brain and severing the spinal cord between the head and the trunk region using a sharp needle) and the liver tissue was removed, washed in ice-cold isotonic sucrose (0.33 M), blotted dry and weighed.

(a) Activity of Acid Phosphatase (ACP) (E.C.3.1.3.2) in Various Subcellular Fractions of Liver Tissue

Liver was homogenised in isotonic sucrose at 0° C (10%). The homogenate was centrifuged at 600 g for 10 min in a high speed refrigerated

centrifuge. The sediment of nuclei, unbroken cells and plasma membrane (nuclear fraction) was separated. The supernatant was again centrifuged at 15000 g for 30 min. The 15000 g sediment (lysosomal fraction) and the nuclear fraction were resuspended in citrate buffer containing 0.2% Brij-35. The 15000 g supernatant (soluble fraction) was diluted with an equal volume of double strength buffer. The activity of acid phosphatase was determined in all these fractions (Plummer, 1987).

(b) Rate of Release of Acid Phosphatase from Lysosomal Fraction or Lysosomal Enzyme Release Assay (LERA)

(i) In vitro Studies

Liver from control fishes was homogenised in isotonic sucrose and the lysosomal fraction was obtained as above. The lysosomal pellet was washed, centrifuged at 15000 g for 10 min and again resuspended in sucrose. A definite volume of this suspension was incubated at room temperature and aliquots were withdrawn at various time intervals of 0, 15 and 30 min. The retrieved fractions were stored at 0°C (Control). In order to study the effect of surfactants on the lysosomal membrane, a definite volume of the lysosomal suspension (Test) was incubated in presence of each of the surfactants such that the final surfactant concentration is 1 ppm. Here also aliquots were withdrawn at time intervals of 0, 15, 30 and 45 min. Both the control and test aliquots were centrifuged at 15000 g for 30 min to separate the unbroken lysosomes and the acid phosphatase activity released into the supernatant was determined. Total lysosomal activity was estimated after adding citrate buffer containing Brij-35. For evaluation of the effect of surfactants on enzyme release, the possible effect of surfactant (inhibition or activation) was also taken into account.

(ii) In vivo Studies

Here the hepatic tissue from control animals and from those exposed to surfactant for 30 days were homogenised separately and centrifuged to obtain the lysosomal fraction as described in 3.2.2(a). The rate of release of acid phosphatase was noted at definite time intervals by withdrawing definite aliquots of the suspension as described in *in vitro* methods in 3.2.2(b)(i).

(c) Determination of Enzyme Activity (Anon, 1963)

0.5 ml of para nitro phenyl phosphate (400 mg%) was mixed with an equal volume of 0.1 M citrate buffer of pH 4.8. The enzyme was added and incubated for 30 min at room temperature. At the end of 30 min, reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The absorbance of the solution was measured at 410 nm in a UV-visible spectrophotometer (Hitachi). The amount of p-nitrophenol liberated by the enzyme per hour per mg protein gives the specific activity. Protein was estimated by the method of Lowry *et al.* (1951).

(d) Statistical analysis

Statistical analysis of results was done by ANOVA followed by Least Significant Difference (LSD) (Zar, 1996).

3.2.3 Results

(a) Subcellular activity of acid phosphatase

The subcellular activity of acid phosphatase is given in Table 3.1 and Figure 3.1.

Table 3.1. Subcellular activity of acid phosphatase exposed to 1 ppm surfactants *in vivo*

Groups	Nuclear activity	Lysosomal activity	Soluble activity	Ratio Lysosomal/soluble
Control	5.789 ± 0.92	11.99 ± 0.91	6.1 ± 0.85	1.965
SDS dosed	10.55 ± 0.52	8.89 ± 0.83	7.18 ± 0.9	1.23
Triton dosed	9.09 ± 0.47	2.48 ± 0.82	12.01 ± 0.892	0.207
CTAB dosed	8.87 ± 0.43	6.41 ± 0.88	10.65 ± 0.88	0.591

Values are the mean ± SD of six separate experiments.
Activity expressed as mg p-nitro phenol liberated/h/mg protein in each fraction.

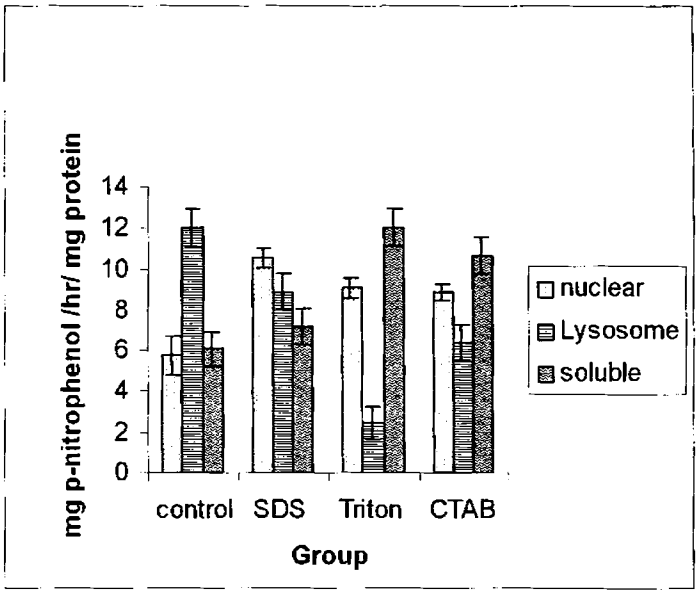


Figure 3.1. Subcellular activity of acid phosphatase in *O. mossambicus*

One way analysis of variance (ANOVA) revealed an overall significant change ($P<0.001$) in the ACP activity in the nuclear ($F = 157.82$) (Table 3.1a), soluble fractions ($F = 156$) (Table 3.1b) and lysosomal ($F = 327.06$) (Table 3.1c) fractions of the experimental animals.

Table 3.1a. ANOVA for nuclear ACP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72.08802	3	24.02934	157.8178	4.34E-14	3.098393
Within Groups	3.0452	20	0.15226			
Total	75.13322	23				

Table 3.1b. ANOVA for soluble ACP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	145.2006	3	48.4002	156.0006	4.85E-14	3.098393
Within Groups	6.205128	20	0.310256			
Total	151.4057	23				

Table 3.1c. ANOVA for lysosomal ACP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	290.8049	3	96.93495	327.0631	3.71E-17	3.098393
Within Groups	5.9276	20	0.29638			
Total	296.7325	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Subsequent comparisons between different groups were done by Least Significant Difference (LSD) analysis, the results of which are given in Table 3.1d.

Table 3.1d. Results of LSD analysis for subcellular acid phosphatase activity

Groups	P value		
	Nuclear	Lysosomal	Soluble
Control x SDS	P < 0.001	P < 0.001	P < 0.001
Control x Triton	P < 0.001	P < 0.001	P < 0.001
Control x CTAB	P < 0.001	P < 0.001	P < 0.001
SDS x Triton	P < 0.001	P < 0.001	P < 0.001
Triton x CTAB	NS	P < 0.001	P < 0.001
SDS x CTAB	P < 0.001	P < 0.001	P < 0.001

NS – Not significant

There was a significant increase (P<0.001) in nuclear and soluble ACP in all the surfactant treated groups when compared to the control. On the other hand a significant decrease was noted in lysosomal ACP of surfactant exposed groups when compared to the control.

Comparison between surfactants revealed no significant differences in the nuclear acid phosphatase activity in animals exposed to Triton X-100 and CTAB, but there were significant differences (P<0.001) between the surfactants with respect to the lysosomal and soluble enzyme activity.

The lysosomal ACP activity in surfactant dosed fishes was lower than that of control but the soluble fraction activity was highly increased. This indicates damage to the lysosomal membrane on exposure to surfactants. The ratio of ACP activity in lysosomal fraction to that in the soluble fraction or Lysosomal stability index (LSI) was the lowest for Triton X- 100 (0.207). It was followed by CTAB which had an LSI of 0.591 and the anionic SDS had an LSI of 1.23. This indicates that the nonionic surfactants are the most damaging followed by the cationic and then the anionic, the lysosomal stability index for the control group was 1.965.

(b) Lysosomal enzyme release assay (*in vitro and in vivo*)

The time dependent release of ACP from lysosomes *in vitro* and *in vivo* is given in Tables 3.2 and 3.3 and Figures 3.2 and 3.3.

Table 3.2. LERA *in vitro*

Time (min)	Control	SDS	Triton X-100	CTAB
0	1.06 ± 0.21 (8.48)	0.957 ± 0.03 (7.66)	1.77 ± 0.06 (14.16)	1.01 ± 0.05 (8.1)
15	1.135 ± 0.02 (9.1)	1.063 ± 0.01 (8.7)	1.915 ± 0.054 (15.32)	1.17 ± 0.01 (19.36)
30	1.21 ± 0.01 (9.68)	1.276 ± 0.02 (10.21)	2.27 ± 0.02 (18.16)	1.33 ± 0.02 (10.64)

Values are the mean ± SD of six separate experiments expressed as mg p-nitrophenol/h/mg protein.
In brackets is represented ACP release as % of total activity.

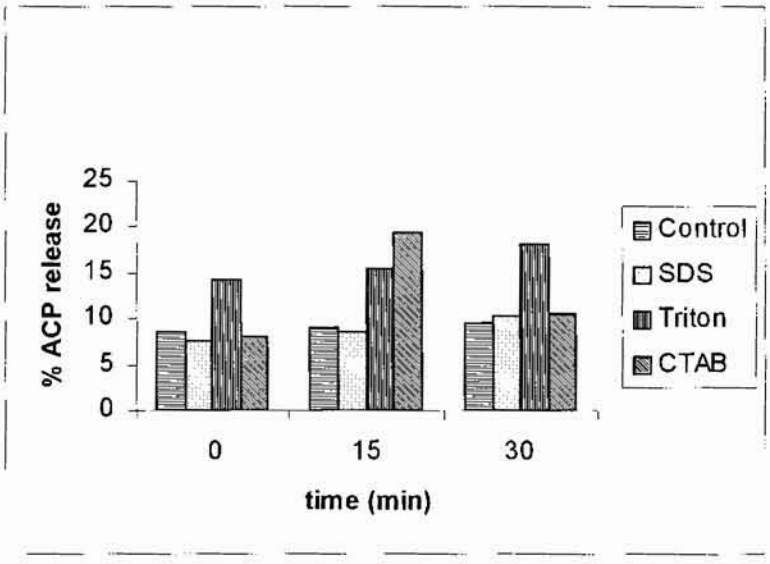


Figure 3.2. Percentage release of ACP from lysosomes *in vitro*

Two way ANOVA (comparing ACP release with time) on *in vitro* studies revealed an overall significant change ($P<0.05$) in the experimental groups (Table 3.2a).

Table 3.2a. Two way ANOVA for LERA *in vitro*

Source of Variation	SS	df	MS	F	P-value	F crit
Between Time	0.006	2	0.003	19.82	0.002	5.15
Between Groups	0.022	3	0.007	51.76	0.0001	4.76
Error	0.001	6	0.0001			
Total	0.0284	11				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Subsequent LSD analysis (Table 3.2b) reflected significant differences ($P<0.05$) in the release of ACP at time intervals of 0-15, 0-30 and 15-30 min.

Table 3.2b. Results of LSD analysis

ACP release with time	P value
0-15	$P < 0.05$
0-30	$P < 0.05$
15-30	$P < 0.05$

Comparison of surfactant treated groups with control and also among themselves is given in Table 3.2c.

Table 3.2c. Results of LSD analysis for *in vitro* studies on LERA

Groups	P value
Control × SDS	P < 0.001
Control × Triton	P < 0.001
Control × CTAB	P < 0.001
SDS × Triton	P < 0.001
Triton × CTAB	NS
SDS × CTAB	P < 0.001

NS – Not significant

The rate of release of ACP was significantly different ($P<0.05$) in all the surfactant treated groups when compared to the control. Also there were significant differences ($P<0.05$) between the surfactant with respect to ACP release.

The results of *in vivo* studies on enzyme release are given in Tables 3.3 and Figure 3.3.

Table 3.3. LERA *in vivo*

Time (min)	Control	SDS	Triton X-100	CTAB
0	0.702 ± 0.23 (5.6)	0.798 ± 0.02 (6.38)	0.922 ± 0.04 (7.38)	0.8 ± 0.095 (6.38)
15	0.936 ± 0.04 (7.5)	0.979 ± 0.11 (7.83)	1.064 ± 0.01 (8.51)	1.596 ± 0.05 (12.77)
30	1.035 ± 0.02 (8.28)	1.138 ± 0.08 (9.11)	0.993 ± 0.05 (7.94)	1.862 ± 0.04 (14.89)

Values are the mean ± SD of six separate experiments expressed as mg p-nitrophenol/h/mg protein.
In brackets is represented ACP release as % of total activity.

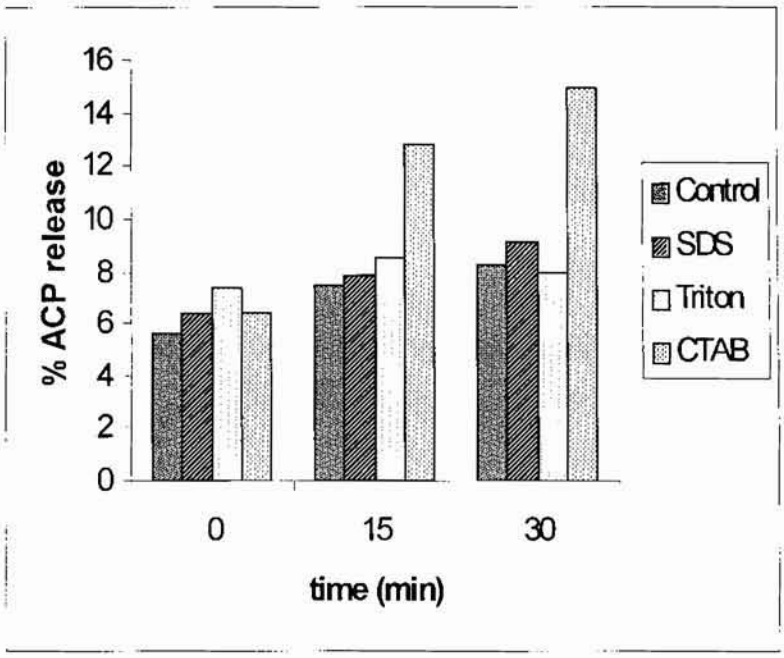


Figure 3.3. LERA *in vivo*

Two way ANOVA on *in vivo* studies showed that there was no significant difference in the enzyme release with time. But the surfactant exposure was found to induce an overall significant change (Table 3.3a).

Table 3.3a. Two way ANOVA for LERA *in vivo*

Source of Variation	SS	df	MS	F	P-value	F crit
Between Time	0.013856	2	0.006928	3.7	0.08967	5.15
Between Groups	0.036192	3	0.012064	6.5	0.026316	4.76
Error	0.011226	6	0.001871			
Total	0.061274	11				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Comparison by LSD analysis (Table 3.3b) revealed that only CTAB exposure caused significant release ($P<0.05$) of ACP when compared to control. Also ACP release in CTAB dosed fish were significantly different ($P<0.05$) from that in animals exposed to Triton X-100 and SDS.

Table 3.3b. Results of LSD analysis for *in vivo* studies

Groups	P value
Control × SDS	NS
Control × Triton	NS
Control × CTAB	$P < 0.05$
SDS × Triton	NS
Triton × CTAB	$P < 0.05$
SDS × CTAB	$P < 0.05$

NS – Not significant

3.2.4 Discussion

The interaction of surfactants with the lysosomal membrane involves two aspects-first there are hydrophobic as well as hydrophilic interactions with membrane lipids and proteins, and second is the interaction with the membrane lipids causing peroxidation.

The concentration of the surfactant plays a major role in the interaction with the cell membrane. The membrane is rendered leaky at a much lower concentration than the one required for complete solubilisation. Also this concentration increases with an increase in the critical micellar concentration (CMC) of the surfactant. Thus a surfactant of low CMC causes release of the cell/ cytoplasmic protein by intercalating into the membrane bilayer without much

solubilisation. Thus the decreasing order of toxicity of surfactants is TX-100 > CTAB > SDS.

Triton X-100, the non-ionic surfactant and CTAB, the cationic surfactant have comparatively much lower CMC than SDS, the anionic surfactant. This might have caused increased release of the acid phosphatase enzyme into the soluble fraction. This is evident from an LSI of 0.207 for Triton, 0.593 for CTAB and 1.23 for SDS.

Results of LERA *in vitro* revealed significant increase in ACP release with time. This demonstrates direct interaction of surfactants with the lysosomal membrane. The effects of Triton were significantly different from that of SDS and CTAB, which were similar in labilising effects. But *in vivo* studies on LERA presented a different result. Here only the cationic surfactant CTAB induced significant membrane labilisation when compared to the control. It could be inferred that the metabolism of the surfactant *in vivo* leads to a change in toxicity pattern.

It has been proved that the toxic effects of surfactants on fishes are manifested through a decrease in the surface tension and that the toxicity of the surfactant depends on the length of the alkyl chain and the chemical structure (Swedmark *et al.*, 1971). The non ionics like Triton and the cationic CTAB have long alkyl chains which results in a rapid reduction of surface tension than the anionic SDS.

In addition, damage via peroxidation of lipids in the cell membrane cannot be overruled. Though lysosomal membrane has only 25% lipids, it is still susceptible to the deleterious effects of peroxidation. The works by Desai *et al.* (1964) indicate that lysosomes treated with methyl linoleate exhibited rapid peroxidation which resulted in 50% release of the aryl sulfatase enzyme in one

hour. Cyto chemical observations on the size and conglomeration of the ACP containing particles in the cortical neurones and the purkinge cells of X-ray irradiated rats also support the sensitivity of the lysosomes to free radicals.

Thus it may be concluded that surfactant interactions with the cell membrane lipids and proteins as well as lipid peroxidation are key factors in the damage caused to the lysosomal membrane *in vitro* whereas metabolism of the surfactant is a key factor in deciding *in vivo* toxicity.

3.3 Studies on Erythrocyte Membrane

3.3.1 Introduction

Haematological profile has played a significant role in assessing the impacts of chemicals or toxicants. The commonly studied parameters include WBC count, haemoglobin content, hematocrit, packed cell volume, RBC membrane stability etc. Of these the stability of the erythrocyte/RBC membrane is a simple and precise method in evaluating the toxicities of pollutants because the release of haemoglobin can serve as a criterion for haemolytic effects in presence of pollutants.

The red blood cell is bounded by a single membrane and it has a low protein to lipid ratio whereas the ratio of cholesterol to lipid is high. Lysis of RBC membrane by surfactants has been studied by Kirkpatrick *et al.* (1974). It was observed that surfactants like Triton X-100 and sodium dodecyl sulfate exerted protective effects against osmotic shock on the membrane at concentrations of 1×10^{-5} to 6×10^{-5} M but at higher concentrations lysis was the rule (Helenius and Simons, 1975).

The present study focuses on the impacts of three surfactants –anionic sodium dodecyl sulfate, cationic cetyl tri methyl ammonium bromide and the non ionic Triton on the red cell membranes of the teleost *Oreochromis mossambicus in vitro* and *in vivo* (30 days exposure).

3.3.2 Materials and Methods

Oreochromis mossambicus of size 15 ± 3 gm and 8 ± 0.5 cm were used for the experiments.

Collection of blood: Blood was collected from the cardinal vein in plastic syringes containing citrate as the anti coagulant (Michael et al., 1994). Fresh saline solutions – isotonic (0.85%) and hypotonic (0.5%) were prepared.

Stock suspension of RBC was prepared after washing the cells thrice with isotonic saline. Then different volumes of the suspension was mixed with distilled water to hemolyse the cells and centrifuged at 1000 g for 5 min. The absorbance of the supernatant was read at 540 nm against distilled water blank. The dilution giving a suitable absorbance for 100% hemolysis was selected. Also a suitable volume of blood giving a suitable absorbance for 100% hemolysis was noted.

The experiment was done with each of the three surfactants as described below.

(1) To 0.1 ml of the stock RBC suspension in a centrifuge tube, 5 ml of isotonic saline was added and incubated for 30 min at room temperature. Then it was centrifuged at 1000 g for 5 min. The absorbance of the supernatant was read at 540 nm. This gives the absorbance of the "blank"(B).

(2) To 0.1 ml of the stock RBC suspension in a centrifuge tube, 4.5 ml of distilled water was added and incubated for 30 min at room temperature. To this 0.5 ml of the surfactant was added (such that the final surfactant concentration was 1 ppm). Then it was centrifuged at 1000 g for 5 min and the absorbance of the supernatant was read at 540 nm. This gives the absorbance corresponding to 100% hemolysis (H).

(3) To 0.1 ml of the stock RBC suspension in a centrifuge tube, 4 ml of hypotonic saline and 0.5 ml of distilled water was added and incubated for 30 min at room temperature. Then 0.5 ml of the surfactant was added (such that the final surfactant concentration was 1 ppm). Then it was centrifuged at 1000 g for 5 min and the absorbance of the supernatant was read at 540 nm. This gives the absorbance of the "control" (C).

(4) To 0.1 ml of the stock RBC suspension in a centrifuge tube, 4 ml of hypotonic saline and 0.5 ml of surfactant was added (such that the final surfactant concentration was 1 ppm) and incubated for 30 min at room temperature. This was followed by the addition of 0.5 ml of distilled water and was centrifuged at 1000 g for 5 min. The absorbance of the supernatant was read at 540 nm and this gives the absorbance corresponding to "test"(T).

Calculations

% Hemolysis in the control(X) = C-B/H-B x 100
% Hemolysis in the test (Y) = T-B/H-B x 100
% labilisation by test = Y-X /X x 100

3.3.3 Results

The results are given in Table 3.4 and Figure 3.4.

Table 3.4. Percentage hemolysis in *O. mossambicus* on exposure to 1 ppm surfactants *in vitro* and *in vivo*

Group	% Hemolysis *in vitro*	% Hemolysis *in vivo*
Control	0	0
SDS	14.99 ± 0.5	10.1 ± 2.5
TRITON	61.45 ± 1.3	64.2 ± 3.5
CTAB	24.40 ± 1.5	32.5 ± 5.0

Values are the mean ± SD of six separate experiments

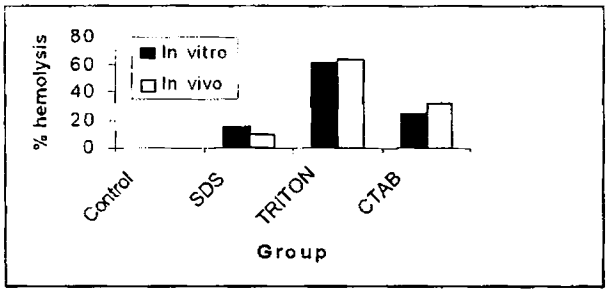


Figure 3.4. **Percentage hemolysis in *O. mossambicus* on exposure to 1 ppm surfactants *in vitro* and *in vivo***

In vitro studies indicated that the exposure to 1 ppm of surfactants had a labilising effect on the RBC membrane (Table 3.4 and Figure 3.4). SDS, the anionic surfactant induced 14.99% labilisation, for the cationic CTAB it was 24.398% and the non ionic Triton produced a labilising effect of 61.45%.

In vivo studies also presented a similar toxicity pattern. Here Triton X-100 was the most damaging producing 64.2% hemolysis. CTAB caused 32.5% and SDS induced 10% hemolysis.

One way analysis of variance revealed that there was an overall significant difference between the surfactants with respect to their haemolytic activity in vitro ($F = 5653.21$, $P<0.001$) (Table 3.4a), as well as in vivo ($F=1343.47$ $p< 0.001$ (Table 3.4b).

Table 3.4a. ANOVA for RBC studies *in vitro*

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7239.94	2	3619.97	5653.21	2.61E-22	3.69
Within Groups	9.61	15	0.64			
Total	7249.54	17				

Table 3.4b. ANOVA for RBC studies *in vivo*

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8866.92	2	4433.46	1343.473	1.21E-17	3.68
Within Groups	49.5	15	3.3			
Total	8916.42	17				

Subsequent comparisons by multiple comparison (LSD) revealed that haemolytic effects of all the three surfactants were significantly different ($P<0.001$) from control and also from one another (Table 3.4c) in both *in vitro* and *in vivo* studies.

Table 3.4c. Results of LSD analysis for *in vitro* and *in vivo* studies

Groups	P value
Control × SDS	P<0.001
Control × Triton	P<0.001
Control × CTAB	P<0.001
SDS × Triton	P<0.001
Triton × CTAB	P<0.001
SDS × CTAB	P<0.001

3.3.4 Discussion

The results indicated that the non ionic surfactant Triton X-100 was the most damaging to the cell membrane followed by the cationic CTAB and then the anionic SDS both *in vitro* and *in vivo*. The cell membrane composition of RBC and the selective property of the detergents are important in the interactions.

RBC membrane is rich in cholesterol, phospholipids, glycolipids etc. So the surfactant having a higher affinity for these lipid moieties would have an upper hand in the solubilising power. SDS is thought to bind with positive hydrophilic groups or to specific hydrophobic receptors and is suggested that it extracted individual proteins and lipids separately and has only little lipid sensitivity. Triton is supposed to solubilise most proteins and lipids in parallel (Partearroyo *et al.*, 1991). CTAB being cationic would have rendered the membrane leaky by interaction with negatively charged lipids and proteins. Thus it may be inferred that initially all the surfactants solubilised protein and later lipids were selectively extracted. More over the peroxidative effects on the cell membrane (rich in lipids) would have had an added effect on the solubilising capacity of the surfactants. Or it may be suggested that the membrane modifications leading to hemolysis may be a sequel to the generation of activated oxygen species.

3.4 Effects of common industrial surfactants on erythrocyte membrane stability of *Oreochromis mossambicus*

A laboratory study was conducted to assess the impacts of commercial surfactants on RBC membrane. The surfactants tested were

1. Surf: a popular high- priced household detergent powder, anionic with linear alkyl benzene sulfonate (LAS) of 12-15%.
2. Wheel: a low-priced anionic (LAS) household detergent powder
3. Teepol: liquid cleaner, anionic containing 39% w/v C9-C13 sodium alkyl sulfates
4. Extran: liquid cleaner, mixture of various non ionics and alkali.
5. DiTallow Di Methyl Ammonium Chloride (DTDMAC): cationic surfactant (C36) used in fabric softeners, shampoos etc.

3.4.1 Materials and methods

Same as in section 3.3.2. Each of the commercial surfactants were tested at 0.1 %.

3.4.2 Results

Results are given in Table 3.5. It was observed that all surfactants were labilising to the RBC membrane. The non ionic extran induced maximum hemolysis of 40%. Cationic DTDMAC induced 25 % hemolysis. The laundry detergents surf and wheel caused 20% and 16.0 % hemolysis respectively. The anionic teepol had 12.1 % hemolytic activity.

Table 3.5. Effects of common industrial surfactants on RBC membrane of *O.mossambicus in vitro*

Surfactant	% hemolysis
Control	0
Surf	20.0 ± 5.0
Wheel	16.0 ± 4.5
Teepol	12.1 ± 3.5
Extran	40.0 ± 5.5
DTDMAC	25.0 ± 2.5

One way analysis of variance revealed an overall significant differences between the surfactants with respect to their hemolytic activity ($F = 97.36$, $P < 0.001$, Table 3.5 a).

Table 3.5a Anova for RBC stability in vitro

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2801.421	4	700.3553	97.36172	7.26E-15	2.75
Within Groups	179.8333	25	7.193333			
Total	2981.255	29				

Subsequent LSD analysis indicated that the hemolytic effects of all surfactants differed significantly from control and from one another ($P<0.001$). Comparison of hemolytic property of industrial surfactants with that of SDS, triton and CTAB has been represented in fig. 3.5 a

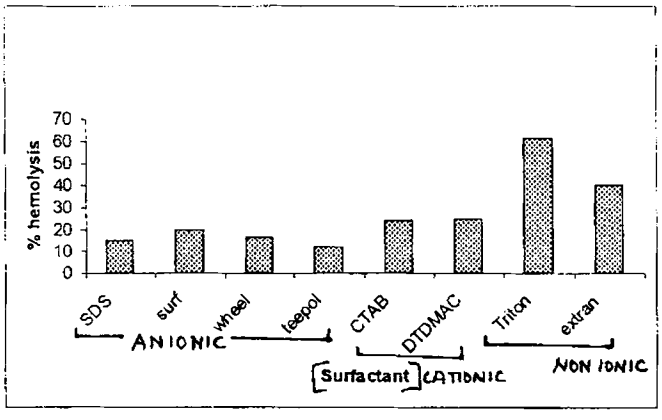


Figure 3.5a. Comparison of hemolytic property of surfactants based on charge

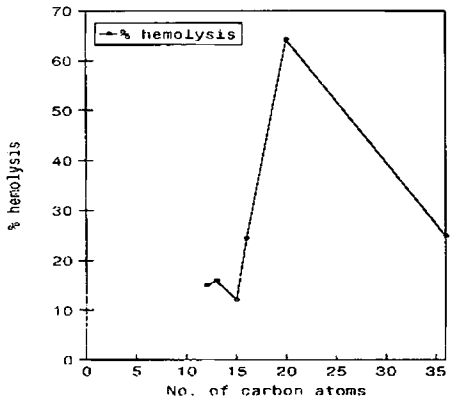


Figure 3.5b. Hemolytic property of surfactants vs carbon chain length

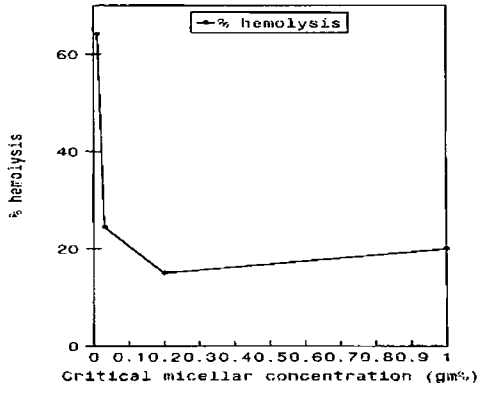


Figure 3.5c. Hemolytic property of surfactants vs critical micellar concentration (CMC)

It was observed that non ionics were the most labilising when comparison is made on the basis of the surfactant charge (fig.3.5 a). Thus, extran > DTDMAC > LAS (Surf, wheel) > SDS> Teepol. It is observed from fig. 3.5 b that as carbon chain length increases, there is an increase in hemolytic potential probably due to a concomitant decrease in critical micellar concentration (CMC) of the surfactants. Hence the decreasing order of toxicity is C20 > C15 > C12 > C36. But beyond C20 it is indicated that an increase in carbon chain has no significant impacts on hemolytic effects So DTDMAC with 36 carbon atoms is the least toxic. Fig 3.5 c indicates relationship between CMC and % hemolysis.

DISCUSSION

It may be inferred from the present study that non ionics are the most labilising to the RBC membrane. The non ionics are capable of both hydrophilic (via ethylene oxide side chain) and hydrophobic (via alkyl chain) interactions with the membrane. On the other hand the interaction of cationics are restricted to the anionic sites and vice-versa. Moreover non ionics show more lipid sensitivity than anionics and cationics. It is to be recalled that RBC membrane has a high lipid : protein ratio. Thus, the damage caused by lipid peroxidation would be the maximum for non ionics compared to cationic and anionic surfactants. Thus it may be concluded that charge of the surfactant, CMC and peroxidative potential are the deciding factors in causing RBC membrane labilisation.

CHAPTER

4

Lipid Peroxidation

4.1 Introduction

The evolution of photosynthetic organisms 2 to 3 billion years ago resulted in large increases in the atmospheric oxygen concentrations. This in turn permitted the concomitant evolution of organisms dependent on oxygen for metabolism (aerobic forms) (Berkner and Marshall, 1964). In aerobic organisms the strong oxidising power of dioxygen is used to couple oxidative degradation of organic substrates to the production of ATP. Fortunately, this process requires activation of oxygen because dioxygen in the ground state is a bi radical (with 2 unpaired electrons). Most of the biomolecules do not bear unpaired electrons and therefore do not react with oxygen. But oxygen can react extremely rapidly with traces of free radicals to produce a variety of peroxides which in turn can initiate a cascade of damaging reactions (Chaudiere, 1994; Chaudiere and Iliou, 1999).

4.1.1 Biological Sources of Free Radicals

Free radicals are critical for the normal operations of a number of biological processes. The catalytic action of many cellular enzymes, electron transport processes and leucocyte activation yield free radical intermediates.

Mitochondrial respiration produces 1 to 5 % of free radicals. The reduction of oxygen to water in mitochondria requires four electrons, but is not a single step reaction and hence intermediate free radicals like superoxide anion free radical ($O_2^{\cdot-}$), hydroxyl ion (OH^-), hydroxyl free radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) etc are produced. The mitochondrial production of H_2O_2 was reported first by

Jensen (1966). The intra mitochondrial concentration of superoxide has been estimated to be 8 X 10^{-12} M (Tyler, 1975). There are species to species and organ to organ differences in the concentrations of components of the mitochondrial respiratory chain which can contribute to differences in major sites and extent of $O_2^{\cdot-}$ and H_2O_2 production by isolated mitochondria from different sources (Turrens *et al.*, 1982).

4.1.2 Intracellular Sources of Free Radicals

Free radicals may be generated in the cell by auto oxidation of small molecules which are capable of undergoing oxidation-reduction reactions in the neutral aqueous environment. These include thiols (Baccanari, 1978), hydroquinones (McCord and Fridovich, 1970), catecholamines (Misra and Fridovich, 1972), flavins (Ballou *et al.*, 1969) and tetrahydropterins (Fisher and Kaufman, 1973). In all cases, superoxide is the primary radical formed by the reduction of oxygen by these molecules and hydrogen peroxide is a secondary product.

Soluble enzymes and proteins also generate free radicals for example, xanthine oxidase generates superoxide during reduction of oxygen to hydrogen peroxide (Fridovich, 1970). Aldehyde oxidase (Rajagopalan, 1980), tryptophan dioxygenase (Hirata and Hayaishi, 1971) and flavoprotein dehydrogenase (Massey *et al.*, 1969) also utilise superoxide during catalysis.

Endoplasmic reticulum and nuclear membrane transport systems also contribute to the formation of free radicals. These membranes contain cytochromes P_{450} and b_5 that oxidise unsaturated fatty acids (Capdevila *et al.*, 1981) and xenobiotics (Chignell, 1979) and reduce dioxygen among other

substrates (Aust, 1972). The flavoprotein containing cytochrome reductases which provide the electrons for the cytochrome P_{450} and b_5 mediated reactions are also capable of auto oxidation to produce superoxide and hydrogen peroxide (Arebakov *et al.*, 1980). Substrates for microsomal cytochrome P_{450} oxidative reactions have been shown to either stimulate (Ullrich and Diehl, 1971) or inhibit hydrogen peroxide formation (Hildebrandt *et al.*, 1973).

Peroxisomes are potent sources of cellular hydrogen peroxide because of high concentrations of oxidases viz. D-amino acid oxidase, urate oxidase, L-hydroxy acid oxidase and fatty acyl Co A oxidase. Peroxisomal catalase is the main scavenger of hydrogen peroxide generated by oxidases. Also around 2% to 11-42% of hydrogen peroxide so generated was also found to diffuse across the membrane into the cytoplasm (Poole, 1975).

Plasma membrane is a critical site of free radical reactions. This is due to the presence of PUFA (20-80% of membrane mass) in the membrane phospholipids, glycolipids, glycerides and sterols and also due to the trans membrane proteins containing oxidisable amino acids (Harold, 1989).

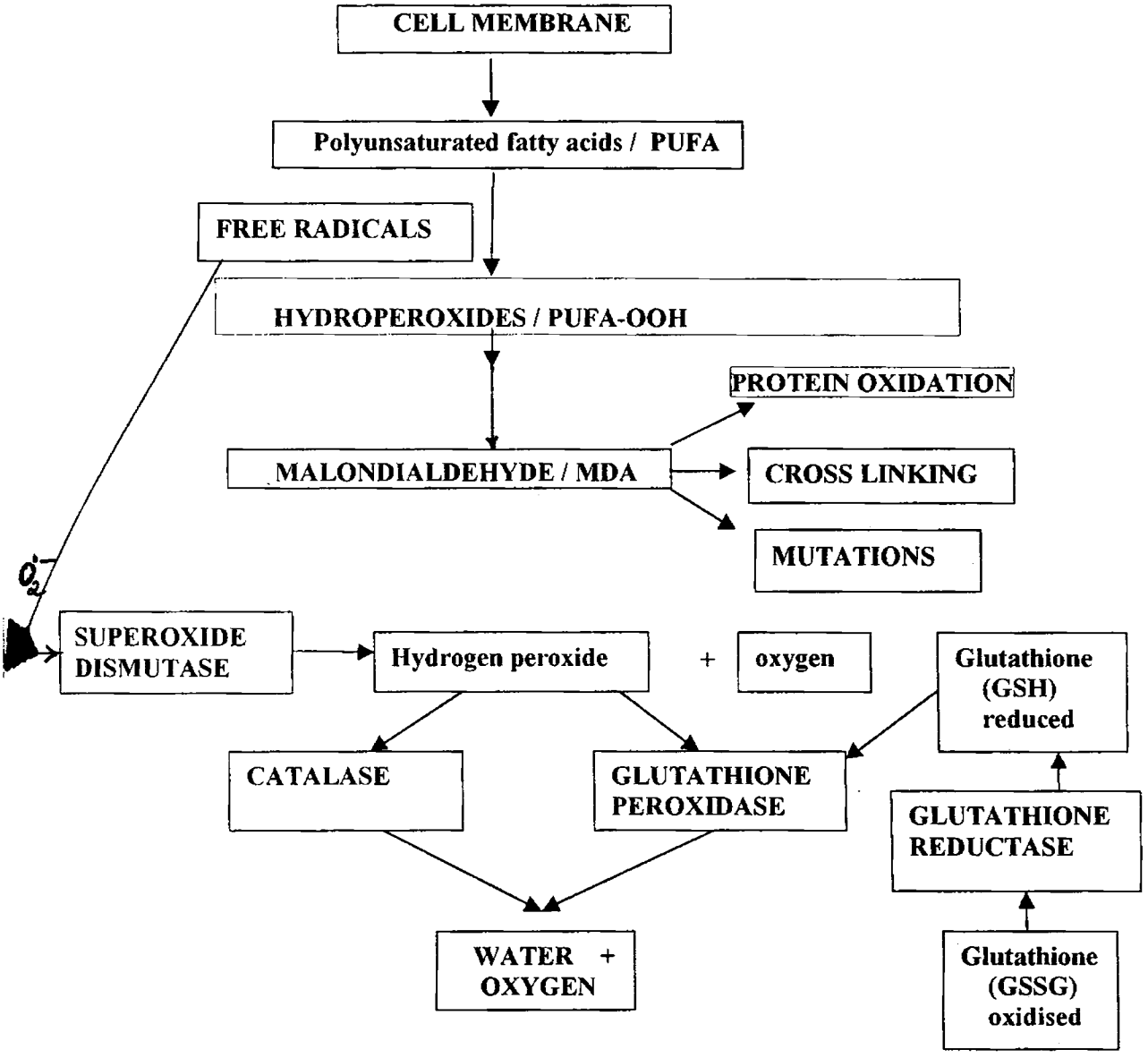
4.1.3 Lipid Peroxidation

The process of oxidative destruction of PUFA is referred to as lipid peroxidation. This is a chain reaction initiated by the attack of free radicals on PUFA. These free radicals can abstract hydrogen atoms from the methylene group of PUFA (initiation phase). The carbon radical so formed is stabilised by molecular rearrangement to produce conjugated dienes which can easily react with oxygen molecule to produce a peroxy radical which can then propagate the chain reaction to give a lipid hydroperoxide (propagation phase). Alternatively peroxy

radical can form cyclic peroxides or endo peroxides which on fragmentation leads to the formation of cytotoxic aldehydes like malondialdehyde. The process of peroxidation proceeds till the PUFA substrate is consumed or until the free radical self annihilates (termination phase).

After peroxidation of membrane fatty acids, the presence of shortened fatty acids with R-OOH, R-COOH, R-CHO and R-OH may seriously affect membrane permeability which is critical to the functions like responses of cells to hormones, neurotransmitters, growth factors, transport of molecules and enzyme activity (Hochstein and Jain, 1981). In addition malondialdehyde (MDA) generated by peroxidation of fatty acids with 3 or more double bonds can cause cross linking and polymerisation of membrane components (Nielsen, 1981). Being diffusible MDA can also react with nitrogenous bases of DNA (Donato, 1981). Thus MDA is beset with mutagenic potential (Mukei and Goldstein, 1976). It is also genotoxic to cultured cells (Bird and Draper, 1980) and carcinogenic (Shamberger, *et al.*, 1974).

Reaction of free radicals with proteins can also generate by products that can amplify cellular damage. Superoxide, hydroxyl free radical and hydrogen peroxide can bring about non-enzymatic hydroxylation of proline and lysine (Trelstad *et al.* 1981). The susceptibility of proteins to free radicals depends upon their amino acid composition (unsaturated and sulphur containing molecules are more reactive, e.g., tryptophan, histidine, phenyl alanine, methionine, cysteine) and location of susceptible amino acids that mediate protein conformation and activity.



SIMPLE SCHEMATIC DIAGRAM OF ANTI-OXIDANT ENZYMES AND GENERATION OF FREE RADICALS BY LIPID PEROXIDATION OF CELL MEMBRANE PUFA

4.1.4 Cellular Defenses Against Peroxidation

The cells have built-in anti oxidant systems to check the deleterious process of peroxidation. There are preventive anti oxidants which decrease the rate of initiation of the chain reaction and they reduce the hydro peroxides to simple molecules, e.g., catalase and glutathione peroxidase.

There is another group of chain-breaking anti oxidants which can trap the free radicals directly. These include superoxide dismutase (SOD), glutathione (GSH), β carotene, vitamin E etc.

(a) Superoxide Dismutase/SOD (E.C.1.15.1.1)

SOD catalyses 1 e^- dismutation of superoxide anion free radicals to hydrogen peroxide and oxygen.

$$2O_2^{\cdot -} + 2H^{\cdot} \rightarrow H_2O_2 + O_2$$

The intracellular concentration of SOD is about 10^{-10} M which supports the concept that superoxide anion is strongly toxic. Superoxide can form complexes with transition metals of enzymes and can cause destruction of the active site. In animal cells Cu-Zn SOD is present in the cytosol whereas the Mn SOD is restricted to the mitochondrial matrix (Steinman, 1982).

(b) Catalase/CAT (E.C.1.11.1.6)

Catalase is a porphyrin containing enzyme of high molecular weight which destroys hydrogen peroxide at high concentrations by catalysing its 2e dismutation to oxygen and water. At low hydrogen peroxide concentrations catalase acts as a peroxidase using co substrates like alcohols, ascorbate and phenols.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase appears to be localised in the peroxisomes where hydrogen peroxide concentration is very high, i.e., $>10^{-4}$ M (De Duve and Baudhuin, 1966, Tolbert, 1981). K_m value of catalase for hydrogen peroxide is very high and is close to 1 M. (Ogura, 1955).

(c) Glutathione Reductase/GR (E.C.1.6.4.2)

In cytosolic and mitochondrial compartments the oxidised glutathione (GSSG) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme GR (Schirmer and Siegel, 1989).

$$GSSG + NADPH + H \rightarrow 2GSH + NADP$$

NADPH is recycled by glucose-6-phosphate dehydrogenase via pentose phosphate pathway of glucose oxidation.

(d) Glutathione (GSH)

GSH is a tri peptide and is gamma glutamyl cysteinyl glycine. It is an important scavenger of free radicals, the intra cellular concentration being 1 to 10 mM. The scavenging property is due to the presence of the sulfhydryl group of pKa 9.2 (Huckerby *et al.*, 1985). Hence only 1.2% of glutathione exists in the thiolate form at pH 7.3. The protonated form serves as a hydrogen donor to the free radical. Also electrophilic xenobiotics are conjugated to this molecule which is nucleophilic, the reaction being mediated by Glutathione-S-Transferases (GST). GSH also serves as the coenzyme for glutathione peroxidase.

(e) Conjugated Dienes (CD) and malondialdehyde (MDA)

The attack of free radicals on PUFA having 3 or more double bonds leads to the production of intermediary conjugated dienes and ultimately the cytotoxic aldehyde-malondialdehyde, the levels of which can serve as an indicator of cell membrane damage.

4.1.5 Fish Anti Oxidant Defenses

Interest in toxicological aspects of oxidative stress has grown in recent years and research is now increasingly focused on mechanistic aspects of oxidative damage and cellular responses in biological systems. Recent researches in fish have focused on the anti oxidant status as an indicator of stress or pollution. It was suggested by Filho (1996) that anti oxidant status in marine fish may be related to oxygen consumption of the tissues and the whole organism whereas in fresh water fish it is related to physical and chemical characteristics of the environment.

Oxidative stress in fish exposed to model xenobiotics like copper chloride, paraquat, dieldrin etc. was studied by Pedrajas *et al.* (1995). Also studies on fish collected from polluted rivers receiving sewage and industrial effluents have been done by Bainy *et al.* (1996). These studies revealed that the marker enzymes like catalase, SOD, GPX and GST etc were higher in fishes from polluted waters when compared to the control animals. Thus anti oxidant defenses are valuable in pollution research and can serve as bio monitors of oxidative stress.

The present chapter focuses on the impacts of 3 surfactants –anionic sodium dodecyl sulfate, cationic cetyl tri methyl ammonium bromide and the non

ionic Triton on the hepatic, renal and cardiac tissues of *Oreochromis mossambicus* as assessed by the anti oxidant status.

4.2 Materials and Methods

The fish species were collected from Rice Research Institute, Vyttila. They were fed on a commercial diet *ad libitum* and were acclimated in aquarium tanks for a month before the experiment. The fishes were of the size range 15 ± 3 g and 8.5 ± 0.5 cm. Six fishes were maintained in 1 ppm of each of the three surfactants viz. SDS, TX-100 and CTAB in aerated fibre glass tanks and a control group was also maintained without any surfactant. The surfactant concentrations were prepared by dissolving the respective surfactants in tap water and diluted to obtain the required concentration of 1 ppm (APHA).

The tap water used had dissolved oxygen content of 7-8 ppm, hardness-below detectable limits, pH 7, temperature 25 ± 3°C and salinity 0 ppt. During the experimental period of 30 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and anti oxidant status. The water in the experimental tanks was replaced every 48 h with water containing fresh surfactant so as to avoid any possible degradation of the surfactant. Fishes were deprived of food 24 h before assay. They were killed by pithing (by damaging the brain and severing the spinal cord between the head and the trunk region using a sharp needle) and the tissues viz. liver, kidney and heart were removed, washed in ice-cold sucrose (0.33M), blotted dry and weighed.

The marker enzymes in lipid peroxidation-catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) were assayed. Also the levels of

the anti oxidant glutathione (GSH) conjugated dienes (CD) and malondialdehyde (MDA) were estimated.

4.2.1 Assay of Catalase

Catalase was assayed by the method of Maehly and Chance (1954). The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 3 ml of 30mM hydrogen peroxide in 0.01M phosphate buffer (pH 7) and 50 microlitre of enzyme extract which was prepared by homogenising the tissue in the phosphate buffer and centrifuging at 600 g). Specific activity was expressed as international units/mg protein, 1IU=Change in absorbance/min/extinction coefficient (0.021).

4.2.2 Assay of SOD

Superoxide dismutase (SOD) was assayed by the method of Kakkar *et al.* (1984). The tissues were homogenised in 0.33M sucrose and subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulfate and after dialysis against 0.0025M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. The reaction mixture contained 0.1 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml of 186 micromol phenazine methosulfate, 0.3 ml of 300 micromol nitroblue tetrazolium and 0.2 ml of enzyme. Reaction was started by addition of 0.2 ml of 780 micromol of NADH. After incubation for 90 seconds at 30 C, the reaction was stopped by adding 1 ml of glacial acetic acid. Then it was shaken vigorously with 4 ml of n-butanol. The mixture was kept undisturbed for 10 minutes, then centrifuged. The chromogen in the butanol layer was read at 560 nm against butanol as blank. The inhibition of the NADH-induced reduction of nitro blue tetrazolium salt by the enzyme was the basis of the assay.

A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the chromogen formation by 50% in one minute under assay conditions. Result was expressed as specific activity in units/mg protein.

4.2.3 Assay of GR

Glutathione reductase (GR) was assayed by the method of Bergmeyer (1974). The decrease in absorbance of a mixture containing 0.12 ml of 1.15% glutathione (GSSG) (oxidised), 0.12 ml of 0.06% NADPH, 0.1 ml of 15mM EDTA, 1.6 ml of 0.067 M phosphate buffer (pH 6.6) and 0.1 ml of enzyme was noted for 3-5 min at 340 nm in a UV-visible spectrophotometer. The controls were run with distilled water instead of GSSG. Enzyme activity was expressed as units/mg protein. One unit was defined as the change in absorbance/minute.

4.2.4 Assay of GSH

Reduced Glutathione (GSH) was assayed by the method of Patterson and Lazarov (1955). The reaction mixture contained 0.5 ml of tissue extract (in 0.5 M phosphate buffer pH 7.5), 0.5 ml alloxan, o.5 ml of 0.5M phosphate buffer and o.5 ml of 0.5N NaOH. After 6 minutes the reaction was stopped by the addition of 0.5 ml of 1N NaOH. The absorbance was read at 305 nm in a UV-visible spectrophotometer. The GSH content was expressed in nanomol/100 gm wet weight of the tissue.

4.2.5 Estimation of Conjugated Dienes

Conjugated dienes was estimated by the method of Retnagal and Ghoshal (1966). The tissue extract in chloroform was prepared (1 in 20). The $CHCl_3$ layer was evaporated to dryness in a water bath at 70°C. The lipid residue was dissolved in 1.5 ml cyclohexane and the absorbance was read at 233 nm in a UV-visible spectrophotometer against cyclohexane blank. The CD levels were

expressed as mmol/ 100 gm wet tissue weight. Extinction coefficient for conjugated diene is 2.52×10^4/M/cm.

4.2.6 Estimation of malondialdehyde

Malondialdehyde was assayed by the method of Nichaus and Samuelson (1968). 1 ml of the tissue homogenate prepared in 0.1 M Tris-HCl buffer (pH 7.5) was combined with 2 ml of 15% w/vTCA: 0.375% w/v of TBA (thiobarbituric acid): 0.25 N HCl (1:1:1) reagent. This was mixed thoroughly and heated for 15 min in a boiling water bath. It was then cooled and centrifuged for 10 min at 600 g. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that contained no tissue extract. The extinction coefficient for malondialdehyde is 1.56×10^{-5}/M/cm. The MDA content was expressed as mmol/100 gm wet tissue weight.

Protein was estimated by the method of Lowry *et al.* (1951).

4.3 Results

The catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione (GSH) conjugated dienes and malondialdehyde (MDA) levels in the hepatic, renal and cardiac tissues are given in Tables 4.1 to 4.6 and Figures 4.1 to 4.6, respectively. The statistical significance of the results was tested by ANOVA followed by LSD analysis (Tables 4.7 and 4.8).

Table 4.1. Catalase activity in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER	KIDNEY	HEART
CONTROL	12.1±1.25	5.29±1.85	10.38±2.5
SDS	32.01±1.52	4.8±1.7	9.74±3.12
TRITON	35.28±3.5	8.3±2.3	13.8±2.95
CTAB	78.31±5.8	17.35±5.5	17.5±3.2

One IU=change in absorbance at 230 nm/min. Extinction coefficient=0.021
Values are the mean ± SD of six separate experiments.

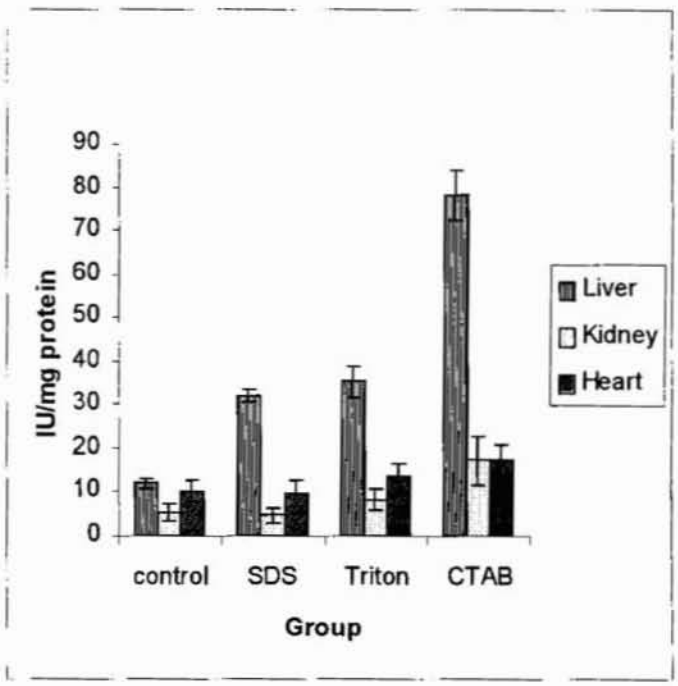


Figure 4.1. Catalase activity in *O. mossambicus*

Table 4.1a. ANOVA for hepatic catalase

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	13927.85	3	4642.618	521.7065	3.76E-19	3.09
Within Groups	177.9781	20	8.89			
Total	14105.83	23				

Table 4.1b. ANOVA for renal catalase

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	609.6	3	203.2	40	1.22×10^{-8}	3.09
Within Groups	101.6	20	5.079			
Total	711.2	23				

Table 4.1c. ANOVA for cardiac catalase

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	230.1316	3	76.71	21.96317	1.54E-06	3.09
Within Groups	69.8538	20	3.49			
Total	299.9854	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Hepatic catalase activity showed an overall significant change ($F=521.7$, $P<0.001$) as obtained by ANOVA (Table 4.1a). The hepatic catalase activity was significantly increased ($P<0.001$) in all the surfactant treated groups when compared to the control. Comparison between the surfactants revealed that SDS and Triton induced similar changes. However CTAB mediated effects were significantly different ($P<0.001$) when compared to the effects of SDS and Triton. The renal enzyme also showed an overall significant change ($F = 40.01$, $P<0.001$, by ANOVA) (Table 4.1b). However multiple comparison by LSD revealed that significant increase ($P<0.001$) was noted only in animals exposed to CTAB when compared to the control. Here too animals exposed to Triton and SDS showed similar enzyme activities which were not significantly different from one another.

Cardiac enzyme also showed an overall significant change ($F=21.96$, $P<0.001$, by ANOVA) (Table 4.1c). The enzyme was significantly ($P<0.001$) elevated in animals exposed to Triton and CTAB. A comparison between the effects of these surfactants was found to be significantly ($P < 0.001$) different from one another.

Table 4.2. Superoxide dismutase activity in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER	KIDNEY	HEART
CONTROL	12.51±0.31	12.11±0.15	15.38±0.41
SDS	16.6±0.26	24.2±0.12	23.7±0.53
TRITON	14.1±0.28	13.9±0.18	20.02±0.36
CTAB	26.6±0.4	24.3±0.3	27.9±0.29

One unit is defined as the amount of enzyme which gives 50% inhibition of the formazan formation/min, expressed as units/mg protein.
Values are the mean ± SD of six separate experiments.

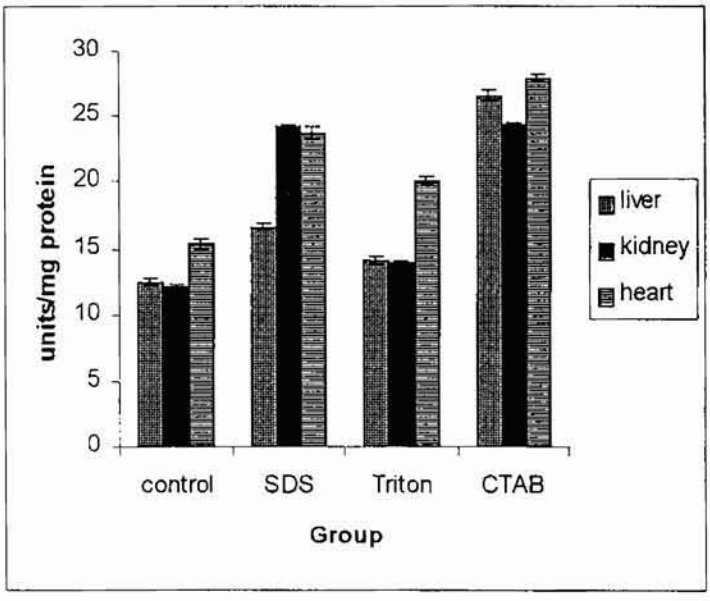


Figure 4.2. Superoxide dismutase activity in *O. mossambicus*

Table 4.2a. ANOVA for hepatic SOD

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	724.6316	3	241.5439	3309.878	3.98E-27	3.09
Within Groups	1.459533	20	0.0729			
Total	726.0911	23				

Table 4.2b. ANOVA for renal SOD

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	768.4728	3	256.1576	16228.77	5.04E-34	3.09
Within Groups	0.315683	20	0.015			
Total	768.7885	23				

Table 4.2c. ANOVA for cardiac SOD

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	511.1688	3	170.3896	2571.142	4.95E-26	3.098393
Within Groups	1.3254	20	0.06627			
Total	512.4942	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

ANOVA showed that there was an overall significant change in superoxide dismutase activity in hepatic ($F=3309.88$, $P<0.001$) (Table 4.2a), renal ($F=16228.77$, $P<0.001$) (Table 4.2b) and cardiac tissues ($F=2571.14$, $P<0.001$) (Table 4.2c). SOD activity in all the surfactant treated groups was significant different ($P<0.001$) when compared to control. Comparison between the surfactants revealed that hepatic and cardiac enzyme activities were significantly different ($P<0.001$) in all the surfactant treated groups. The renal enzyme activity in fish exposed to CTAB and SDS did not show significant differences when compared with each other, but there were significant increases ($P<0.001$) in the

renal enzyme activity in animals exposed to Triton when compared with SDS and CTAB dosed groups.

Table 4.3. Glutathione Reductase (GR) × 10^{-3} Units in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER	KIDNEY	HEART
CONTROL	2.4±0.13	4.1±0.11	2.3±0.2
SDS	6.8±0.17	1.9±0.14	5.2±0.21
TRITON	4.4±0.19	6.5±0.15	3.5±0.18
CTAB	4.1±0.2	2.2±0.18	2.4±0.61

Unit is defined as the change in absorbance at 340 nm/min/mg protein
Values are the mean ± SD of six separate experiments.

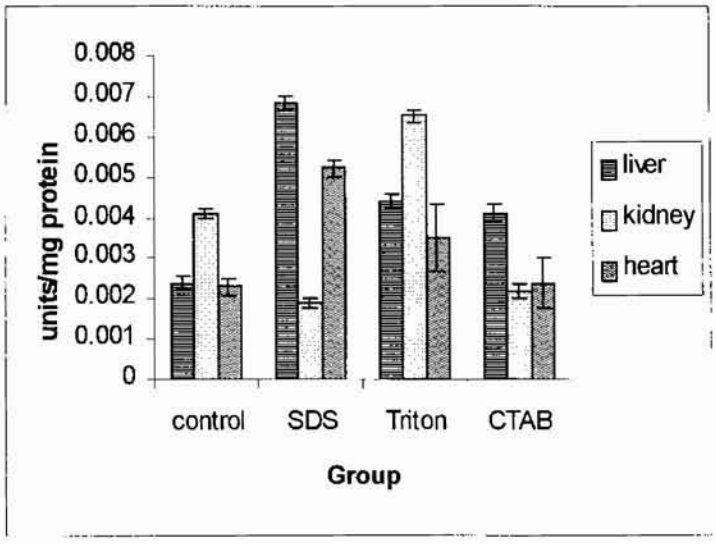


Figure 4.3. Glutathione reductase activity in *O. mossambicus*

Table 4.3a. ANOVA for hepatic GR

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	59.085	3	19.69	18.99	4.52×10^{-6}	3.098
Within Groups	20.7328	20	1.04			
Total	79.8178	23				

Table 4.3b. ANOVA for renal GR

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	84	3	28	86.24	1.29×10^{-11}	1.098
Within Groups	6.49	20	0.32			
Total	90.49	23				

Table 4.3c. ANOVA for cardiac GR

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	32.7	3	10.9	36.57	2.6×10^{-8}	3.098
Within Groups	5.96	20	0.298			
Total	38.66	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Glutathione reductase also showed an overall significant change in hepatic ($F=18.99$, $P<0.001$) (Table 4.3a), renal ($F=86.24$, $P<0.001$) (Table 4.3b) and cardiac ($F=36.58$, $P<0.001$) (Table 4.3c) tissues. The enzyme activity was significantly increased ($P<0.001$) in the hepatic, renal and cardiac tissues (except cardiac tissues of CTAB) of surfactant treated animals when compared to control. The effects of Triton and CTAB on the enzyme levels in hepatic and cardiac tissues were not significantly different from one another whereas SDS mediated effects were significantly ($P<0.001$) different from that of Triton and CTAB ($P<0.001$). The renal enzyme was influenced alike by all the surfactants.

Table 4.4. Glutathione (GSH) content in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER	KIDNEY	HEART
CONTROL	1256±48.6	1302±52.5	750±50.2
SDS	2534±50.2	3706±63.8	1650±52.9
TRITON	1920±65.7	2280±59.7	1000±48.9
CTAB	2110±68.6	3040±63.6	1580±60.5

Values are the mean ± SD of six separate experiments expressed as nmoles/100 g wet tissue.

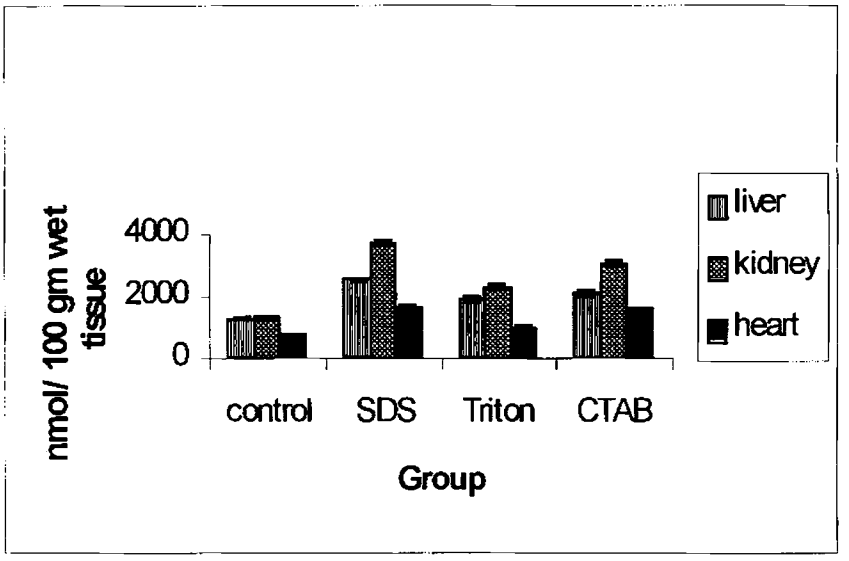


Figure 4.4. Glutathione content in *O. mossambicus*

Table 4.4a. ANOVA for hepatic GSH

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4909552	3	1636517	32.78	6.46×10^{-8}	3.098
Within Groups	998342	20	49917.11			
Total	5907894	23				

Table 4.4b. ANOVA for renal GSH

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	19210858	3	6403619	4626.89	1.41×10^{-28}	3.098
Within Groups	27679	20	1383.99			
Total	19238537	23				

Table 4.4c. ANOVA for cardiac GSH

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3487800	3	1162600	1022.523	4.8E-22	3.098
Within Groups	22739.82	20	1136.991			
Total	3510540	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

There was an overall significant change in reduced glutathione content in hepatic ($F=32.78$, $P<0.001$) (Table 4.4a), renal ($F = 4626.89$, $P<0.001$) (Table 4.4b) and cardiac ($F=1022.52$, $P<0.001$) (Table 4.4c) tissue by ANOVA. There were significant increases ($P < 0.00\ 1$) in hepatic, renal and cardiac tissue levels of reduced glutathione in all the surfactant treated groups when compared with the control group. SDS dosed fish had the highest and the Triton dosed fish had the lowest hepatic glutathione content. The renal and cardiac glutathione content in all the three surfactant treated animals were significantly different ($P<0.001$). But there were no significant differences between the hepatic glutathione levels in animals exposed to Triton and CTAB.

Table 4.5. Levels of Conjugated Dienes (CD) in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER
CONTROL	60.22 ± 9.8
SDS	98.61 ± 5.5
TRITON	150.39 ± 10.5
CTAB	100.79 ± 9.5

Values are the mean ± SD of six separate experiments expressed as mmoles/100 g wet tissue.

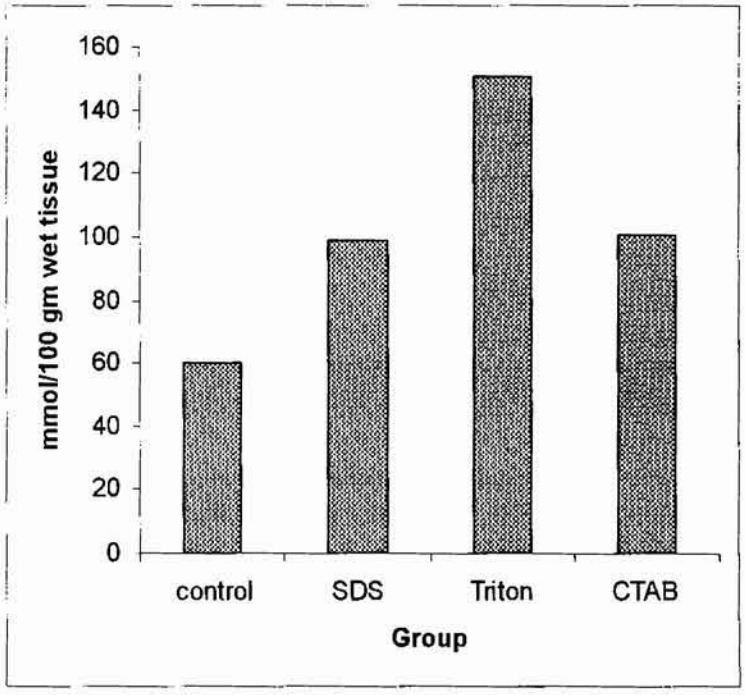


Figure 4.5. Levels of conjugated dienes

Table 4.5a. ANOVA for hepatic CD

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	24613.39	3	8204.46	249.99	5.11E-16	3.098
Within Groups	656.382	20	32.82			
Total	25269.772	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

There was an overall significant change ($P<0.001$) in the levels of conjugated dienes in hepatic tissues of experimental animals (Table 4.5a). Subsequent LSD analysis (Table 4.8) showed that hepatic levels of conjugated dienes in all the three surfactant exposed animals were significantly different ($P<0.001$) from control. Comparison between the surfactant treated groups reveal that CD levels in TX-100 were significantly different ($P<0.001$) from SDS and CTAB, which had similar levels of conjugated dienes.

Table 4.6. Malondialdehyde (MDA) levels in control and surfactant exposed *O. mossambicus*

GROUPS	LIVER
CONTROL	0.0627±0.015
SDS	0.097±0.032
TRITON	0.295±0.017
CTAB	0.0809±0.027

Values are the mean ± SD of six separate experiments expressed as mmol/100 g wet tissue.

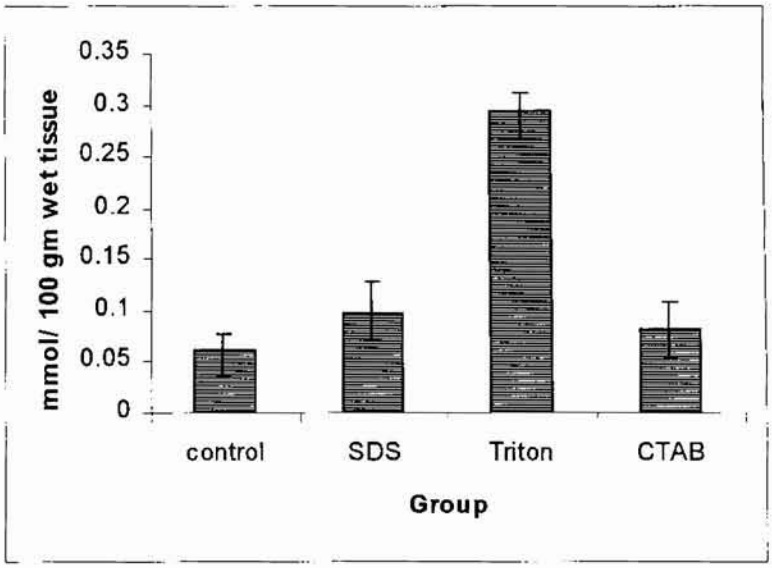


Figure 4.6. Malondialdehyde levels in hepatic tissues of *O. mossambicus*

Table 4.6a. ANOVA for hepatic MDA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.21116	3	0.070387	310.4831	6.17E-17	3.098393
Within Groups	0.004534	20	0.000227			
Total	0.215694	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

ANOVA showed an overall significant change in the hepatic malondialdehyde levels ($F=310.48$, $P<0.001$) (Table 4.6). However group comparisons by LSD test revealed that malondialdehyde levels were significantly different ($P<0.001$) from the control group only in hepatic tissues of animals exposed to Triton. The animals exposed to SDS and CTAB had malondialdehyde levels comparable to that of control. Also there were no significant differences between SDS and CTAB with respect to the hepatic levels of malondialdehyde.

Table 4.7. Results of LSD analysis for catalase, SOD, GR and GSH in the hepatic renal and cardiac tissues of *O. mossambicus*

Groups	Parameters	P values		
		Liver	Kidney	Heart
Control vs. SDS	CAT	P<0.001	NS	NS
	SOD	P<0.001	P<0.001	P<0.001
	GR	P<0.001	P<0.001	P<0.001
	GSH	P<0.001	P<0.001	P<0.001
Control vs. Triton	CAT	P<0.001	NS	P<0.001
	SOD	P<0.001	P<0.001	P<0.001
	GR	P<0.001	P<0.001	P<0.001
	GSH	P<0.001	P<0.001	P<0.001
Control vs. CTAB	CAT	P<0.001	P<0.001	P<0.001
	SOD	P<0.001	P<0.001	P<0.001
	GR	P<0.001	P<0.001	P<0.001
	GSH	P<0.001	P<0.001	P<0.001
SDS vs. Triton	CAT	NS	NS	P<0.001
	SOD	P<0.001	P<0.001	P<0.001
	GR	P<0.001	NS	P<0.001
	GSH	P<0.001	P<0.001	P<0.001
Triton vs. CTAB	CAT	P<0.001	P<0.001	P<0.001
	SOD	P<0.001	P<0.001	P<0.001
	GR	NS	NS	NS
	GSH	NS	P<0.001	P<0.001
SDS vs. CTAB	CAT	P<0.001	P<0.001	P<0.001
	SOD	P<0.001	NS	P<0.001
	GR	P<0.001	NS	P<0.001
	GSH	P<0.001	P<0.001	P<0.001

NS – Not Significant

Table 4.8. Results of LSD analysis for MDA and CD

Groups	MDA	CD
Control vs. SDS	NS	P<0.001
Control vs. Triton	P<0.001	P<0.001
Control vs. CTAB	NS	P<0.001
SDS vs. Triton	P<0.001	P<0.001
Triton vs. CTAB	P<0.001	P<0.001
SDS vs. CTAB	NS	NS

NS – Not significant

4.4 Discussion

Toxicity of oxygen is due to the production of oxygen derived free-radicals, the most common ones being superoxide (O_2^-), hydroxyl free radical (OH^-) and the singlet oxygen. Under normal conditions also free radicals are produced during several physiological processes. The most important ones include mitochondrial respiration which produces 1-5% (Wei, 1998) and immune response by activated phagocytes (Babior *et al.*, 1993). These normal levels of free radicals are scavenged by the normal amounts of anti oxidant enzymes. But a substantial increase in the levels of these highly reactive radicals occurs when the animal is subjected to stress conditions like environmental chemicals/ pollutants (Pedragas *et al.*, 1993). This is reflected in the increased production of the anti oxidant enzymes.

The anti oxidant profile in the surfactant-dosed fishes revealed significant increase in the levels of catalase, super oxide dismutase and glutathione. The antioxidant enzymes viz. catalase and superoxide dismutase showed the highest increase on exposure to CTAB. Also the glutathione content and glutathione reductase were significantly increased, but malondialdehyde levels

were not significantly high. This could be due to the increase in glutathione which can prevent the formation of MDA (Christophersen, 1986). It is well documented that the cationic surfactant interacts with the cell membrane in two possible ways –hydrophobic interactions with the hydrophobic residues and hydrophilic interactions with the ionic groups of membrane proteins and lipids (Lichtenberg *et al.*, 1983). The negative charge on the phospholipids might also have resulted in enhanced interactions. In addition, the surfactant is highly polar and it is thought that the fish species does not metabolise it (Attwood and Florence, 1983).

Triton X-100 was found to resemble CTAB in its toxic effects but differs in that it is non ionic and also subjected to metabolism. The levels of catalase and superoxide dismutase were lower than in CTAB group, but MDA was significantly higher. It was also observed that the glutathione content was the lowest in the Triton-dosed group. The increased MDA in this group might be due to the decreased glutathione content. This depletion of GSH might be due to its increased conversion to oxidised glutathione (GSSG) by the enzyme glutathione peroxidase or/and utilization for conjugation reactions by the hepatic biotransformation enzyme-glutathione-S-transferase (GST). Also studies have shown that nonionics like alkyl phenol ethoxylates are metabolised by the fish species *in vivo* to 4-nonyl phenols which are excreted as glucuronide conjugates and hydroxylates (Augustine *et al.*, 2000). Gadagbui *et al.* (1996) also support the view that tilapia is more likely to excrete xenobiotics as glutathione conjugates or mercapturic acids because of its high GST activity. Thus increased GSH utilization and comparatively lower levels of catalase and superoxide dismutase could account for increased oxidative stress and increased MDA in this group. Being non ionic Triton X-100 is capable of hydrophobic interactions with the cell

membrane through its long alkyl chain, and also hydrophilic interactions through its ethylene oxide chain.

SDS, the anionic surfactant, is a short chain alkyl sulfate. The levels of catalase and superoxide dismutase were comparatively lower in this group than in the other two surfactant-treated groups. But the levels of glutathione reductase and GSH were significantly increased in all the tissues studied. The levels of malondialdehyde were comparable with that of control. These factors together imply that SDS-induced stress in these fishes might be overcome to a large extent by an increased production of the chain-breaking antioxidant GSH as well as increases in the levels of catalase, superoxide dismutase and glutathione reductase. Being anionic SDS interactions with the cell membrane are limited to the cationic sites on the cell membrane lipids and proteins. Also negative charge of SDS may repel similarly charged phospholipids. The surfactant may also be metabolised to some extent by beta or omega oxidation in the hepatic tissues and excreted as carboxylic acid derivatives (Attwood and Florence, 1983).

Thus it may be inferred from the statistical data analysis that the extent of peroxidative damage induced is in the order CTAB>TRITON>SDS.Though Triton is non ionic and extensively metabolised it induces more damage than the anionic SDS as is revealed from the high MDA and low GSH. CTAB is observed to be more toxic than Triton, possibly because it is not metabolised

Thus, from the present study it is inferred that the exposure to surfactants is stressful. The increases in the levels of malondialdehyde coupled with the increased production of catalase, superoxide dismutase, glutathione reductase and glutathione reflect the cell membrane-directed toxicity of the surfactants used and essentially serve as bio monitors of surfactant-induced oxidative stress.

CHAPTER

5

Hepatic Enzymes and Other Biochemical Parameters

5.1 Introduction

Liver plays a pivotal role in metabolism. The organ is endowed with different aspects of carbohydrate metabolism (glycogenolysis, glycogenesis, gluconeogenesis), lipid metabolism (fatty acid oxidation, fatty acid biosynthesis) and protein metabolism (transamination). In addition to these basic functions, liver also plays a major role in xenobiotic metabolism. Liver is the site of detoxification reactions like conjugation with glucuronic acid, glutathione, etc.

Studies on the uptake and metabolism of surfactants have proved that liver is only next to gall bladder in the accumulation and concentration of surfactants (Kimerle *et al.*, 1981; Kikuchi *et al.*, 1980). Hence deviations from the normal metabolic /detoxifying functions can be taken as an indicator of stress.

5.1.1 Transaminases and Phosphatases

Transamination reactions in liver are of great significance.The enzymes of importance are alanine transaminase (ALT) and aspartate transaminase (AST) (Zubay, 1997).

ALT (E.C.2.6.1.2) catalyses the conversion of alanine to pyruvate by transfer of the amino group to ∝-keto glutarate thereby converting it to glutamate.

Alanine + α-keto glutarate → Pyruvate + Glutamate

AST (E.C.2.6.1.1) catalyses the conversion of aspartate to oxaloacetate and the transfer of amino group to keto glutarate gives glutamate.

Aspartate + α-keto glutarate → Oxaloacetate + Glutamate

These reactions involve the conversion of an amino acid to a keto acid by transfer of amino group to give a new amino acid and a new keto acid. It has been reported that pollutants like metals, pesticides, carbon tetra chloride and chloroform could induce the synthesis of these transaminases. Also under conditions of severe hepatic injury the necrosis of the liver tissue occurs and the enzymes get released into the blood stream. Thus the levels of transaminases in tissues and blood stream can serve as indicators of stressful situations. Mild stress would only elevate tissue levels of these enzymes whereas severe injury to the tissue would result in release of these enzymes into the blood stream by the damage of the tissue (Wieser and Hinterleitnar, 1980).

Alkaline phosphatase (ALP) (E.C.3.1.3.1) is involved in membrane transport and is a good indicator of stress in biological systems (Verma *et al.*, 1980). Phosphatases are concerned with carbohydrate metabolism (Milter and Grave, 1961), oxidative phosphorylation (Goodman and Rothstein, 1957) and growth and differentiation (Barker and Alexander, 1958). Increase in ALP occurs in response to the release of corticosteroids (Murphy 1964). It was also suggested by Rudel and Kinel (1972) that the release of acid and alkaline phosphatases is hormone-dependent. Thus phosphatases can serve as indicators of stressful situations (Gupta and Dhillon, 1983).

5.1.2 Other Biochemical Parameters

Glycogen, Protein and Lipid

The levels of glycogen, lipid and protein of the tissues has also served as useful indices to monitor stressful conditions. It has been reported that various pollutants affect these reserve biochemical constituents can indicate a stressful condition (Verma *et al.*, 1984; Sastry and Rao, 1984).

5.2 Materials and Methods

Same as in 3.2.3 of Chapter 3.

Preparation of enzyme: The liver tissue was homogenised in 0.33 M sucrose solution (10%) and was centrifuged in a refrigerated centrifuge for 15 minutes at 800 g. The supernatant obtained was used as the source of enzyme for the estimation of AST, ALT and ALP.

5.2.1 Estimation of Alanine Transaminase (ALT) (Mohun and Cook, 1957)

Reagents

1. Buffered substrate: [100 mmol/L phosphate buffer and 2 mmol /L 2-oxo glutarate.]- Dissolved 1.5 g di potassium hydrogen phosphate, 2 g potassium di hydrogen phosphate and 300 mg 2-oxo glutarate in 600-700 ml distilled water. Then 17.8 g DL-alanine was added. The pH of the solution was adjusted to 7.4 using 0.4 N NaOH.
2. 2, 4 Dinitro phenyl hydrazine (DNPH): 1 mmol/L in 1 N HCl.
3. NaOH: 0.4 N

4. Pyruvate standard: 2 mmol/L

5. Working standard: 1 in 20 dilution of the stock standard.

Procedure

Pipetted out 1 ml of buffered substrate into two test tubes marked 'test' and 'control.' The enzyme (0.2 ml) was added to the tube labelled 'test' and incubated at 37°C for 30 min. After incubation, 0.2 ml of the enzyme extract was added to the control tube. This was followed by the addition of 1 ml of 2, 4-dinitro phenyl hydrazine reagent to both the tubes and incubated for 20 min. This was followed by the addition of 10 ml of 0.4 N NaOH.The absorbance of the solution was read at 525 nm after 5 min in a spectrophotometer.

5.2.2 Estimation of aspartate transaminase (AST) (Mohun and Cook, 1957)

Reagents

1. Buffered substrate: 100mmol/L phosphate buffer, 2 mmol/L 2-oxo glutarate. Dissolved 1.5 g dipotassium hydrogen phosphate, 2 g potassium di hydrogen phosphate and 300 mg 2-oxo glutarate in 600-700 ml distilled water. Then 15.7 g/L Aspartate mono sodium salt (or 13.2 g/L aspartic acid) was added. The pH of the solution was adjusted to 7.4 using 0.4 N NaOH.

2. 2, 4 Dintro phenyl hydrazine (DNPH): 1 mmol/L in 1 N HCl.

3. NaOH: 0.4 N

4. Pyruvate standard: 2 mmol/L

5. Working standard: 1 in 20 dilution of the stock standard.

Procedure

Pipetted out 1 ml of buffered substrate into two test tubes marked 'test' and 'control.' The enzyme (0.2 ml) was added to the tube labelled 'test' and incubated at 37°C for 30 min. After incubation, 0.2 ml of the enzyme extract was added to the control tube. This was followed by the addition of 1 ml of 2, 4-dinitro phenyl hydrazine reagent to both the tubes and incubated for 20 min. This was followed by the addition of 10 ml of 0.4 N NaOH. The absorbance of the solution was read spectrophotometrically at 525 nm after 5 min.

5.2.3 Estimation of alkaline phosphatase (ALP) (Kind and King, 1954)

Reagents

1. Substrate-Disodium phenyl phosphate: 10 mmol/L. Dissolved 2.18 g anhydrous salt in distilled water and made up to 1 L. Boiled, cooled and added little chloroform and then refrigerated.
2. Buffer-sodium carbonate-bicarbonate buffer 100 mmol/L. Dissolved 6.36 g anhydrous sodium carbonate and 3.36 g sodium bicarbonate in distilled water and made up to 1 L.
3. Buffered substrate-Mixed equal volumes of substrate and buffer, pH 10.
4. Stock phenol: 100 mg% in 0.1 N HCl.
5. NaOH: 0.5 N
6. $NaHCO_3$: 0.5 N
7. 4-amino anti pyrine: 6 g/L.
8. Potassium ferri cyanide: 24 g/L

Procedure

Pipetted out 2 ml of buffered substrate into test tubes marked 'test' and 'control.' Added 0.1 ml enzyme to the tube labelled 'test' and incubated for 15 min at 37°C. Then 0.8 ml of 0.5 N NaOH was added followed by 1.2 ml of $NaHCO_3$. Then 0.1 ml enzyme was added to the control tube. Next 1 ml of 4-amino antipyrine was added followed by 1 ml of potassium ferricyanide. The absorbance of the solution was read at 520 nm in a spectrophotometer.

5.2.4 Estimation of Glycogen

The glycogen content was determined by the method of Carroll *et al.* (1956). The liver tissue was homogenised at 0°C with appropriate volume of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 2000 g for 15 min and the supernatant was filtered through an acid washed paper. The residue was homogenised and the supernatant filtered again. The filtrate was made up to a known volume with TCA. To an aliquot of the filtrate (1 ml) 5 ml ethanol was added. The tubes capped with clean rubber stoppers were allowed to stand at room temperature overnight. After the completion of precipitation these were centrifuged at 3000 g for 15 min. The supernatant was decanted off and the tubes were allowed to drain in an inverted position for 10 min. The precipitate was dissolved in distilled water. A reagent blank (2 ml water) and a standard (glucose 100 mg %) were also prepared. 10 ml of anthrone reagent (0.05% anthrone and 1% thiourea in 72% by volume of sulphuric acid) was added into each tube with constant mixing. The tubes were capped with glass marbles and placed in cold water, they were immersed in a boiling water bath for 15 min and then removed to

a cold water bath, cooled to room temperature and the absorbance was read in a spectrophotometer at 620 nm.

5.2.5 Estimation of Protein

Protein was estimated by the method of Lowry *et al.* (1951). The liver tissue from experimental animals was blotted dry, weighed and then homogenised in isotonic sucrose (10%). The homogenate was mixed with equal volume of 10% TCA to precipitate the protein. It was then centrifuged at 600 g for 10 min and the supernatant was discarded. The precipitated protein was redissolved in 0.1 N NaOH. A suitable dilution was then prepared and the protein content was estimated.

5.2.6 Estimation of Lipid

Total lipid was estimated by the phospho vanillin method (Frings and Dunn, 1970).

Reagents

(1) Conc. H_2SO_4

(2) Vanillin 0.6%

(3) Phospho vanillin reagent – 0.6% vanillin dissolved in 100 ml o-phosphoric acid

(4) Standard olive oil 1 g % in C_2H_5 OH.

Procedure

The liver tissue from experimental animals was weighed and then taken in a homogeniser and 10 ml chloroform-methanol was added, mixed well and

filtered. To the filtrate 2 ml of sodium chloride was added and mixed well. It was then kept overnight at 4°C for lipid extraction. The lower phase containing the lipid was made up to 10 ml and then a definite aliquot of this was dried in vacuum desiccator overnight. To the tubes 0.5 ml of sulphuric acid was added and kept in a boiling water bath for 10 min. 0.2 ml of this acid digest was taken and 5 ml phosphovanillin reagent was added and incubated for 15 min. The absorbance was read at 520 nm. The lipid content was expressed in milligram/100 g wet tissue.

5.3 Results

The AST, ALT and ALP activities of control and surfactant dosed groups are given in Tables 5.1-5.3 and Figures 5.1-5.3.

Table 5.1. Activity of AST (IU/L) in *O. mossambicus*

Group	AST activity
Control	1.248 ± 0.089
SDS	1.88 ± 0.067
Triton	0.88 ± 0.259
CTAB	1.69 ± 0.069

Values are the mean ± SD of six separate experiments.

Table 5.2. Activity of ALT (IU/L) in *O. mossambicus*

Group	ALT activity
Control	3.748 ± 0.212
SDS	5.556 ± 0.089
Triton	2.777 ± 0.093
CTAB	9.12 ± 0.10

Values are the mean ± SD of six separate experiments.

Table 5.3. Activity of ALP (KA units) in *O. mossambicus*

Group	ALP activity
Control	15.00 ± 1.58
SDS	17.50 ± 2.02
Triton	20.00 ± 2.85
CTAB	34.17 ± 1.81

Values are the mean ± SD of six separate experiments.

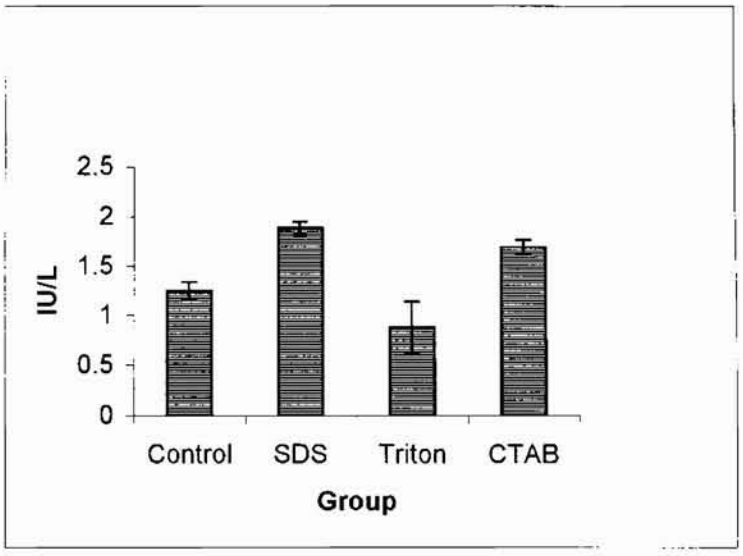


Figure 5.1. AST activity in *O. mossambicus*

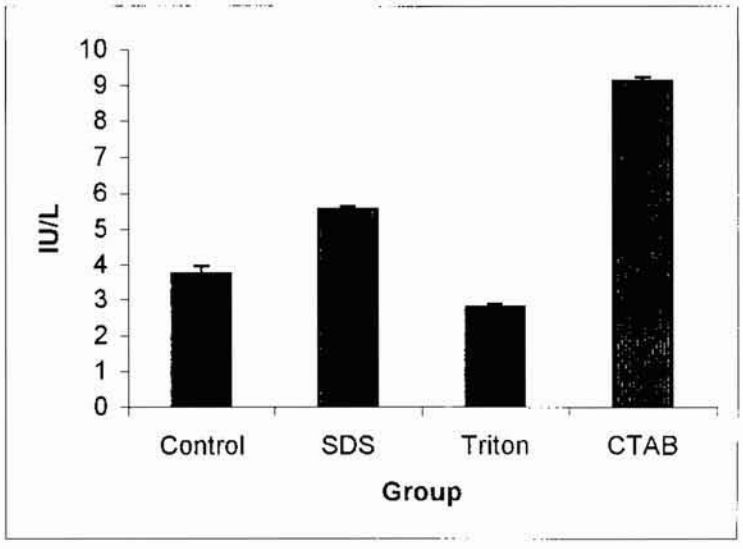


Figure 5.2. ALT activity in *O. mossambicus*

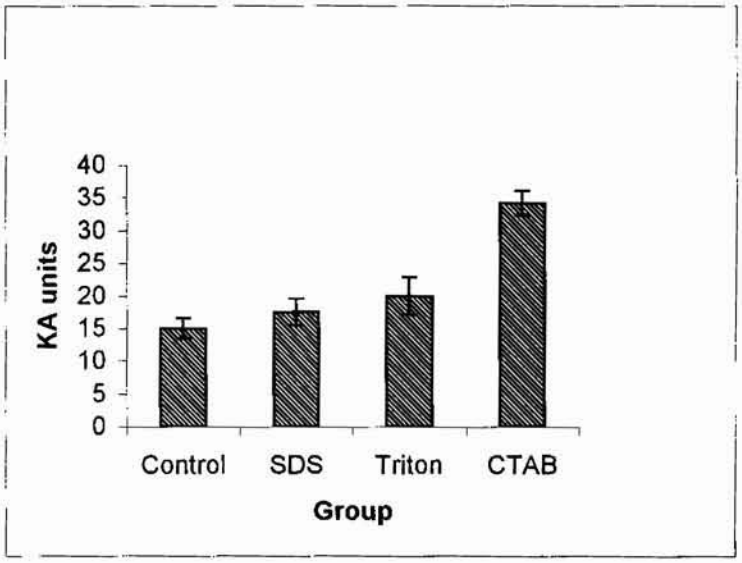


Figure 5.3. ALP activity in *O. mossambicus*

Table 5.1a. ANOVA for AST

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.642033	3	1.214011	60.49387	3.28E-10	3.098393
Within Groups	0.401367	20	0.020068			
Total	4.0434	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Aspartate transaminase activity showed an overall significant change ($p<0.001$) in experimental groups of animals (Table 5.1a). The highest activity was noted in SDS group (150.64% of control) followed by CTAB group (135.42% of control). But the animals exposed to Triton had significantly lower levels (70.5%) of aspartate transaminase. Subsequent LSD analysis revealed significant differences ($P<0.001$) in the enzyme activity in surfactant treated groups when compared to control and also among themselves (Table 5.5).

Table 5.2a. ANOVA for ALT

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	140.57	3	46.86	2316.35	4.2E-26	3.098
Within Groups	0.36	20	0.02			
Total	140.93	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

There was an overall significant change in alanine transaminase activity ($P<0.001$) in experimental groups (Table 5.2a). LSD analysis showed significant ($P<0.001$) differences in the surfactant treated groups when compared to control (Table 5.5). The enzyme activity was the highest for fishes exposed to the cationic surfactant (243.33% of control activity). The animals exposed to SDS showed the next highest increase in alanine transaminase activity (148.24% of control). However the fishes exposed to the non ionic surfactant Triton exhibited a significant decrease in the activity (73.91% of control). There were significant differences between the individual surfactants with respect to the induction of alanine transaminase ($P<0.001$) (Table 5.5).

Table 5.3a. ANOVA for ALP

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1325	3	441.67	98.35	3.82E-12	3.098
Within Groups	89.81	20	4.49			
Total	1414.81	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Alkaline phosphatase activity showed an overall increase in the experimental groups of animals ($p<0.001$) (Table 5.3a). The highest enzyme activity was noted in CTAB dosed group (227.78% of control) followed by Triton exposed animals (133.33% of control). In the animals exposed to the anionic SDS, the enzyme concentrations were 116.665% of that of the control group. Subsequent comparisons by LSD analysis revealed significant increase in enzyme activity in all surfactant treated groups when compared to control. The effects of SDS and Triton were almost similar with respect to the induction of alkaline phosphatase (Table 5.5).

Table 5.5. Results of LSD analysis for hepatic enzyme activity

Groups	P value		
	AST	ALT	ALP
Control × SDS	P < 0.001	P < 0.001	P < 0.05
Control × Triton	P < 0.001	P < 0.001	P < 0.001
Control × CTAB	P < 0.001	P < 0.001	P < 0.001
SDS × Triton	P < 0.001	P < 0.001	P < 0.05
Triton × CTAB	P < 0.001	P < 0.001	P < 0.001
SDS × CTAB	P < 0.001	P < 0.001	P < 0.001

Other biochemical parameters

Glycogen, lipid and protein content in hepatic tissues of control and surfactant treated animals are given in Table 5.6.

Table 5.6. Hepatic glycogen, protein and lipid content in *O. mossambicus*

Group	Glycogen	Protein	Lipid
Control	406.66 ± 11.54	29.3 ±2.59	621.8 ±10.91
SDS	367.6 ±10.84	14.01 ±3.87	287.3 ±13.21
Triton	111.1 ± 9.53	34.5 ±1.87	621.9 ±11.68
CTAB	261.6 ± 12.7	16.3 ±4.6	283.3 ±9.85

Values are the mean ± SD of six separate experiments
Expressed in mg/100 g wet wt.

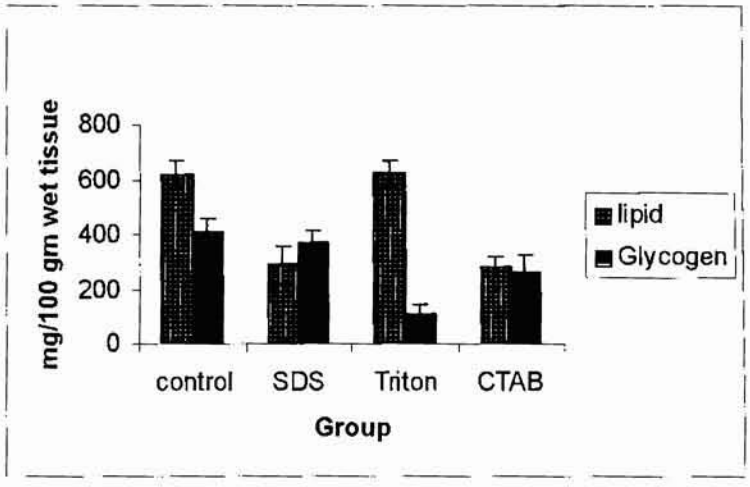


Figure 5.5. Glycogen and lipid content in *O. mossambicus*

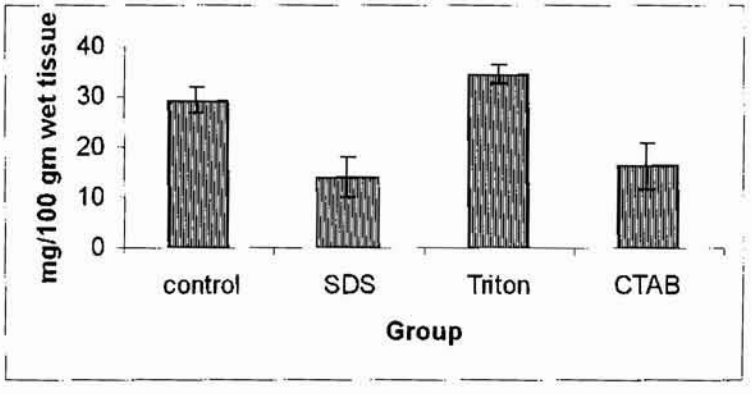


Figure 5.6. Protein content in *O. mossambicus*

Table 5.6a. ANOVA for Glycogen

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	314403.5	3	104801.2	2084.4	4.01E-25	3.098
Within Groups	1005.58	20	50.28			
Total	315409	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

There was an overall significant (P<0.001) change in glycogen on exposure to the surfactants for 30 days (Table 5.6a). The maximum decrease in glycogen was observed in fishes exposed to the non ionic Triton. This was followed by CTAB dosed and lastly the SDS dosed animals. Comparison by LSD analysis revealed significant (P<0.001) decrease in glycogen content in all surfactant treated groups when compared to control. Also there were significant (P<0.001) differences between the surfactant treated groups when compared among themselves.

Table 5.6b. ANOVA for Protein

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1702.56	3	567.52	41.33	9.24E-09	3.098
Within Groups	274.63	20	13.73			
Total	1977.19	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Protein content in hepatic tissues of control and surfactant exposed animals are given in Table 5.6. The fishes exposed to the cationic surfactant CTAB showed the maximum decrease in protein content. On the contrary protein content

was increased in animals exposed to the non ionic Triton and anionic SDS. The changes in the protein content were found to be significant (P<0.001) (Table 5.6b). Comparison by LSD analysis revealed significant (P<0.001) differences in protein content in all surfactant treated groups when compared to control. Also there were significant (P<0.001) differences in protein content of CTAB dosed fish when compared to SDS and Triton. But protein content in SDS and Triton dosed fish did not differ significantly.

Table 5.6c. ANOVA for Lipid

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	679643.4	3	226547.8	4299.01	293E-28	3.098
Within Groups	1053.95	20	52.69			
Total	680697.4	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Lipid content of control and surfactant exposed animals are given in table 5.6. There was an overall significant change in the lipid content of animals (P<0.001) as obtained from ANOVA (Table 5.6c). LSD analysis revealed significant differences (P< 0.001, Table 5.7) in lipid content of SDS and CTAB treated groups when compared to the control. However the lipid content in animals exposed to the non ionic Triton had lipid levels not significantly different from the controls.

Table 5.7 Results of LSD analysis for glycogen, protein and lipid

Groups	P value		
	Glycogen	Protein	Lipid
Control vs SDS	P < 0.001	P < 0.001	P<0.001
Control x Triton	P < 0.001	NS	P < 0.001
Control x CTAB	P < 0.001	P < 0.001	P < 0.001
SDS x Triton	P < 0.001	P < 0.001	NS
Triton x CTAB	P < 0.001	NS	P < 0.001
SDS x CTAB	P < 0.001	P < 0.001	P < 0.001

NS – Not significant

5.4 Discussion

Transaminases have an important role in the metabolic adjustments in response to stress. Under normal conditions there is a base line activity of these enzymes. But when the organism is subjected to stress, the levels of these enzymes are significantly increased in order to meet the increase in ATP demands. Thus the activity of transaminases would convert amino acids to keto acids like pyruvate and oxalo acetate, which could be used as intermediates in Krebs' cycle or directed into the gluconeogenic pathway.

The alkaline phosphatase levels were also significantly increased in animals exposed to surfactants (except in the Triton dosed group). It has been suggested that increase in this enzyme occurs in response to corticosteroids (Murphy 1964; Rudel and Kinel, 1972).

. It has also been suggested by Walden and Farzaneh (1990) that under normal conditions corticoids are necessary to stimulate the synthesis of phenyl ethanolamine-N-methyl transferase which is involved in the conversion of nor epinephrine to epinephrine.

Glycogen was mobilised to the maximum extent in fishes exposed to the non ionic surfactant Triton. The level of protein in this group was higher than that of control where as the lipid content was comparable to that of the control group. It appears that Triton-induced stress had largely affected the carbohydrate metabolism of the animals compared to protein and lipid metabolism.

It was observed that Triton induced maximum mobilisation of glycogen. This increased mobilisation of glycogen in Triton-stressed fish could also be the result of interaction of the surfactant with β-adrenergic receptors of the hepatic cells. The interactions between hormone receptors and adenylate cyclase depends upon membrane fluidity (Cuatracas, 1974) and the enzyme activity is affected by the phospholipid composition (Warren and Metcalfe, 1976). The non ionic surfactant might have increased the membrane fluidity and thus altered the mobility or distribution of receptors or/and adenylate cyclases within the membrane.

In SDS dosed fish, the lipid reserve was extensively mobilised to produce ATP whereas the utilisation of glycogen was only secondary. The increased lipolysis might have been effected through epinephrine. This probably might have led to the production of fatty acids which on β-oxidation would yield acetyl Co A for supply into Kreb's cycle and ATP production. The glycogen was also mobilised in SDS dosed fish to a much lesser extent than the other two surfactant

exposed groups. The protein content was significantly increased in this group when compared to the controls.

In CTAB dosed animals, there was extensive mobilisation of glycogen, lipid and protein. This indicated that the cationic surfactant induced maximum stress which demanded large amounts of ATP. Hence there was an increase in the rate of metabolic reactions like Krebs' cycle, transamination and probably fatty acid oxidation.

Similar findings of decrease in glycogen in the liver, gill and kidney has been observed in fingerlings and yearlings of *Cirrhina mrigala* and *Puntius sophore* respectively on exposure to linear alkyl benzene sulphonate (Misra *et al.*, 1991). Maruthanayagam *et al.* (1997) has also reported significant decreases in the glycogen, lipid and protein content in the hepatopancreas, muscle and gill tissue of fresh water prawn *Machobrachium lammarei* on exposure to detergents.

Thus it may be deduced that surfactant exposure is stressful to the fishes. This is reflected clearly from the increased metabolic rate and metabolic adjustments to increase ATP production.

In the non ionic Triton dosed fishes, glycogenolysis was greatly enhanced which would supply glucose and then acetyl Co A for reactions of Krebs' cycle.

In SDS dosed fish, lipid mobilisation and possible β-oxidation of fatty acids derived from extensive lipolysis would generate acetyl Co A to be fed into Krebs' cycle.

In animals exposed to the cationic surfactant CTAB, in addition to mobilisation of lipids and glycogen, extensive proteolysis was also observed. This would lead to an increase in the amino acid pool. These amino acids may be utilised for ATP production via 2 ways. They could be converted to keto acids via

transaminases and then fed into tricarboxylic acid cycle. Or they could be channelled into gluconeogenic pathway and then subsequently into Krebs' cycle.

Thus it may be inferred that in all the cases of surfactant exposure -whether cationic, anionic or non ionic, the energy metabolism of the animals was significantly affected. There was increased mobilisation of reserve glycogen, lipid and protein to varying degrees in all the surfactant exposed animals. However in all these cases of chronic exposure to surfactants, Krebs' cycle or aerobic oxidative pathway formed the convergence point of all the metabolic reactions. Here it may be recalled that acute toxic responses are generally anaerobic in nature whereas chronic toxicity is defended by an increase in aerobic metabolism (Raju *et al.*, 1994).

CHAPTER

6

Effects of Surfactants on Branchial ATPases

6.1 Introduction

Gills are highly vulnerable to aquatic pollutants as they are directly and continuously exposed to the pollutants, and also their large surface area makes them immediately susceptible to the adverse conditions generated by the pollutants. In addition to the prime function of respiration, gills also play an equally important role in osmoregulation. Hence the aquatic vertebrates differ from the terrestrial ones in that stress causes frequent and often pronounced disturbances in the hydro-mineral balance of the former group. The membrane-bound enzymes, sodium-potassium (E.C.3.6.3.9) and magnesium ATPases (E.C.3.6.3.1), play a major role in this respect. The sodium-potassium ATPase enzyme has a key role in the branchial uptake of sodium ions. The teleost fresh water fishes engage in active ion uptake to maintain homeostasis. This is mediated with the help of mitochondria-rich cells, viz., chloride cells or ionocytes (Perry, 1997). In addition to Na^+,Ca^{2+} and Cl^- are also taken up by these cells (Foskett *et al.*, 1983). The chloride cells have an extensive tubular membrane system which lodges the bulk of branchial ATPase. The functional unit of ATPase consists of a catalytic α subunit non-covalently linked with a glycoprotein β subunit. The activity of ATPase is also the driving force for the secondary transport of Na^+-Ca^{++} exchanger which plays a crucial role in Ca^{2+} transport and homeostasis of the cells. Perry and Laurent (1993) have reported that in fresh water fish, chloride cell density is directly correlated with Na^+-K^+ ATPase activity.

To maintain homeostasis during exposure to pollutants the animal activates a number of biochemical and physiological processes aimed at detoxifying or/and restoring the branchial ion uptake machinery. Hence effects of pollutants on gills may be studied by monitoring the activity of ATPases. The ATPase system has been evidenced as a target for the toxic action of a variety of toxicants in different organisms (Philips and Wells,1974; Desaiah and Koch 1975; Verma *et al.*, 1979). The present chapter deals with the effects of the anionic sodium dodecyl sulfate, non ionic Triton and the cationic cetyl tri methyl ammonium bromide on the activities of the branchial Na^{+}-K^{+} ATPase and Mg^{2+} ATPase.

6.2 Materials and Methods

Collection, acclimation and experimental conditions are same as in 3.2.3 of Chapter 3. After 30 days the fish were sacrificed and the gill tissue was dissected out, washed in ice-cold isotonic 0.33 M sucrose, blotted dry and weighed. A 10% homogenate was prepared in 0.33 M sucrose.

6.2.1 Extraction of the Enzyme

The homogenate was centrifuged at 3000g for 15 min and the supernatant so obtained was centrifuged at 12000g for 30 min. The supernatant was again subjected to further centrifugation at 35000g for 30 min. The pellet so obtained corresponds to the heavy microsomal fraction (Davis, 1970). The pellet was then resuspended in 0.33 M sucrose and used as the enzyme source.

6.2.2 Enzyme Assay

Na^{+}-K^{+} activated, Mg^{2+} dependent ATPase (Total ATPase) was determined using the reaction mixture containing 60 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5) and 2.5 mM Tris-ATP. The Mg^{2+} ATPase was measured by substituting sucrose in place of NaCl and KCl. A control was maintained in the absence of sodium, potassium and magnesium ions for estimating non-specific phosphatases and other ATPases. The reaction mixture was incubated at 37°C for 15 min. The Na^{+}-K^{+} ATPase activity was calculated in terms of the difference between total and Mg^{2+} ATPase values. After incubation 2 ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture, and the supernatant separated off by centrifugation at 1300 g for 10 min. The inorganic phosphate liberated from ATP was estimated by the method of Fiske and Subbarow (1925). The specific activity of Na^{+}-

K^+ ATPase was defined as micro moles of P_i /mg of enzyme protein/h. The protein was estimated by the method of Lowry *et al.* using bovine serum albumin as standard.

6.3 Results

The activities of ATPases are given in Table 6.1 and Figures 6.1 and 6.2.

Table 6.1. Gill Na^+-K^+ ATPase and Mg^{2+} ATPase Activities in control and surfactant exposed *O. mossambicus*

GROUP	TOTAL ATPase (Corrected value)	Na^+-K^+ ATPase	Mg^{2+} ATPase (Corrected value)
Control	44.06 ± 5.6	21.56±0.8	22.5±1.3
SDS	41.28 ± 4.5	21.89±1.5	18.39±0.9
TRITON	36.105 ± 4.1	18.05±1.6	18.05±0.8
CTAB	33.12 ± 5.2	17.23±1.1	15.89±1.4

Activity expressed as μ moles of inorganic phosphate (P_i) liberated/h/mg protein
Values are the mean ± SD of six separate experiments.

Table 6.1a. Total ATPase Activities in control and surfactant exposed *O.mossambicus*

Sample	Total ATPase (1)	Non specific phosphatases + ATPases (2)	Corrected value (1-2)
Control	46.57	2.51	44.06
SDS	43.48	2.2	41.28
TRITON	37.995	1.89	36.105
CTAB	35.22	2.1	33.12

Activity expressed as μ moles of inorganic phosphate (P_i) liberated/h/mg protein

Table 6.1b. Magnesium ATPase + other non-specific phosphatase activities in control and surfactant exposed *O.mossambicus*

Sample	Mg ATPase + other phosphatases (1)	Non specific phosphatases + ATPases (2)	Corrected value (1-2)
Control	24.25	1.75	22.5
SDS	20.18	1.79	18.39
TRITON	20.14	2.09	18.05
CTAB	17.79	1.9	15.89

Activity expressed as μ moles of inorganic phosphate (P_i) liberated/h/mg protein

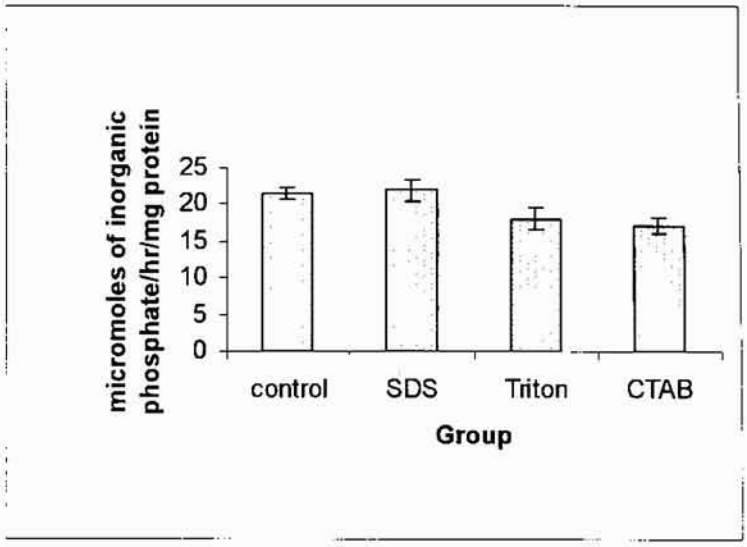


Figure 6.1. Na^{+}-K^{+} ATPase activity in *O. mossambicus*

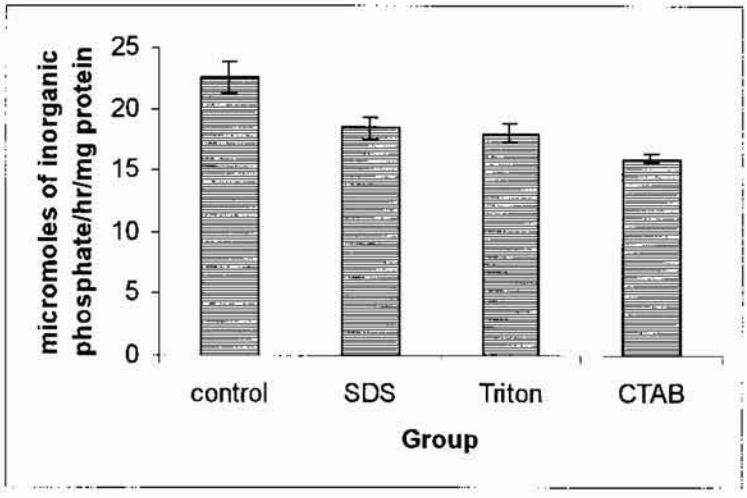


Figure 6.2. Mg^{2+} ATPase activity in *O. mossambicus*

Table 6.1a. ANOVA for Na^+-K^+ ATPase

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	77.4173	3	25.80575	4.581445	0.013424	3.098393
Within Groups	112.653	20	5.632667			
Total	190.071	23				

Table 6.1 b ANOVA for Mg^{2+}-ATPase

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	137.1268	3	45.70895	89.62539	9.06E-12	3.098393
Within Groups	10.2	20	0.51			
Total	147.3268	23				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Table 6.1c. Results of LSD analysis for ATPases

Groups	Na^+–K^+ ATPase	Mg^{2+} ATPase
Control x SDS	NS	P<0.05
Control x Triton	NS	P<0.05
Control x CTAB	NS	P<0.05
SDS x Triton	P<0.05	NS
Triton x CTAB	NS	NS
SDS x CTAB	P<0.05	NS

NS – Not significant

There was an overall significant change in the activity of the branchial Na^+-K^+ ATPase ($F = 4.58$, $P<0.01$, Table 6.1a) in presence of surfactants. It was observed that from subsequent LSD analysis (Table 6.1c) that the enzyme activity in all the three surfactant exposed animals were similar when compared to the control. SDS induced an insignificant activation of the enzyme when compared to

the control. The enzyme activity in Triton and CTAB dosed animals were significantly different ($P<0.05$) from that in SDS.

There was an overall significant change ($F = 89.62$, $P<0.001$, Table 6.1b) in Mg^{2+} ATPase activity) in all the experimental animals. Comparison of the surfactant exposed groups with control revealed significant ($P<0.05$) inhibition of the enzyme in all the three surfactant treated groups. However there was no significant difference between the surfactants with respect to inhibition (Table 6.1c). The total ATPase values were significantly lower for all the three surfactants. This might be due to the inhibition effect of surfactants or /and the damage to the gill membranes. CTAB dosed fish had the least values for total ATPase, followed by Triton and then SDS.

6.4 Discussion

It was observed that the activities of both the ATPases were inhibited by all the surfactants tested except SDS which caused an insignificant stimulation of Na^+-K^+ ATPase. The cationic surfactant CTAB was the most inhibitory to both the ATPases and its effect on Mg^{2+} ATPase was more pronounced than on Na^+-K^+ ATPase. SDS, the anionic surfactant caused significant inhibition of Mg^{2+} ATPase and insignificant activation of Na^+-K^+ ATPase.

It was observed that all the surfactants interfered with the branchial ATPases even at sub-lethal concentration of 1 ppm. ATPase is an integral membrane protein and requires specific lipids for its activity. It therefore may be inferred that surfactant-induced damage to the cell membrane lipids and proteins resulted in enzyme inhibition. The damage via peroxidation can also disrupt cell membrane integrity.

LITERATURE REVIEW FOR SECTION 1

Nature of study	Type of surfactant	Organism studied	Effects observed	Reference
physiological	ABS	*Salmo gairdneri*	Blocking effect on olfaction	Sutterlin *et.al* (1971)
	SLS	*Coregonus clupeaformis*	Depressed olfactory response	Hara& Thompsun (1978)
	LABS	Tilapia, *Diaptomus forbesi, Branchiura*	Feeding	Chattopadhyay & Konar (1985)
	Alkyl ethoxylates	*Lepomis machrochirus*	Respiration	Maki (1979)
	Triton X-100, SLS, Tween-20	*Tautoglobarus adspersus*	Bradycardia	Kiceniuk *et al* (1978)
	LAS,ABS, LES, NP10 EO, TAE	*Pimephales, Gadus, Corbicula*	Developmental abnormalities and avoidance reactions	
	LABS	Veliger larvae	Decreased survival rate	Hidu (1966)

Nature of study	Type of surfactant	Organism studied	Effects observed	Reference
Behavioral response	ABS	salmonids	avoidance	Srague & Drury (1969)
	LAS, AS, ABS	*Plecoglossus*	avoidance	Tatuskawa & Hidaka, 1978
	ABS	*Gadus, Salmo*	avoidance	Hoglund, 1976
	Alkyl phenol ethoxylates (EO:10)	Cod, mussel	Increased swimming activity and avoidance	Hoglund, 1976

Nature of study	Type of surfactant	Organism studied	Effects observed	Reference
Histological	Sodium dodecyl benzene sulfonate	*Rita*	Delipidation from gill epithelium and club cells	Roy, 1990
	Zephiran (cationic detergent)	*Salmo*	Lesions on gill tissue, necrosis and lamellar fusion	Byrne et al, 1989
	LABS	*Cirrhina*	Distorted gill lamellae	Misra et al, 1987
	LABS	*Arenicola*	Papillae damage, decreased olfaction and rupture of intestinal wall.	Emilio conti, 1987
	LABS	Rainbow trout	Skin degeneration	Pohlagubo & Adam, 1982
	Ariel (commercial detergent powder)	*Oreochromis mossambicus*	Degeneration of respiratory lamellae, atrophy	Raju et al, 1994
	SDS	*Clarias*	Enlarged mucus cells of epidermis	Garg & Mittal, 1993
	SDS	*Sparus auratus*	Tubular and renal corpuscle retraction, damage to spleen and increased infiltration of WBC and RBC	Rosety et al, 1985

Nature of study	Type of surfactant	Organism studied	Effects observed	Reference
Studies on cell membrane	ABS, Nonyl phenol	*Onchorhyncuss*	Decreased gill viability	Part et al, 1985
	Idet 5L,Swanic 6L, Swascofix (ABS)	*Channa punctatus*	Insignificant activation of Na-K ATP ase and inhibition of K ATP ase in brain and gill	Verma et al, 1983
	Sodium deoxy cholate, Lubrol	Bovine brain	Activation of Mg-Ca ATPase	Roufogallis, 1973
	Sodium alkyl aryl sulfonate	*Ctenopharyngodon idella*	Decreased plasma sodium	Rosas et al, 1988
	SDS, Triton X-100 , deoxycholate	Erythrocyte membrane	destabilisation	Kirkpatrick et . al, 1974
	Zephiran	Erythrocyte membrane	Crenation of RBC and labilisation of membrane	Byrne et al, 1989
	SDS, Triton, CTAB	Gills of trout	Solubilisation of membrane bound enzymes	Partarroyo et. al, 1991
	Span 80	Hen RBC	fusion	Attwood & Florence, 1983
	Nonyl phenol 40, SDS, DOC, TX-100,CPB	Lymphocytes of tonsillar tissue	Increase in glucose oxidation by DOC, drastic damaging effect by CPB	Hrabat et.al, 1982
	SDS,CTAB	*Geodia cydonium*	Inhibition of phospholipase A2 release	Vgarakovic et. al, 1991
	SDS, Tween	Rat lysosomes	Increased release of lysosomal acid phosphatase	Tabata et.al, 1990
	Anionic, cationic , non ionic and amphoteric surfactants	Rabbit corneal epithelial cells	Cytotoxic to ocular epithelium, causes irritancy	Lewis et.al 1993, Grant et. al,1992

	Polyoxy ethoxylates	Human RBC	Inhibits transport of 2,4 dinitro phenyl glutathione out of the cell	Board 1993
	Various non ionics	Egg yolk cell membrane	Leakage of amino acids and other ions	Regen et.al, 1989

Nature of study	Type of surfactant	Enzyme studied	Effects observed	References
Enzymes studied *in vitro*	SDS, TritonX-100, quarternary ammonium compounds (QAC)	Bone alkaline phosphatase	Increase in activity probably due to detergent binding at active sites	Joao et.al, 1987
	Octa ethylene glycol	renal Na-K ATPase of dog	Dissociation of the enzyme	Mimura et.al, 1993
	SDS	*Aspergillus niger* catalase	activation	Jones et.al, 1987
	SDS	Bovine catalase	Inhibition	Jones et.al, 1987
	SDS	urease	inhibition	Diane & Christensen, 1982
	Triton X-100	Rat liver mitochondrial LCAT	activation	Dygas et.al, 1989
	Triton X100, Non ionics (Mryj 52, 59 and Tween 20, 80)	Human elastase and cathepsin G	Activation of cathepsin Inhibition of elastase	Wenzel et.al 1990
	Non ionics	*Chromobacterium* lipase	activation	Yamada et.al, 1993
	Triton	ATPase (plasma membrane)	activation	Sandstrom & Cleland, 1989

	SDS	Acid phosphatase, succinic dehydrogenase of rat liver and kidney	Activation of Acid phosphatase and inhibition of succinic dehydrogenase	Gupta & Dhillon, 1983

Nature of study	Type of surfactant	**Enzymes studied in vivo**	**Effects observed**	Reference
Enzymes studied in vivo	Emulgen	Heme oxygenase, cytochrome p 450 and b5 (rat liver)	Decrease in cytochrome p450 and b5 activities and increase in heme oxygenase	Ariyoshi et.al, 1991
	Swascofix (ABS), SDS	Na-K ATPase in brain of *Mystus and Cirrhina* sp	inhibition	Verma et.al, 1979
	Swascofix (ABS), SDS	Liver Acid phosphatase of *Mystus and Cirrhina* sp	Moderate inhibition	Verma et.al, 1979
	Swanic, Idet	ATP ases of *Cirrhina* sp	Activation of Na-K ATPase at low concentration , inhibition of Mg ATPase	Verma et.al, 1979
	Idet 20	Acetyl choline esterase, glucose-6-phosphate dehydrogenase, 5-nucleotidase *(Clarias sp.)*	Inhibition of all enzymes	Verma, 1984
	Sodium deoxy cholate	5-nucleotidase of rat liver	activation	Emmelot & Boss, 1965, Konopka 1972

	Ariel	Succinic dehydrogenase and lactate dehydrogenase (gill, liver, kidney and brain of *Oreochromis mossambicus)*	Activation of Succinic dehydrogenase and inhibition of lactate dehydrogenase	Raju et.al, 1994

Nature of study	**Type of surfactant**	**Organism studied**	**Pattern of metabolism**	**Reference**
Metabolism and bioaccumulation	Nonyl phenol	*Salmo gairdneri*	Metaboliseed to glucuronide and hydroxylate conjugates , excreted in bile and urine	Augustine et. al, 2000
	Cationics (QAC, CPB)	*Pimephales*, clams and tadpoles	Highest bioaccumulation in the gills due to negatively charged polysaccharide secretions in gill	Knezovich et. al 1989
	DiTallow Di Methyl Ammonium Chloride (DTDMAC)	*Lepomis*	Viscera showed maximum bioaccumulation	Lewis & Wee 1983
	SDS	Fish and mammals	Surfactant is subjected to beta oxidation and converted to butyric acid-4-sulfate. It is then desulfated and butyro lactone is formed	Tjeerdema 1993
	Corexit	*Salmo gairdneri*	Surfactant is metabolized by lipase to give fatty acids	Payne 1982

	LABS	*Lepomis*	Highest bioaccumulation in gall bladder	Kimerle et al 1981
	Sodium dodecyl tri oxy ethylene sulfate	*Cyprinus carpio*	Order of bioaccumulation is gills> hepatopancreas>gall bladder	Kikuchi et.al 1980

Nature of study	**surfactant**	**Other pollutants**	**Organism studied**	**Effects observed**	**Reference**
Synergistic studies	ParnolJ (anionic)	Crude oil	*Tilapia*	Decrease in growth rate, maturity index and increase in gastrosomatic index	Panigrahi, 1986
	Pluronic (non ionic)	metal	*Caenorhabditis elegans*	Metal in combination with the surfactant is more toxic than metal alone.	Dennis et.al 1997
	SDS	nickel		Increased lipid peroxidation and higher tissue accumulation of nickel in presence of SDS.	Mathur 1992

SECTION 2

Studies on *Synechocystis salina* Wislouch

CHAPTER

7

Studies on the Marine Cyanobacterium *Synechocystis salina* Wislouch

7.1 Introduction

The phytoplankton constitute an important component of any ecosystem. These mainly include algae classified into various classes based on the pigment present (Chlorophyceae, Cyanophyceae, Rhodophyceae, etc.), diatoms and the like. With the help of their pigment system which may be chlorophyll *a, b, c* or both, the algae constitute the important primary producers of the ecosystem and manufacture carbohydrate for the rest of the community. Phototrophic primary producers encompass a wide variety of cyanobacteria and eukaryotic algae. Of these the cyanobacteria abound in the world water bodies, constitute more than 50% of the autotrophic biomass and are responsible for the total primary production in the euphotic zone of the open ocean. Cyano bacteria are characterised by the presence of chlorophyll a, biliproteins gram negative cell wall, share photosystems I, II and are capable of oxygenic photosynthesis (Boekema *et al.*, 2001).

The toxicities of detergents to aquatic flora have been studied in both fresh water and marine conditions. Reported data include the production of algal blooms following the input of phosphate containing detergents into the water bodies (eutrophication) and the disturbance of the nitrogen : phosphorus ratio (Mukherjee *et al.*, 1994). The lethal concentration for fresh water species for 72-96 hr were of the range 0.9-300 ppm for C_{10-14} LAS whereas for marine species LC_{50} was between 0.025-10 ppm. For sodium alkyl sulfates, the lethal concentration for 24-48 h was 4-32 ppm for marine species. Cationic surfactant toxicity for alkyl

(C_{12-18}) tri methyl ammonium chloride was 0.1-6.1 ppm (lab *in situ* studies) and for acetyl tri methyl ammonium bromide 0.03-2.6 ppm for fresh water species for 72-96 h whereas data are lacking for the toxicities of cationics and non ionics on marine species (Kutt and Martin, 1974). The commonly tested species for toxicity studies were *Selanastrum, Microcystis, Chlorella, Navicula* etc.

The present study deals with the evaluation of toxicities of three surfactants namely sodium dodecyl sulfate (anionic), Triton X-100 (non ionic) and cetyl tri methyl ammonium bromide (cationic) on the marine cyanobacterium *Synechocystis salina* Wislouch.

7.2 Materials and Methods

Surfactants – used were the anionic sodium dodecyl sulfate (SDS), non ionic Triton X-100 and the cationic cetyl tri methyl ammonium bromide (CTAB).

Test algae - The species used for the experiment was the marine cyanobacterium *Synechocystis salina* Wislouch belonging to the order Chroococcales (Waterbury, 1989). These are small spherical cells of diameter 3 microns with bluish-green colour. They are rarely seen in pairs and jerky movements are characteristic of the cells. Within each cell the thylakoids are peripheral in 4-5 concentric layers which contain chlorophyll *a*. On outer surface of the thylakoid there are regular rows of electron dense structures that carry phycobilisomes lodging the phycobilins viz. phycocyanin and phycoerythrin. Also polyhedral polyphosphate bodies are seen. In addition small structural granules representing lipid inclusions are seen scattered among the thylakoids. These reproduce by binary fission and divisions occur in 2 or 3 planes at right angles to one another or in irregular planes.

7.2.1 Culture Medium

The cells were cultured in Allen and Nelsons medium (1910)

Solution A	Potassium nitrate 2 M	
Solution B	Di Sodium hydrogen phosphate.12 H_2O	4 g
	Calcium chloride.6 H_2O	4 g
	Con HCl	2 ml
	Distilled water	80 ml

Autoclaved A and B, added 2 ml A and 1 ml B to 1 litre sea water (sterilised) of salinity 30 ppt. The pH was adjusted to 6 as it was found to be the most optimum for algal growth in preliminary experiment.

Preparation of the test solutions: The surfactant solutions were prepared in the culture medium and the pH was adjusted to 6 ± 0.2 with 0.1 N HCl or 0.1 N NaOH. The surfactant concentrations tested include 0.2, 0.4, 0.6, 0.8 and 1 ppm of all the three surfactants. These were sub lethal concentrations which were approx. 1/6 or 1/10 of the LC_{50} of the surfactants.

Inoculation: The inoculum was taken from a culture in the exponential phase of growth so as to avoid any lag period in growth. A definite volume of the culture was taken and the cells counted using a hemocytometer. The same volume of inoculum was added to the control as well as the surfactant added test tubes. The test and control cultures were maintained in test tubes of the size 12 × 18 mm. These tubes were kept in a slanting manner and illuminated constantly from above with fluorescent light of 3000 ± 100 lux intensity. The duration of the experiment was 14 days.

The biochemical parameters studied included:

1. Estimation of Growth
2. Estimation of chlorophyll *a*
3. Estimation of protein
4. Estimation of carbohydrate
5. Estimation of lipid

7.2.2 Estimation of Growth

Monitoring of algal growth was achieved by 2 ways- by cell count using a hemocytometer and by fluorescence of algal chlorophyll. A high degree of correlation (r = 0.99) was obtained between cell count and fluorescence in preliminary experiments. These measurements were made every 24 h after inoculation and at the end of the experimental duration. The growth inhibition was evaluated as the ratio of the growth in the surfactant dosed tubes to that in the control tubes and expressed as percentage.

7.2.3 Estimation of Chlorophyll *a*

The photosynthetic efficiency was assessed indirectly by estimating the amount of chlorophyll *a* by the method of Strickland and Parson (1972). The volume of cultures in the control and the surfactant dosed tubes was noted. The cultures were then filtered through a 4.5 cm Whatman GF/C paper, the filter was drained completely under suction before removing it from the filtration equipment. The peripheral excess of the paper was trimmed using a scissors. The filter was placed in a 15 ml centrifuge tube and 8 ml of 90% acetone was added. The tube was then stoppered and the filter was dissolved by shaking the tube vigorously. The pigments were allowed to get extracted by placing the tubes in a refrigerator in complete darkness for about 20 h. The tubes were then removed from the

refrigerator and allowed to warm up in the dark nearly to room temperature. Then 90% acetone was added so as to make up the volume of acetone in the tubes to 10 ml. The tubes were then centrifuged for 5-10 min. The clear supernatant liquid was drained into a test tube. The absorbance of this liquid was read at 665, 645 and 630 nm. The chlorophyll content was calculated from the equation

$$\text{Chlorophyll a} = 11.85 \times (E_{665}) - 1.54 \times (E_{645}) - 0.08 \times (E_{630})$$

The chlorophyll content was expressed as microgram/litre of the culture volume.

7.2.4 Estimation of Protein (Lowry *et al.*, 1951)

At the end of the experimental period, the control and surfactant dosed tubes were centrifuged at 600 g to obtain a pellet of the cultured cells. These were then transferred to small glass bottles and dried at 80°C till a constant dry weight was obtained (Abou-Waly *et al.*, 1991). The dry weights of the control and surfactant dosed culture tubes were noted.

The dried cell mass was mixed with 5 ml of 1 N NaOH and finely ground in a mortar. The alkaline solution was transferred into centrifuge tubes and kept in a water bath at 60-70°C with intermittent agitation for 20 min. Later the tubes were transferred to ice-cold water and cooled. The volume of the solution was measured and readjusted to 5 ml with distilled water. This was then subjected to centrifugation for 10 min at 600 g. 1 ml of the supernatant so obtained was used for protein estimation. The protein content was estimated from a calibration curve prepared by using bovine serum albumin as standard. The protein content was expressed in μg/mg dry weight.

7.2.5 Estimation of Carbohydrate (Carroll *et al.*, 1956)

The dry cell mass for the estimation was prepared as described earlier in 7.2.4. The weight of dry cell mass was noted. To this, 5 ml of 2.5 N HCl was added and hydrolysed by keeping in a boiling water bath for 3 h. It was then cooled to room temperature and neutralised with anhydrous sodium carbonate till effervescence ceased. Then the volume was made up to 10 ml and centrifuged. The supernatant after suitable dilution was used for analysis. To 1 ml supernatant, 4 ml anthrone reagent was added and placed in a boiling water bath for 10 min after capping with glass marbles. The absorbance was read spectrosphotometrically at 620 nm. The carbohydrate content was expressed in μ gm/mg dry weight.

7.2.6 Estimation of Lipid (Frings and Dunn, 1970)

The dried tissue was prepared as in 7.2.4 and the dry weight was noted. The dry cell mass was taken in a homogeniser and 10 ml chloroform - methanol was added, mixed well and filtered. To the filtrate 2 ml of sodium chloride was added and mixed well. It was then kept overnight at 4°C for lipid extraction. The lower phase containing the lipid was made up to 10 ml and then a definite aliquot of this was dried in vacuum desiccator overnight. To the tubes 0.5 ml of sulphuric acid was added and kept in a boiling water bath for 10 min. 0.2 ml of this acid digest was taken and 5 ml phosphovanillin reagent was added and incubated for 15 min. The absorbance was read at 520 nm. The lipid content was expressed in μ gm /mg dry weight.

7.3 Results

Growth inhibition studies: The results are given in Table 7.1. Of the 3 surfactants tested, the cationic surfactant CTAB was the most inhibitory. There was only 56.87% growth at 0.8 ppm of the cationic whereas at the corresponding concentration there was 81.38% and 94.46% growth in anionic and the non ionic surfactants respectively.

Table 7.1. Percentage growth in control and surfactant exposed *S. salina*

Concentration	SDS	TRITON	CTAB
0 ppm (Control)	100	100	100
0.2 ppm	87.22 ± 5.2	97.19 ± 6.5	87.36 ± 3.8
0.4 ppm	88.69 ± 7.3	95.47 ± 8.1	72.46 ± 4.1
0.6 ppm	83.25 ± 6.5	94.76 ± 7.2	63.39 ± 7.4
0.8 ppm	81.38 ± 4.1	94.46 ± 6.3	56.87 ± 5.6
1 ppm	79.71 ± 3.8	94.05 ± 4.7	26.99 ± 8.5

Values are the mean ± SD of six separate experiments expressed as %.

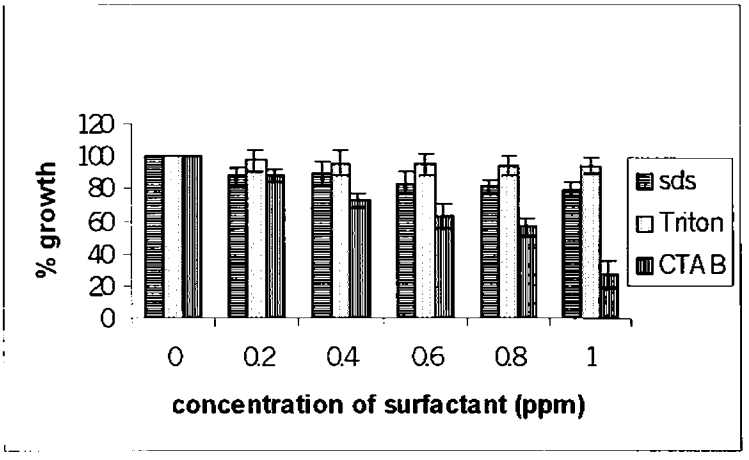


Figure 7.1. Percentage growth in *S. salina*

Table 7.1a. ANOVA for growth

Source of Variation	SS	df	MS	F	P-value	F crit
Between concentrations	1938.154	5	387.6309	2.418243	0.109999	3.33
Between surfactants	2467.982	2	1233.991	7.698276	0.009465	4.11
Error	1602.945	10	160.2945			
Total	6009.081	17				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Two way ANOVA showed that there was an overall significant difference between the surfactants with respect to growth inhibition ($F = 7.698$, $P<0.01$) (Table 7.1a). Subsequent LSD analysis (Table 7.1b) revealed that growth in CTAB treated cultures was significantly different from that of control ($P<0.01$) as well as SDS and TX-100 ($P<0.05$). However SDS and TX-100 caused similar inhibitions on growth which were insignificant.

Chlorophyll: The chlorophyll content in various cultures exposed to the surfactants are given in Table 7.2 and Figure 7.2.

Table 7.2. Chlorophyll content in control and surfactant exposed *S. salina*

Concentration	SDS	TRITON	CTAB
0 ppm (Control)	1.66 ± 0.01	1.66 ± 0.01	1.66 ± 0.01
0.2 ppm	1.45 ± 0.03	2.03 ± 0.04	1.47 ± 0.03
0.4 ppm	1.47 ± 0.02	1.51 ± 0.02	1.19 ± 0.02
0.6 ppm	1.38 ± 0.01	1.27 ± 0.03	1.17 ± 0.01
0.8 ppm	1.35 ± 0.04	0.88 ± 0.01	1.01 ± 0.02
1 ppm	1.32 ± 0.03	1.51 ± 0.03	0.77 ± 0.01

Values are the mean ± SD of six separate experiments expressed as µg/litre

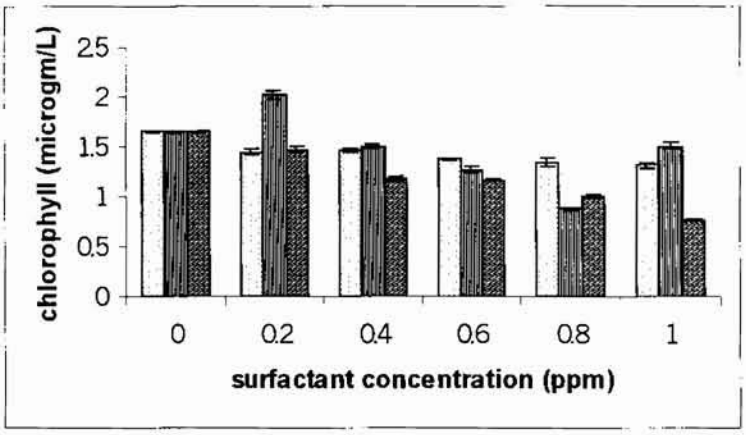


Figure 7.2. Chlorophyll content in *S. salina*

There was an overall significant change in the chlorophyll content between various concentrations of surfactants (Table 7.2a). LSD analysis (Table 7.6) revealed significant decrease in the chlorophyll content only at higher concentrations (0.8 and 1 ppm) of the surfactants ($P<0.05$) when compared to control. But there was no significant difference between the surfactants with respect to the decrease in chlorophyll content. Cationic surfactant was the most inhibitory where 50% reduction of chlorophyll was obtained between 0.8 and 1 ppm. However the reduction in chlorophyll content was not strictly concentration–dependent. For example in case of Triton dosed cells, there was only 53% chlorophyll at 0.8 ppm whereas at 1 ppm there was more of the pigment (90%).

Table 7.2a. ANOVA for chlorophyll

Source of Variation	SS	df	MS	F	P-value	F crit
Between concentrations	0.824494	5	0.164	3.254655	0.05299	3.325
Between surfactants	0.041144	2	0.020	0.40604	0.67679	4.102
Error	0.506656	10	0.050			
Total	1.372294	17				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Protein: Protein content in the control and the surfactant treated cultures are given in Table 7.3 and Figure 7.3.

Table 7.3. Protein content in control and surfactant treated *S. salina*

Concentration	SDS	TRITON	CTAB
Control (0 ppm)	20.6 ± 4.3	20.6 ± 4.3	20.6 ± 4.3
0.2 ppm	13.55 ± 2.3	9.74 ± 2.8	16.85 ± 3.1
0.4 ppm	16.76 ± 3.1	8.22 ± 1.8	14.4 ± 2.2
0.6 ppm	27.67 ± 4.2	7.1 ± 2.4	12.75 ± 3.0
0.8 ppm	5.28 ± 0.2	2.23 ± 0.1	11.59 ± 2.6
1 ppm	13.62 ± 3.1	3.23 ± 0.3	8.95 ± 3.1

Values are the mean ± SD of six separate experiments expressed as μg / mg dry weight

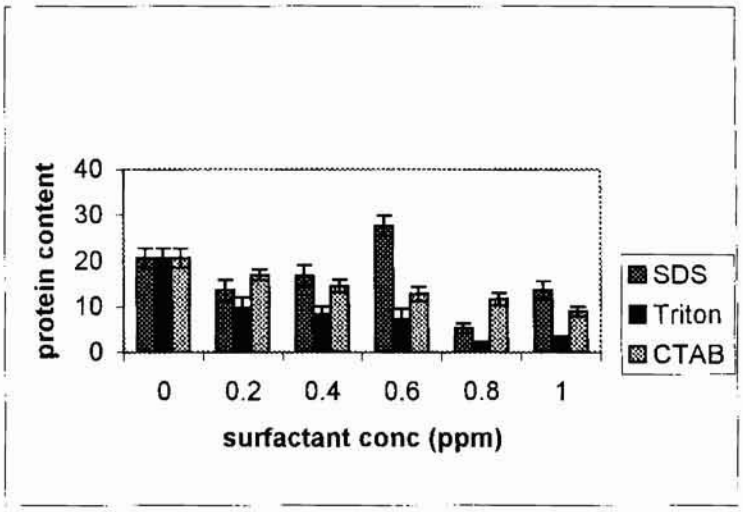


Figure 7.3. Protein content in *S. salina*

In the cationic surfactant dosed cells there was a significant concentration dependent decrease in the protein content (Table 7.3a). In SDS dosed cultures there was stimulation in protein content at 0.6 ppm and maximum inhibition was noted at 0.8 ppm, but here the inhibition was not concentration dependent. Triton dosed cultures showed no specific trend with respect to the protein content and cultures exposed to 0.8 ppm had the least protein content.

Table 7.3a. ANOVA for protein

Source of Variation	SS	df	MS	F	P-value	F crit
Between concentrations	388.0377	5	77.60754	3.925969	0.03135	3.325837
Between surfactants	192.1603	2	96.08016	4.860452	0.033525	4.102816
Error	197.6774	10	19.76774			
Total	777.8754	17				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Two way ANOVA reflected significant differences between the surfactants as well as the various concentrations with respect to protein content ($P<0.05$) (Table 7.3a). LSD analysis (Table 7.6) revealed significant differences ($P<0.05$) in protein content in all surfactant treated cultures (except SDS) when compared to control. Protein content in Triton exposed cultures were significantly decreased ($P<0.05$) when compared to SDS and CTAB. It was also observed that only higher surfactant concentrations of 0.8 and 1 ppm caused significant inhibition ($P<0.01$) of protein synthesis.

Carbohydrate: Carbohydrate content in the cultures are given in Table 7.4.

Table 7.4. Carbohydrate content in *S. salina*

Concentration	SDS	TRITON	CTAB
Control (0 ppm)	854.88 ± 29.58	854.88 ± 29.58	854.88 ± 29.58
0.2 ppm	1055.77 ± 45.6	279.12 ± 13.59	1120.65 ± 51.1
0.4 ppm	537.72 ± 30.6	332.97 ± 15.7	1453.63 ± 50.7
0.6 ppm	388.97 ± 38.9	504.72 ± 21.8	883.68 ± 28.8
0.8 ppm	295.79 ± 29.9	551.39 ± 25.7	1024.57 ± 32.3
1 ppm	395.98 ± 40.2	677.83 ± 33.9	992.51 ± 31.6

Values are the mean ± SD of six separate experiments expressed as µg / mg dry weight

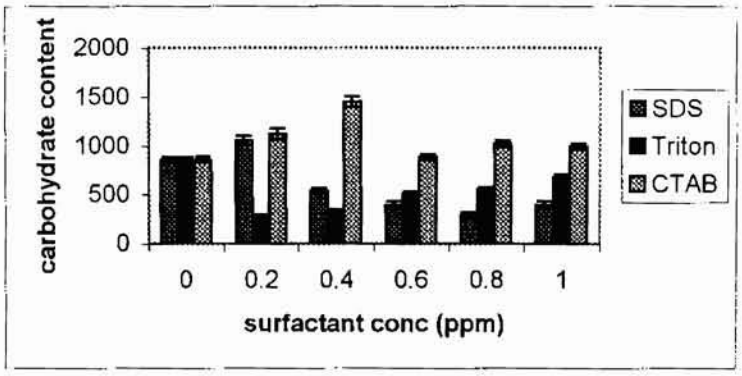


Figure 7.4. Carbohydrate content in *S. salina*

Here there was no concentration dependent decrease. On the contrary a stimulation was noted in Triton treated cultures at concentrations of 0.2, 0.4, 0.6, 0.8 and 1 ppm. In SDS dosed cultures, there was a decrease in 0.4, 0.6, 0.8 and 1 ppm which was not concentration dependent whereas there was stimulation at 0.2 ppm.

Table 7.4a. ANOVA for carbohydrate

Source of Variation	SS	df	MS	F	P-value	F crit
Between concentrations	171561.2	5	34312.24	0.45719	0.79	3.32
Between surfactants	985719.7	2	492859.9	6.56706	0.01	4.10
Error	750503.1	10	75050.31			
Total	1907784	17				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

It was observed from two way ANOVA (Table 7.4a) that there were significant differences between the surfactants with respect to the carbohydrate content ($P<0.01$). Carbohydrate content in CTAB was significantly higher

($P<0.05$) than that in control, SDS and TX-100. But there were no significant changes between various concentrations of the surfactants with respect to the carbohydrate content.

Lipid: Lipid content in control and surfactant treated cultures are given in Table 7.5 and Figure 7.5.

Table 7.5. Lipid content in *S. salina*

Concentration	SDS	TRITON	CTAB
Control (0 ppm)	542.22 ± 31.2	542.22 ± 31.2	542.22 ± 31.2
0.2 ppm	589.98 ± 35.6	85.29 ± 31.1	275.66 ± 35.1
0.4 ppm	741.89 ± 40.1	83.77 ± 29.9	481.11 ± 25.6
0.6 ppm	456.26± 21.6	1486.38 ± 68.2	272.09 ± 11.8
0.8 ppm	254.99 ± 13.7	1511.05 ± 52.5	351.9 ± 13.5
1 ppm	193.07 ± 10.7	1501.94 ± 50.7	160.76 ± 10.7

Values are the mean ± SD of six separate experiments expressed as μg / mg dry weight

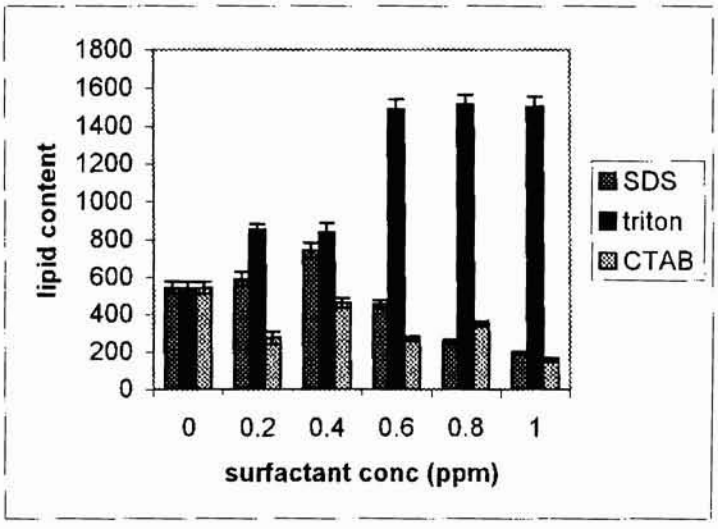


Figure 7.5. Lipid content in *S. salina*

Table 7.5a. ANOVA for lipid

Source of Variation	SS	df	MS	F	P-value	F crit
Between concentrations	91893.8	5	18378.76	0.16	0.970	3.32
Between surfactants	2095724	2	1047862	9.16	0.005	4.10
Error	1143841	10	114384.1			
Total	3331458	17				

SS – sum of squares, df – degrees of freedom, MS – mean of squares.

Two way ANOVA revealed an overall significant change in lipid content in experimental cultures (Table 7.5a). But no significant changes were noted between various concentrations of surfactants. Lipid content was significantly increased ($P<0.01$) in Triton treated cultures when compared to control, SDS and CTAB.

Table 7.6. Results of LSD analysis for growth, chlorophyll, carbohydrate, protein and lipid in *S. salina*

Groups	Growth	Chlorophyll	Carbohydrate	Protein	Lipid
Control vs SDS	NS	P<0.05	P<0.05	P<0.01	NS
Control x Triton	NS	P<0.05	NS	P<0.01	P<0.01
Control x CTAB	P<0.01	P<0.05	NS	P<0.01	NS
SDS x Triton	NS	NS	NS	P<0.05	P<0.01
Triton x CTAB	P<0.05	NS	P<0.01	P<0.05	P<0.01
SDS x CTAB	P<0.05	NS	P<0.01	NS	NS

NS – Not significant

7.4 Discussion

It may be deduced that among the three surfactants CTAB inhibited the growth of the cyanobacterium in a significant and concentration dependent

manner. But there was no significant inhibition of growth in anionic and non ionic surfactant treated cultures.

Photosynthesis in cyanobacteria is principally carried out by the major pigment chlorophyll *a*. The integrity of chlorophyll-lipid-protein complexes in the thylakoid membranes of the autotrophic species is inevitable for effective conversion of light energy to carbohydrate. In the present study, chlorophyll *a* levels were significantly decreased in a concentration-dependent manner in all concentrations of surfactant-treated groups with maximum of 46.54% inhibition in 1 ppm of CTAB. Whereas in Triton X-100, a stimulation of chlorophyll a levels was observed in cultures containing 0.2 ppm surfactant. Here the decrease in chlorophyll a was not concentration -dependent. Maximum decrease was noted in 0.8 ppm. In the anionic SDS the maximum inhibition was noted at 1 ppm though the decrease in chlorophyll content was not concentration-dependent.

This inhibition of photosynthesis might be due to the internalisation of the surfactants which can then impact the photosynthetic processes by making the chlorophyll-protein matrix more water soluble (Hicks and Neuhold, 1966; de Alda *et al.*, 1995). The penetration of the lamellae of the thylakoids by the surfactants would result in their orientation with the similarly charged polar chlorophylls and phospholipids. Transport of light energy may then be blocked as a result of their incorporation in the chlorophyll molecules, thus causing photooxidation.

Protein content was significantly decreased at higher concentrations of all surfactant treated cultures. In CTAB dosed cultures, there was concentration-dependent decrease in protein content with maximum inhibition at 1 ppm. In Triton dosed cultures, the maximum inhibition was noted at 0.8 ppm and 1 ppm. In the anionic and non ionic dosed cultures, the decrease in the protein content was not concentration-dependent. It is thought that this decrease in protein content

might be due to the membrane damage caused by the surfactants. Similar decrease in protein content was observed by Chawla *et al.* (1988) in Nostoc filaments grown in LAS in the range of 1-50 ppm.

There was an increase in the carbohydrate content in all concentrations of CTAB dosed cultures with maximum increase at 0.4 ppm. In Triton dosed cultures there was a concentration dependent increase in the carbohydrate content. But in SDS treated cultures there was a stimulation of the carbohydrate content at 0.2 ppm but decrease in all other concentrations. The increase in carbohydrate might be the response to meet the stress imposed due to surfactant exposure.

Lipid content was significantly decreased in SDS dosed cultures at all concentrations except 0.4 ppm where an increase was observed. In Triton dosed cultures, there was a reduction in the lipid content at 0.2 and 0.4 ppm whereas a highly significant increase was noted at higher concentrations of 0.6, 0.8 and 1 ppm. In CTAB exposed cultures there was a decrease in the lipid content at all concentrations, the maximum decrease was noted at 1 ppm. However this decrease was not linear with increasing concentrations of the surfactant. A change in the sterols of the red algae *Porphyridium purpureum* on exposure to detergents was observed by Nyberg and Saranpaa (1989). They have reported significant decrease in 22-dehydro cholesterol in the algal cultures exposed to the anionic LAS. And exposure to non ionic Triton X-100 at concentrations of 10 ppm resulted in an increase in cycloartenol. Here too the results were not linear with increasing detergent concentrations. This non linear pattern obtained for various biochemical parameters studied seems to be a feature often encountered in algae exposed to surfactants.

Extensive studies of toxicity tests on non ionics like polyoxy alkylene block co polymers was done by Smulikowska (1984) on the alga *Scenedesmus*

quadricauda and bacteria *Sphaerotilus natans*. A wide concentration range of 0.1 to 10000 ppm was studied. The results were difficult to interpret as it was not linear with respect to the concentrations. Thus very often at higher concentrations, lesser inhibition rates were obtained or sometimes stimulation.

The increased toxicity of cationic surfactants to algae has been attributed to the interaction of these chemicals with negatively charged moieties on the cell surface. In addition, lipid concentration in algal cell wall has a significant role in toxicity as more hydrophobic surfactants are readily solubilised in algal lipids. A positive correlation between surfactant hydrophobicity and increased algal toxicity has been demonstrated (Ukeles, 1965).

The cyanobacteria being Gram negative has an outer lipopolysaccharide (LPS) layer. The lipid constituents of LPS might have facilitated the entry of the hydrophobic surfactant viz. CTAB. Thus it may be inferred that the cationic surfactant was the most inhibitory to the cyanobacterium under study.

LITERATURE REVIEW FOR SECTION 2

TYPE OF SURFACTANT	ORGANISM STUDIED	EFFECTS OBSERVED	REFERENCE
Linear alkyl benzene sulfonate (LABS)	Freshwater and marine algae	LC 50 was 0.9-300 ppm for fresh water and that for marine- 0.025-10 ppm	Holt et.al,1992
Alkyl sulfates	Freshwater and marine algae	LC 50 was 4-30 ppm for freshwater and that for marine 1-2 ppm	Holt et.al,1992
Linear alkyl ethoxylates	bacteria	Adaptive response on prolonged exposure	Ventullo et.al, 1989
Anionics (ABS, AES, AOS)	*Selanastrum, Microcystis, Nitzschia*	Reduction of specific growth rate, Reduction in growth not always linear with the surfactant concentrations tested	Atsuko et.al, 1984
Anionic surfactant	*Gymnodinium breve*	Decrease in growth rate	Kutt & Martin, 1974
Cetyl pyridinium bromide (CPB)	Freshwater algae	inhibition of growth	Matulova , 1964
LABS, Triton X-100, Betaine	*Porphyridium*	Decrease in 22-dehydro cholesterol	Nyberg & Saraanpa, 1989
LABS	*Rhodomonas salina, Skeletonema costatum*	Decreased rate of photosynthesis	Petersons & Kokusk, 2000
Corexit	*Isochrysis galbana*	Increases bioavailability of naphthalene from water accommodated fraction of crude oil	Wolfe et al, 1998
Triton X-100	*Synechococcus* WH 7805, WH 8103	Growth activated at lower concentrations and inhibited at high concentrations	Waterbury & Ostroumov, 1994
Alcohol ethoxylates	Algae, microbes, protozoa, invertebrates	Inhibition of growth	Belanger et.al, 2000
LAS	*Nostoc*	Decrease in protein content	Chawla et.al, 1988
Poly oxy ethylene co block polymers	*Scenedesmus*	stimulation or inhibition of growth independent of the concentrations.	Smulikowska 1984

CHAPTER

8

Summary

Surfactants are gaining relevance as major aquatic pollutants as they are now the most abundant group of anthropogenic pollutants. The present study investigated the biochemical impacts of three surfactants viz. anionic sodium dodecyl sulfate (SDS), cationic cetyl trimethyl ammonium bromide (CTAB) and non ionic Triton X-100 on a freshwater adapted euryhaline teleost *Oreochromis mossambicus* and the marine cyanobacterium *Synechocystis salina* Wislouch.

8.1 Studies on *Oreochromis mossambicus*

Exposure to surfactants was found to be highly stressful to the animals. Studies on lysosomal and erythrocyte membranes revealed that interaction of surfactant with the cellular membranes depends on the structure of the surfactant, critical micellar concentration and hydrophile-lipophile balance. It was observed that lysosomal stability index (LSI), decreased significantly on surfactant exposure both *in vitro* and *in vivo*. The control animals exhibited an LSI of 1.965. Triton exposure caused maximum release of acid phosphatase from lysosomes as reflected in an LSI of 0.23. Cationic surfactant CTAB was next to Triton in labilising effects with an LSI of 0.591. SDS, the anionic surfactant, caused minimum labilisation as evident from an LSI of 1.23. *In vitro* studies on the release of ACP at definite time intervals from the lysosomes also recorded significant increases in the release of the enzyme from surfactant-incubated lysosomes than from control whereas in *in vivo* studies only CTAB dosed animals exhibited significant increase in ACP compared to the control.

Studies on erythrocytes *in vitro* also confirmed the membrane damaging effects of the surfactants. Triton induced maximum hemolysis of 61.59%. CTAB caused 24.99 % and SDS induced 14.99% hemolysis.

Studies on peroxidation potential of surfactants revealed that severe oxidative stress was experienced by fish exposed to these compounds. The anti-oxidant enzymes like catalase, superoxide dismutase and glutathione reductase were significantly increased particularly in hepatic tissues of all the surfactant-treated groups. The levels of reduced glutathione significantly increased in SDS-treated animals whereas those exposed to Triton had comparitively less amounts of this non-enzymatic anti oxidant. The levels of conjugated dienes and malondialdehyde were significantly increased in hepatic tissues of Triton and CTAB dosed groups.

Thus interaction of surfactants with the biological membranes has severe consequences as obtained from studies on lysosomes, erythrocytes and peroxidation studies. The lysosomal and erythrocyte studies revealed that the non ionic surfactant, Triton, had maximum membrane damaging potential. Here the long unbranched, non ionic and hydrophobic structure of the surfactant together with its low critical micellar concentration (CMC) is responsible for the maximum labilising action. CTAB, the cationic one, has the second lowest CMC but is branched and ionic when compared to Triton. Hence its interaction with the membrane is limited due to the steric factors and the positive charge, which limits interaction with only the negative charges. SDS, the anionic surfactant, is a short chain alkyl sulfate and has the highest CMC compared to Triton and CTAB. Moreover its interaction is limited to cationic sites on the membranes. Thus it causes least damage compared to the other two surfactants.

Thus surfactant interaction with the cellular membranes depends on structure of the surfactant, charge, critical micellar concentration and hydrophile-lipophile balance. Results from membrane stability studies on lysosomes and erythrocytes *in vitro* indicate that the order of toxicity is non ionic > cationic > anionic for membrane labilising effects. But on examining the results of lipid peroxidation studies it is found that cationic surfactant imparted the maximum oxidative stress. Here it must be taken into account that *in vivo* metabolism of the surfactant also plays a key role in peroxidation potential. Cationic surfactant is not at all metabolised by fish and hence is the most toxic. Triton is mainly excreted as glutathione conjugates. This depletes glutathione levels which makes the animals exposed to Triton more prone to peroxidation. SDS, the anionic surfactant, is largely metabolised in hepatic tissues by β and ω oxidation and thus causes decreased peroxidation. This is reflected in very low malondialdehyde levels comparable to controls in SDS-treated fish. Thus it is evident that surfactant metabolism is a major determinant of the extent of toxicity.

Influence of surfactants on the branchial membrane-bound enzymes like Na^+-K^+ ATPase and Mg^{2+} ATPase revealed significant differences in the susceptibility of these enzymes to the surfactants. Na^+-K^+ ATPase was insignificantly activated by the anionic SDS whereas Triton and CTAB caused insignificant inhibitions. Mg^{2+} ATPase was significantly inhibited by all the three surfactants in a similar manner. It is thought that the surfactants interfered with the ATPase activities by affecting the membrane integrity. It is evident that membrane lipids play a critical role in the activity of membrane-bound enzymes. The ability of surfactants to cause peroxidation can result in lipid damage and subsequent inactivation of these enzymes.

Metabolic activities of the animals subjected to surfactants exhibited significant changes when compared to the control group. A typical stress-adaptation pattern was reflected in all the key biochemical processes. Hepatic enzymes like alanine transaminase and aspartate transaminase were significantly increased on surfactant exposure. Alkaline phosphatase also showed significant increase compared to control. Reserve energy stores viz. glycogen, lipid and protein were significantly mobilised in surfactant exposed groups. This clearly points out the fact that surfactant exposure is stressful. Stress calls for an increased ATP demand which is evident from reserve food mobilisation. Also transaminases play an important role in stress because they can convert amino acids to keto acids which can be chanelled into Krebs' citric acid cycle.

Thus it may be concluded that surfactants even at sub lethal concentrations are stressful.

8.1.1 Conclusion

The action of surfactants on the cell membranes largely depends on their structure, critical micellar concentration, charge and hydrophile-lipophile balance. In addition ability for oxidative damage via peroxidation also is a critical factor affecting membrane interaction. The peroxidation potential in turn is largely dependent on surfactant metabolism. On the other hand it may be understood that

inhibition of branchial enzymes and readjustments of metabolic machinery to defend the stressful situation is largely controlled by the piscine stress hormones.

Thus the cationic surfactant CTAB is hydrophobic with a low critical micellar concentration and is not at all metabolised and thus causes maximum stress to the organism. Triton, the non ionic surfactant, is the second toxic as damage via oxidative stress is aggravated by excretion of this compound as glutathione conjugates and this in turn depletes glutathione. The low critical micellar concentration of Triton and its hydrophobicity add to its toxicity. SDS, the anionic surfactant, is more hydrophilic, has higher critical micellar concentration and is metabolised by β and ω oxidation which makes it less toxic compared to the cationic and the non ionic surfactants.

8.2 Studies on *Synechocystis salina* Wislouch

Exposure to surfactants was found to be stressful to the cyanobacterium though the responses were not strictly concentration-dependent. Studies on growth inhibition revealed that the cationic surfactant was the most inhibitory where a concentration-dependent decrease in growth was observed. In case of both the anionic and non ionic surfactants the growth inhibition was insignificant. The chlorophyll content was found to decrease significantly especially at higher concentrations of all the surfactant-treated cultures. The maximum decrease in chlorophyll content was noted at higher concentrations tested viz. 0.8 ppm and 1 ppm. Here too the cationic surfactant was found to cause maximum decrease in chlorophyll content at higher concentrations. Studies on protein estimation revealed that there was significant decrease in the protein content of all the cultures exposed to the surfactants. The decrease in protein content was found to be dose-dependent and was significant at higher concentrations of all the

surfactants tested. The carbohydrate content showed fluctuating trend irrespective of the concentration in surfactant-treated cultures. Cultures exposed to CTAB showed a significant increase in carbohydrate. Lipid content of the cultures also showed irregular changes which were independent of the dose of the surfactant. SDS and CTAB treated cultures showed an overall decrease in lipid content whereas Triton treatment resulted in higher lipid levels at higher concentrations.

8.2.1 Conclusion

It may be deduced that among the surfactants CTAB inhibited the growth of the cyanobacterium in a significant manner. The chlorophyll content was found to be decreased only at higher surfactant concentrations which could be due to the fact that solubilisation of chlorophyll-lipid-protein complexes required higher surfactant concentration. The protein content was decreased significantly in surfactant treated groups which implied membrane damage in the protein synthesizing machinery. The carbohydrate content showed wide fluctuations in surfactant- treated cultures. In CTAB treated cultures, there was an increase in carbohydrate at all concentrations tested. The increase in carbohydrate might be an adaptive mechanism to meet the stress imposed by the surfactant. Lipid content decreased in SDS and CTAB dosed cultures whereas Triton treated ones showed a significant increase. The increase in Triton dosed cultures suggest that probably the surfactant is used as a carbon source by the cyanobacterium and utilized for lipid synthesis.

Thus studies on this cyanobacterium reveal that biochemical processes like protein and chlorophyll synthesis are more or less affected in a dose dependent manner i.e., at higher concentrations there is maximum inhibition. Whereas carbohydrate and lipid content showed fluctuations irrespective of the

concentrations. Regarding the relative toxicity, it may be concluded that the cationic surfactant is the most damaging. This could be due to selective solubilisation and increased penetration of the hydrophobic moiety in the lipo polysaccharide cell wall of this gram-negative species.

Thus the major findings are

- Surfactants at sub lethal concentrations are stressful.
- Order of toxicity for *O. mossambicus* is Cationic (CTAB) > non ionic (Triton) > anionic (SDS), and for *S. salina* cationic is the most toxic.
- Main target of surfactant action is the cellular membranes. Damage to membranes is largely caused by peroxidation of lipids thus inducing oxidative stress.
- Stress-oriented metabolic readjustments are made [illegible] the hepatic enzyme profile and other hepatic biochemical parameters.

8.3 Future Perspectives in Research

- Oxidative stress potential of cationic and non ionic surfactants is clearly established from the present study.
- Oxidative stress can lead to carcinogenesis and mutagenesis.
- A study on carcinogenic and mutagenic effects of surfactants is relevant in this regard.
- And piscine cell lines can then substitute mammalian cell lines in *in vitro* studies on carcinogenicity.

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