AQUATIC SAFETY ASSESSMENT FOR CATIONIC AND AMPHOTERIC SURFACTANTS

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Abstract

Many toxic xenobiotics entering aquatic environments exert their effects through redox cycle. Oxidative stress, incorporating both antioxidant defenses as well as oxidative damage, is a common effect in organisms exposed to xenobiotics in their environment. The present study proposed to evaluate the acute effects of benzenthonium chloride (quaternary ammonium compound with cationic proprieties) and cocamidopropyl betaine *(*zwitterionic compound with amphoteric proper) on antioxidant defense mechanisms and lipid peroxidation in *Cyprinus carpio* organs (liver, intestine, kidneys and gills). We assessed the level of oxidative stress biomarkers (superoxide dismutase

– SOD, catalase - CAT, glutathione peroxidase - GPx, glutathione reductase -GR, glutathione S transferase - GST, glucose -6- phosphate dehidrogenase – G6PDH) and the levels of lipid peroxidation - LPO through malondialdehide -MDA of 1mg/l toxic substance nominal concentration after 96h exposure period. LPO and antioxidative enzymatic mechanisms displayed different responses in the investigated tissues. We observed that the action of toxic substances is selective at organs levels. The liver and gills were the most affected by toxic action of both tested surfactants compared with the controls. At liver, gills and kidneys level, after 96h toxic exposure, significant modifications were observed in case of CAT, G6PDH, GPx, GST and Gred. In all organs LPO installation was observed which indicate oxidative damage of tissues induced by accumulation of reactive oxygen species (ROS). The biochemical analysis led to the conclusion that antioxidant enzymes showed deficit in ROS balance at all tissues levels which induce oxidative stress in fish organs.

Keywords: benzenthonium chloride, cocamidopropyl betaine, oxidative stress, antioxidant enzymes, toxicity

AIMS

Nowadays the surfactants are the most used compounds with different applications for cleaning in industry and household. Due to their favorable physical chemical properties, the surfactants are the essential ingredients in the most household laundry products and industrial cleaners as well as in personal care and cosmetic products. After utilization, they are mainly discharged into the environment compartments by the wastewater pathway. Based on hydrophobic and hydrophilic tails of these synthetic chemicals, the main classes of surfactants are anionic, nonionic, cationic and amphoteric. Because these compounds are produced in big amounts it is not surprising that they may have toxic effect on aquatic organisms.

Surfactants can damage the lipid component of cell membranes through decrease of surface tension of the ambient water witch leads to increased hydration an enlargement of the cell volume. Higher concentration can cause a suppression of metabolic processes in the cells. These effects are observed specially at gill respiratory epithelium. Also can damage the protective layer of mucus on the skin that led to decrease of the resistances of the fish to infection **(Zdenaka S., 1993).**

According to the specialty literature studies, the antioxidant enzymes are affected by different pollutions which can affect the enzymatic activity at the level of different organs by their emphases or inhibition, comparatively to control group **(J. H. Jee, 2005).** Aerobic organisms have developed an antioxidant defense system against the dangers of oxygen radicals resulting in pollutants metabolism which can prevent excess oxidation and damage of cells structures. A disturbance in the balance between the prooxidants (oxygen radicals) and antioxidants leading to a physiological process named oxidative stress. Indicators of oxidative stress include changes in antioxidant enzyme activity, damage DNA bases, protein oxidation products and lipid peroxidation products**.** Antioxidant enzymes facilitate the removal of reactive chemical intermediates resulting reactive oxygen species **(Bethanie C.A, 2008).**

The paper will confer special attention to evaluate the effect on metabolic systems of aquatic organisms of cationic surfactants (frequently use in laundry and dish detergents, balm and also in biocide products), as well as to amphoteric surfactants (used in hair shampoo and balm, liquid soap and cleaning lotion). Scientific literature is relatively poor concerning these informations. Also the cationic and amphoteric surfactants control was not legislated until the implementation of European Regulation 648 (**2004**), due to the absence of a standard method – EN European or ISO international norm or European directive method associate for chemical quantitative assessment for this type of active substances;

Depending on the charge of surfactant, antioxidant and metabolic profile *in vivo*, they can be arranged in order of decreasing toxicity: cationic> neionic> anionic>amfoteric **(Bindu P.C. et al., 2001).**

Quaternary ammonium compounds, which include cationic surfactants, determinate disturbance of the balances of bacterial lipid membranes that ultimately lead to cell death (**Erlend Bore et al., 2007).**

Amphoteric surfactants can induce a concentration-dependent increase in expression of enzymes producing cholesterol and ceramides, while transcription enzyme producing fatty acid is affected **(Tianling Wei et al., 2006).**

Effect of surfactants on antioxidant defense mechanisms of aquatic organisms is not really understood either.

The present study proposed to evaluate the acute effects of benzenthonium chloride (quaternary ammonium compound with cationic proprieties) and cocamidopropyl betaine *(*zwitterionic compound with amphoteric proprieties) on antioxidant defense mechanisms and lipid peroxidation in *Cyprinus carpio* organs (liver, intestine, kidneys and gills). The main studied antioxidant biomarkers were superoxide dismutase – SOD, catalase - CAT, glutathione peroxidase - GPx, glutathione reductase -GR, glutathione S transferase - GST, glucose -6- phosphate dehydrogenase – G6PDH. Also we intended to evaluate the lipid peroxidation (LPO) through malondialdehide –MDA.

EXPERIMENTAL

Chemicals:

Cationic surfactant - CAS: 121-54-0, IUPAC:-*N*-benzyl-*N*, *N*-dimethyl-2-{2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethoxy}ethanaminium chloride (benzenthonium chloride monohydrate), $C_{27}H_{42}ClNO_2$,commercial name HYAMINA 1622**,** active substance >96%, Fluka (53752);

Amphoteric surfactant - CAS: 4292-10-8, IUPAC: 2-[3-(dodecanoylamino)propyl-imethylazaniumyl] acetate (laurilamidopropyl betaine / cocamidopropil betaine - CAPB*),* $C_{19}H_{38}N_2O_3$, commercial name AMFODAC LB, active substance 34,6%;

Fish maintenance and treatments

The aquatic organisms utilized in acute toxicity biotests were represented by one-year-old juvenile carp**,** *Cyprinus carpio sp***.,** with health and origin certificate, taken from selected lots of the S.C. PISCICOLA IEZER S.A., from experimental tanks populated with juvenile fish from healthy animals. After

acquisition, the fishes with similar length, weight and age, these were acclimatised in laboratory conditions, in maintenance aquaria within INCD-ECOIND Bucharest.

We selected to perform an acute toxicity test with a fixed doze (1mg/l for each surfactant) because no mortality was observed in previous acute toxicity test (classical LC_{50} test) but the biochemical investigated parameters, such as oxidative stress biomarker and antioxidant defence system, were affected.

A number of 80 fish (length 15 ± 2cm, weight 56 ± 5 g) uniformly distributed in 2 aquaria (100 L), were acutely intoxicated with a theoretical concentration about 1mg/l from both benzenthonium chloride and cocamidopropil betaine for 96h in a semi static test according with OECD C01 procedure. In the same time we performed a control test without toxic with 40 fish to a comparative analysis. No feeding in the test period.

The analytical concentrations of surfactants in test solutions were: 0.54 – 0.67mg/l for benzenthonium chloride (DIN 38409/20-1989) and 0.55-0.69 mg/l for cocamidopropil betaine **(Borteux method, 1984).**

The mean values of water quality parameters in the experimental tanks were: pH 6.96 -7.86 (SR ISO 10523:2009); temperature 20-21^{o}C, dissolved oxygen 3-4mgO_2/l (SR EN 25814-1999); suspended matters 5.2 – 12.4 mg/l (SR EN 872-2005) and chemical oxygen demand 14.4 – 259.2 mg/l (SR ISO 6060:1996).

Sampling and preparation of protein extract

The mortality at 96h was 0-10% for each tested surfactant and also for control test. At 24h, 48h and 96h 5 fish were sacrificed for each tested substance for sampling of the interest organs: liver, intestine, kidneys and gill for biochemical analysis (the samples were immediately preserved by freezing at -80^{o}C).

From each organ sample replicate and control were weighed 0,3 g of tissue in 1,5ml eppi tubes. The fish tissues were mechanically homogenized using metal balls or knifes and taken in a 1ml TRIS-HCl- EDTA buffer pH 7.4, vortexing and mixing in a homogenizer RETCH MA 301 for 2 min. and frequency 16/S. Samples were transferred in anther tubes with 2 ml TRIS-HCl-EDTA buffer pH 7.4, incubation 60 min. at 4^{o}C, centrifugation for 30 min., 8000-10000 rpm at 4^{o}C and clear supernatant takeover.

Biochemical analyses

The level of LPO (lipid peroxidation) was measured via the thiobarbituric acid (TBA) color reaction for malondialdehyde (MDA) and MDA- TBA complexes existing in samples were measured by fluorimetry according to the method of **Draper and Hadley (1990)**. The reaction volume was obtained of 200µl sample, 700 µl HCl 0.1 N, incubation 20 min. at room temperature, 900µl TBA 0.025 M, , incubation 65 min. at 37^{o}C and 400µl lyses buffer (Tris / HCl 0.1M – EDTA 5mM pH 7.4). The fluorescence of final volume reaction was measured at λ excitation = 520nm and λ emission= 549nm. An MDA solution 1mM was used as a standard. The results were calculated as nmole of MDA per mg protein and expressed as percentage from controls.

Antioxidant enzyme activity assay

Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by a spectrometric method described by **Paolletti and Mocali (1990)**. The method is based on the oxidation of NADH in the presence of superoxide anion generated by EDTA, $MnCl_2$ and β-mercaptoethanol. In the presence of SOD enzyme the NADH oxidation is inhibited. At high concentration of SOD in the sample, the 340nm absorbance remains unchanged while for the control (sample without SOD) the absorbance decrease with the decrease of NADH concentration. The reaction medium contained 20µl sample, 160µl TDB (triethanolamine buffer 100mM - diethanolamine 100mM, pH 7.4), 5µl EDTA 100mM / 50mM $MnCl_2$ pH 7, 20 µl lyses buffer and 8µl NADH 7.5 mM. The decrease in absorbance at 340nm took for 21 min. (30 cycles with 42sec. each) to allow NADPH oxidation at TECAN multireder. One unit (1U) of SOD activity was defined as the amount of enzyme which inhibited the oxidation of NADPH in the control by 50%.

Catalase (CAT) (EC 1.11.1.6) activity was measured by monitoring the disappearance of H_2O_2 at 240nm, according to method of **Aebi (1984).** The reaction medium contained 50 µl sample, 350µl H_2O_2 0,059M and 600µl potassium phosphate buffer pH 7.1. At 1 min after hydrogen peroxide addition the absorbance at 240nm was measured. One unit of CAT activity decomposes 1 µmol of H_2O_2 per min. at 25^{o}C.

Total glutathione peroxidase (GPX) (EC 1.11.1.9) activity was assayed by using the method described by **Beutler (1984)** based on indirect monitoring of GSSG (oxidized glutathione) which is continuously reduced by glutathione reductase (GR) generating a constant level of reduced glutathione (GSH). The reaction mixture contained 10 µl sample, 100µl lyses buffer, 798µl distillated water, 20 µl GSH 0.1M, 60 µl NADPH 2mM, 1 µl glutathione reductase and 10µl *tert*- butyl-hydroperoxide 7mM for start the reaction. Thereby the conversion of NADPH to $NADP^+$ was followed by recording the changes in adsorption intensity at 340nm, and one unit of GPX activity was expressed as 1 µmol of NAPH consumed per min., using a molar extinction coefficient of $6.22x10^3 M^{-1}cm^{-1}$.

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined according to the method of **Goldberg and Spooner (1983)** based on monitoring of GSSG reduction with participation of NADPH. The reaction mixtures contained 0.1 M potassium phosphate buffer pH 7.8, 2mM NADPH, 33mM GSSG, distillated water and sample. The speed of reaction is determined by decrease of absorbance at 340nm due to NADPH oxidation. One unit of GR activity oxidizes 1 µmol NADPH per min. at 25^{o}C.

Glutathione transferase (GST) (EC 2.5.1.18) activity was assayed using the method of **Habig et al. (1974)** based on measuring of formation rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugated with GSH, spectrophotometrically evidenced at 340nm. The final reaction volume contained 0,1M potassium phosphate buffer pH 7.1, 25mM CDNB, 20mM GSH, distillated water and sample. One unit of GST activity was defined as the formation of 1µmol

conjugated product per min, using a molar extinction coefficient for CDNB of 9.6 $mM^{-1}cm^{-1}$.

Glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) activity was measured using a spectrophotometric method described by **Lohr and Waller (1974)** based on monitoring of rate formation of NADPH evidenced at 340nm. The reaction volume contained 50mM HEPES buffer pH 7.5, 40 mM glocoso-6 phosphate, 30mM NADP and sample. One unit of G6PDH activity was expressed as 1µmol $NADP^+$ reduced per min.

The protein content was determined using method of **Lowry et al. (1951)**, with bovine serum albumin as standard. All the enzymatic activities were reported to protein concentration to be expressed in terms of U/mg protein and as percentage from controls.

All data are expressed as means (n=3) ± standard error and differences were considered significant at $p<0.05$.

Most contaminants from water produce intracellular reactive oxygen species (ROS) **(Prieto et al., 2007, Sun et al., 2007, Monferran et al., 2008)**. As a result of oxidative stress, the fish and other vertebrate, trying to reduce the caused changes by the use of antioxidant defense system including SOD, CAT, GPX, GR enzymes and other no enzymatic antioxidants. These systems prevent the ROS formation which can react with biological macromolecules producing lipids hidroperoxidation and protein carbonilation **(Cazenave et al., 2006, Vieira et al., 2008).**

The G6PDH enzyme is involved in the production of NADPH, molecules that provide equivalent reduction of antioxidant enzymes mentioned above. G6PDH is often measured in research studies focused on antioxidant enzyme because these molecules are essential to their function **(Halliwell and Gutteridge 1999).**

Because of the inhibitory effects of oxiradicals formation, enzyme SOD-CAT system is the first line of defense against oxygen toxicity and therefore it is usually used as a biomarker of ROS production (**Ballesteros et al., 2009).** CAT and GPX cooperative act as scavenger enzymes of hydrogen peroxide and other hydro peroxides **(Gate et al., 1999).** GR enzyme plays an important role in antioxidant protection of cells and adjustment of metabolic pathways processes **(Li et al., 2009b).** Antioxidant defenses are developed mainly in the liver as a result of the central role of this organ in detoxification of xenobiotics and in metabolic degradation products processing **(Bethanie C.A, 2008).**

Environmental effects of different pollutants may be evident in all levels of the ecosystem, biocenosis, population and individual. Even if recently, a lot of data concerning cause – effect relationships of xenobiotics exposure of aquatic organisms were accumulated, these aspects are still little understood. Also the relationship between exposure to toxic and antioxidant response is unclear concerning the most antioxidant parameters **(van der Oost et al. 2003).**

The toxic response of aquatic organisms is dependent of species, nutritional status, annual variations, life cycle and toxic substance type (**Linde et al. 1998, Meyer et al. 2005, Ruas et al. 2007).**

Sub lethal exposure of freshwater fish (Oreochromis mossambicus) at concentrations of 1mg/l anionic, cationic and nonionic surfactants has led to severe oxidative stress in liver, kidney and heart muscle. If in the liver the

catalase activity increase significantly for all tested surfactants, in kidney these activities increase to the action of cationic surfactant and in heart significantly increase of enzymatic activities is observed in case of cationic and nonionic surfactants. The SOD and GPX activities increase in the liver and kidneys **(Bindu et al., 2001).**

The cationic surfactants proved to have antibacterial properties since they generate superoxide ions and hydrogen peroxide which induce oxidative stress **(K. Nakata et al., 2010).**

RESULTS AND DISCUTIONS

From our biochemical analysis we observed variation of specific enzymatic activities of the enzyme involved in oxidative stress. The toxic action of tested substances (benzenthonium chloride and cocamidopropil betaine) is organ selective and also has different impact at enzyme system level on the behavior of fish exemplars.

In case of ***benzenthonium chloride*** intoxication we observed that liver and gills were the most affected organs with very significant variations of enzymatic activity compared with controls. SOD enzyme activity levels remained within the limits of controls for all of the organ samples. At liver level were observed significant and very significant changes of enzymatic activities after 96h of intoxication for CAT, GPX, GST and Gred (**figure no. 1**).

Biochemical analysis of antioxidant enzymes in the gills revealed, after 96h of intoxication, fluctuations of enzyme activity for GPX, GST, Gred and G6PDH (**figure no. 2**) Kidneys show highly significant decrease in specific enzymatic activity of GPX and Gred after 96h, and CAT activity increase after 48h and return in limits of controls after 96h (**figure no. 3**). At intestine level no significant effect were observed (**figure no. 4**).

In all organ samples with cationic surfactant intoxication lipid peroxidation was observed (**figure no. 5 - 8**). In the case of kidneys and gills this effect becomes very obvious after 48h of intoxication and after 96h the effect decrease but not outside of the impacted area. Majority of xenobiotics toxicity studies performed to evidence the oxidative stress in fish were based on research of enzymatic antioxidant system and lipid peroxidation**,** but the research data have failed to establish a general available biomarker **(Bainy et al. 1996)**. Lipid peroxidation is a useful used biochemical indicator of oxidative disturbances, as has been observed for all analyzed sample and is correlated with the decrease activity of CAT and GST enzymes, which cause accumulation of toxic products of peroxidation.

The biochemical analysis showed oxidative stress and lipid peroxidation installation in all tested organs (liver, gills, kidneys and intestines) which led to a significant increase of reactive oxygen species (ROS) that caused lipid and protein degradation.

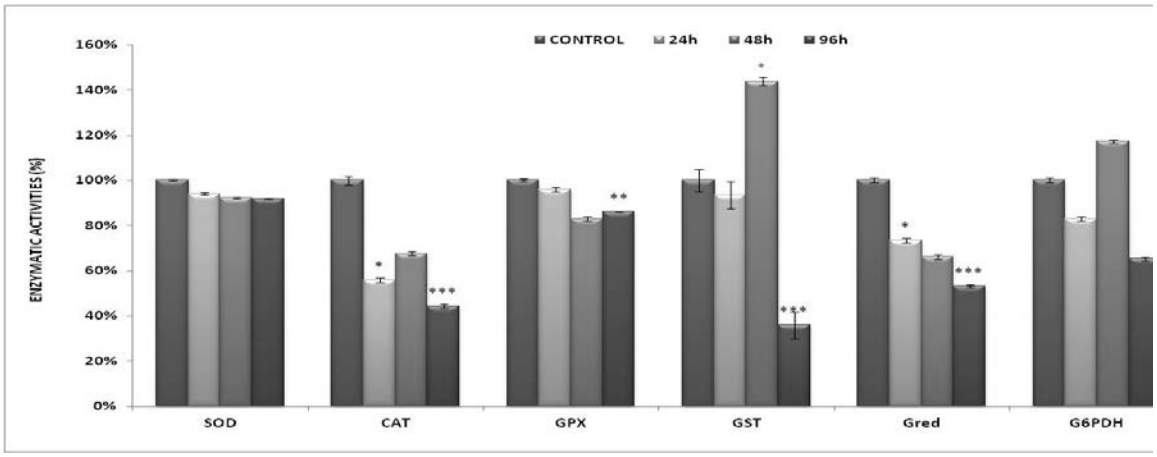

Fig. 1 Effect of benzenthonium chloride (cationic surfactant) on the liver SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01 *** very significantly different from controls at p<0,001)

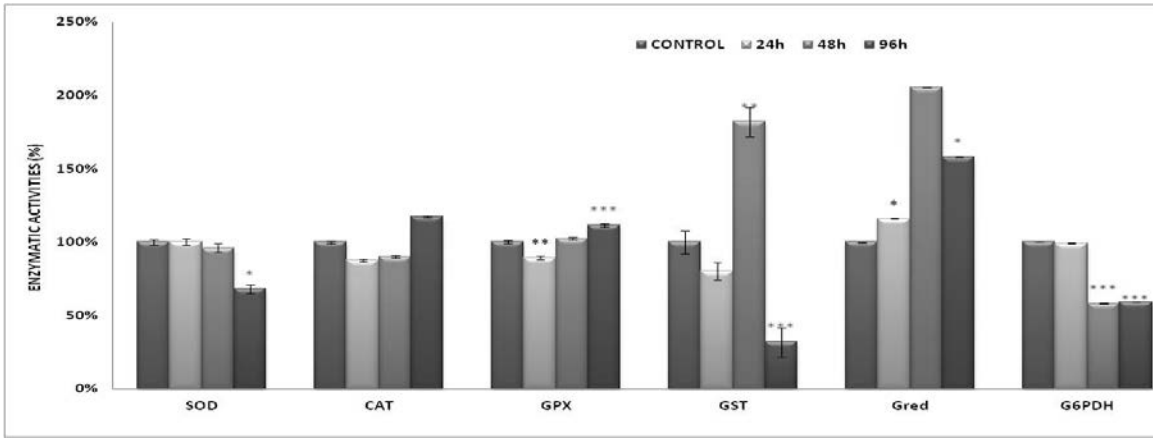

Fig. 2 Effect of benzenthonium chloride (cationic surfactant) on the gill SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01 *** very significantly different from controls at p<0,001)

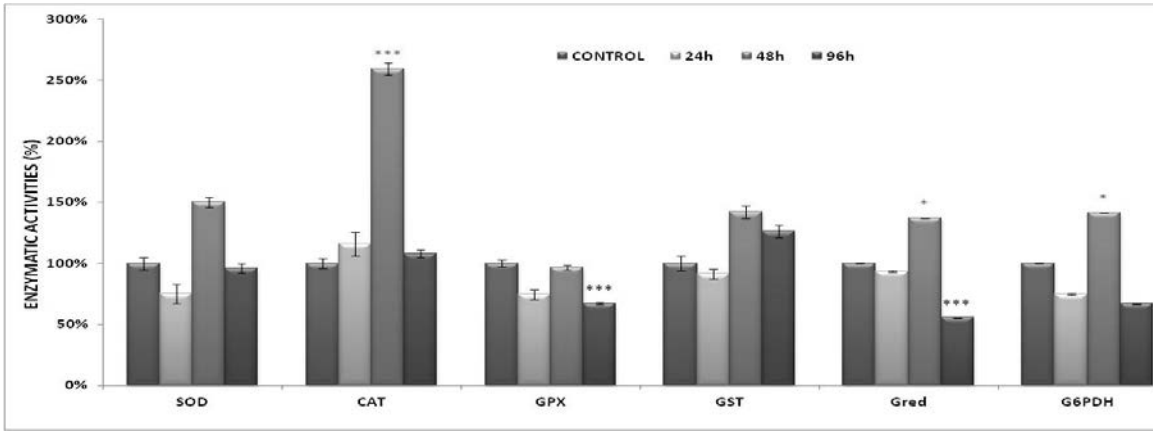

Fig. 3 Effect of benzenthonium chloride (cationic surfactant) on the kidneys SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01 *** very significantly different from controls at p<0,001)

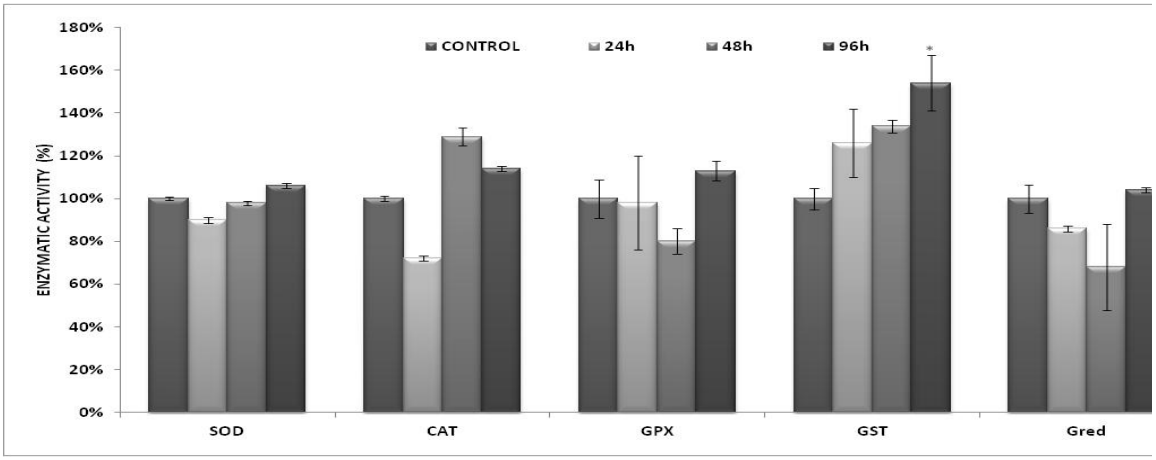

Fig. 4 Effect of benzenthonium chloride (cationic surfactant) on the intestines SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01 *** very significantly different from controls at p<0,001)

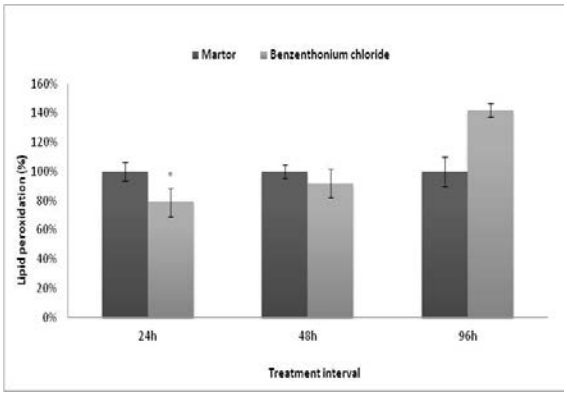

Fig. 5 Effect of benzenthonium chloride (cationic surfactant) on the liver LPO (*significantly different from controls at p<0.05)

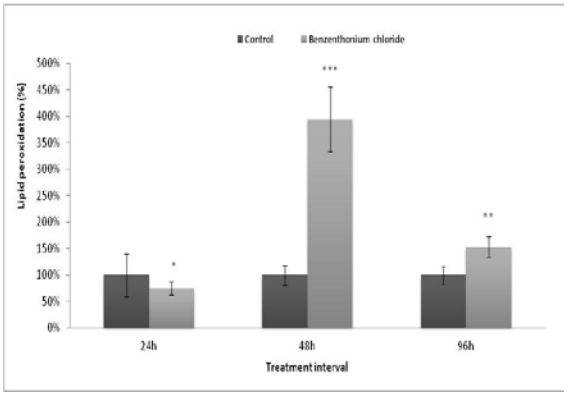

Fig. 6 Effect of benzenthonium chloride (cationic surfactant) on the gill LPO (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01, ***very significantly different from controls at p<0,001)

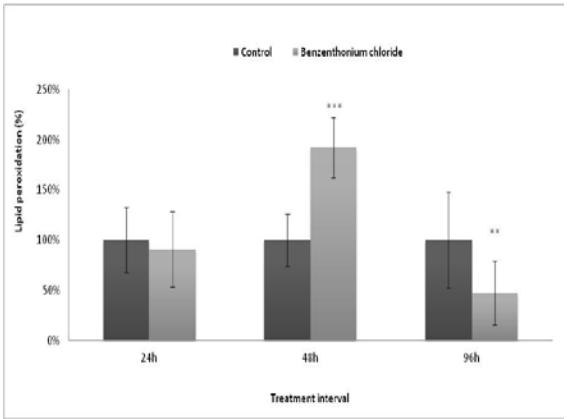

Fig. 7 Effect of benzenthonium chloride (cationic surfactant) on the kidney LPO (**distinct different from controls at p<0.01, ***very significantly different from controls at p<0,001)

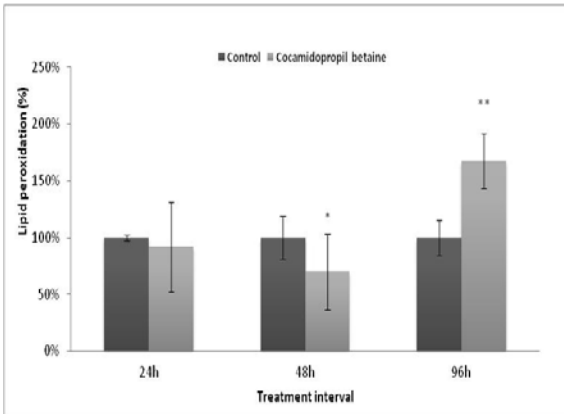

Fig. 8 Effect of benzenthonium chloride (cationic surfactant) on the intestine LPO (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01)

In case of ***cocamidopropil betaine*** intoxication, we observed significant effect on antioxidant enzyme system in all organs. The SOD activity level remained in controls limits in case of intestine and varied for liver, kidneys and gills (accentually increased for liver and distinct significant decreased for gills). Other research studies specified that SOD activity increased in carp erythrocytes when the fish are exposed to 10 mg/l paraquat, the maximum effect occurring at 12h and at 48h -96h decrease below the control limits. This led to the conclusion that enzyme is inhibited by its products - H2O2 and $^{\cdot}$OH **(Ivan S., 2010).** In the liver and gill sample have been observed very significant changes of SOD, CAT, Gred and G6PDH activities involved in organism detoxification (**figure no. 9 and 10**). The protein extract obtained from kidney and intestine showed very significant modifications of CAT, GPX and Gred activities, enzymes which act together for hydrogen peroxide demand and water production (**figure no. 11 and 12**).

From the point of view of lipid peroxidation this biochemical process was observed in all organs samples but less in case of intestine (**figure no. 13 – 16).** Lipid peroxidation increase significant about 2 - 8 fold than controls at all intoxication intervals. The result analysis show that lipid peroxidation affect the organs in the followed order: kidney>liver>gill>intestine.

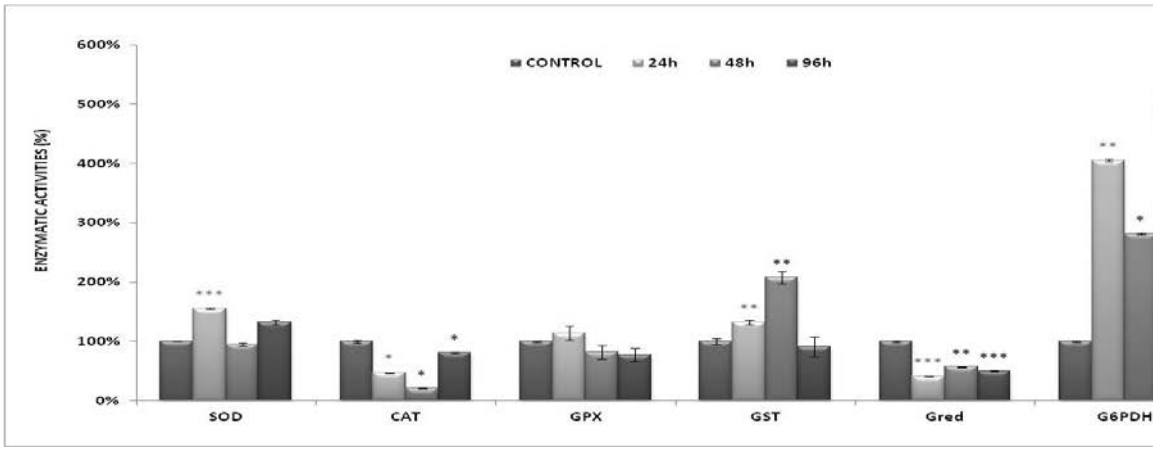

Fig. 9 Effect of cocamidopropil betaine (amphoteric surfactant) on the liver SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01, *** very significantly different from controls at p<0,001)

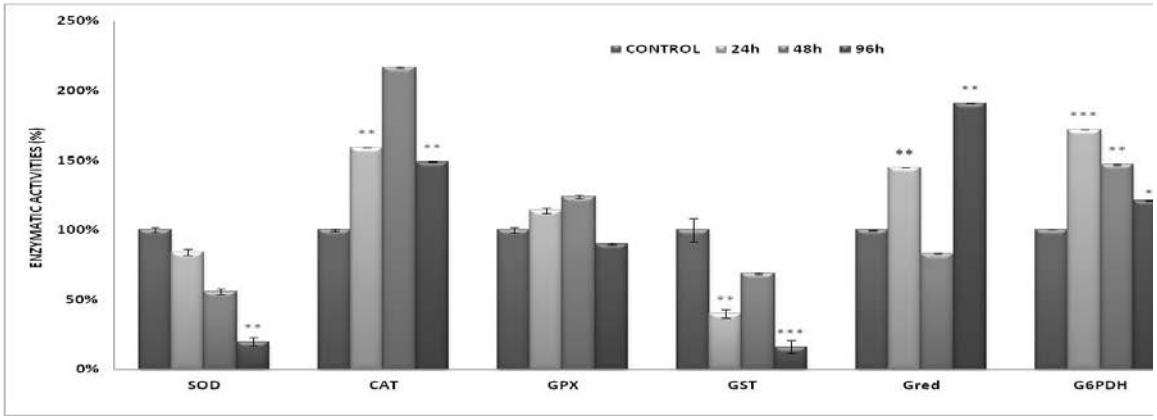

Fig. 10 Effect of cocamidopropil betaine (amphoteric surfactant) on the gills SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01, *** very significantly different from controls at p<0,001)

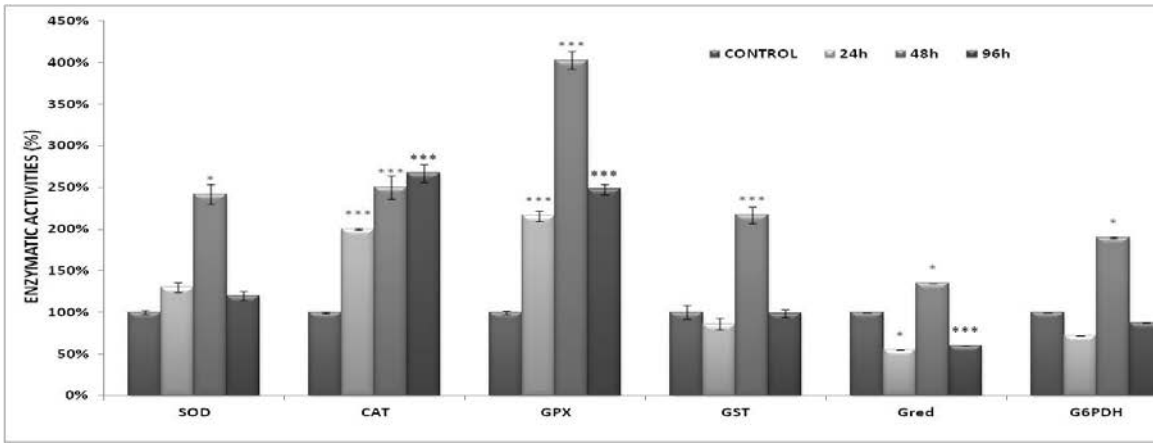

Fig. 11 Effect of cocamidopropil betaine (amphoteric surfactant) on the kidneys SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01, *** very significantly different from controls at p<0,001)

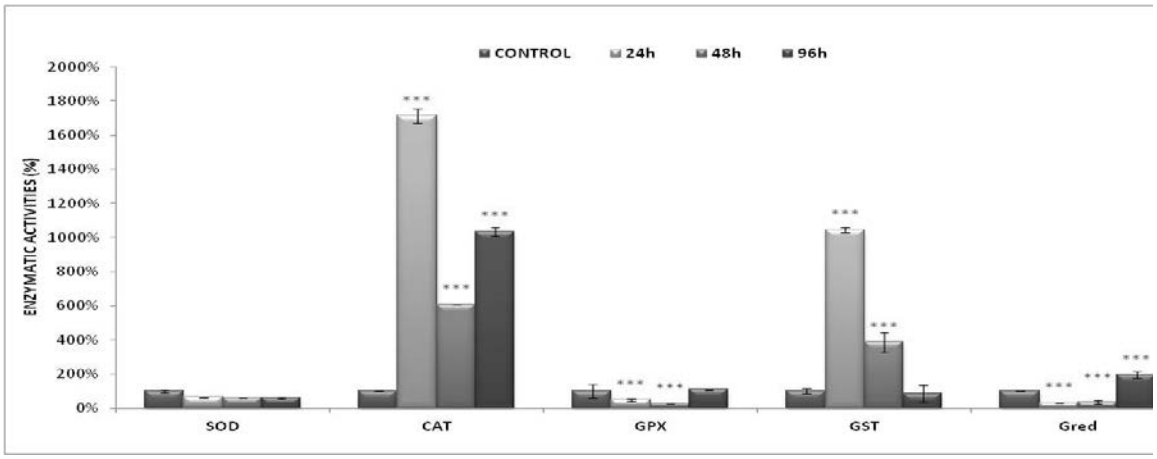

Fig. 12 Effect of cocamidopropil betaine (amphoteric surfactant) on the intestine SOD, CAT, GPX, GST, Gred, G6PDH activities (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01, *** very significantly different from controls at p<0,001)

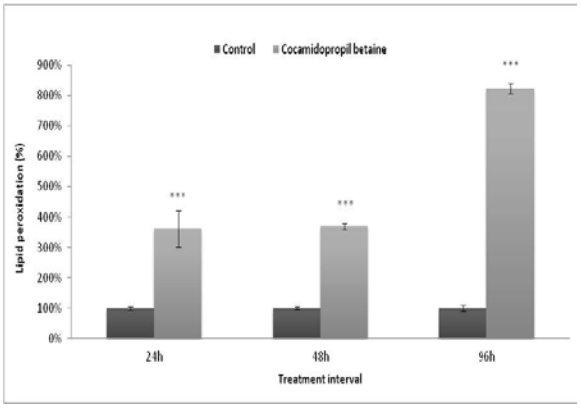

Fig. 13 Effect of cocamidopropil betaine (amphoteric surfactant) on the liver LPO (***very significantly different from controls at p<0,001)

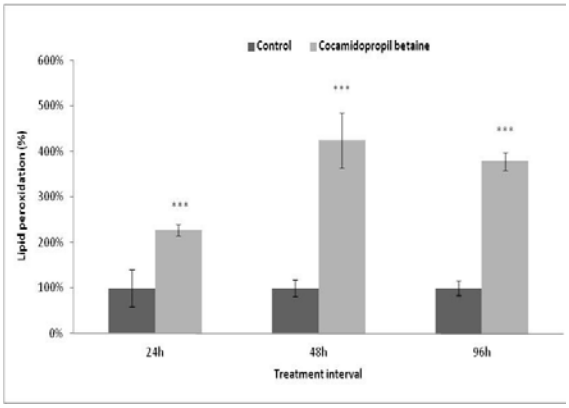

Fig. 14 Effect of cocamidopropil betaine (amphoteric surfactant) on the gill LPO (***very significantly different from controls at p<0,001)

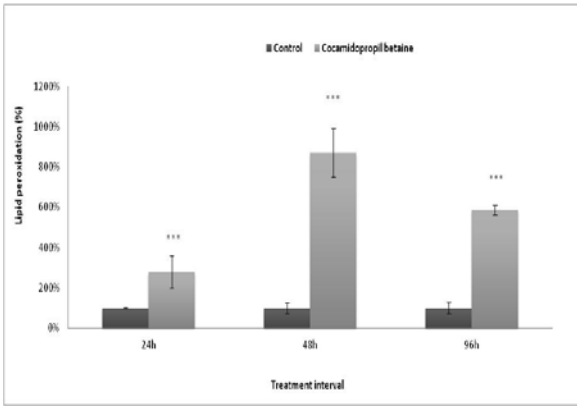

Fig. 15 Effect of cocamidopropil betaine (amphoteric surfactant) on the kidney LPO (***very significantly different from controls at p<0,001)

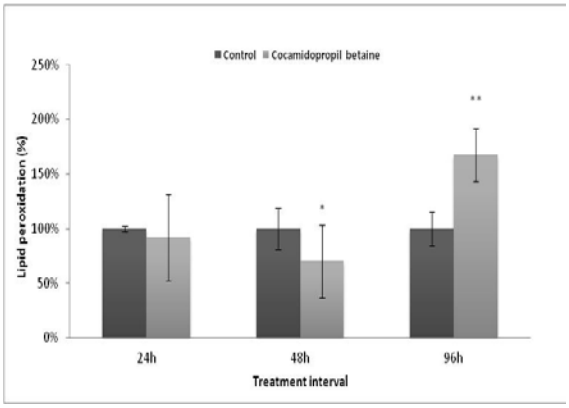

Fig. 16 Effect of cocamidopropil betaine (amphoteric surfactant) on the intestine LPO (*significantly different from controls at p<0.05, **distinct different from controls at p<0.01)

CONCLUSIONS

Our study revealed the effect of two surfactants (cationic and amphoteric) on antioxidant defense system and the establishment of oxidative stress occurred as an impact of toxicity in the liver, kidney, gill and intestine of *Cyprinus carpio sp*. (carp). The difference of enzymatic changes observed at organs level may be correlated with the specific metabolic pathways of these tissues.

Also is known that the liver is the central organ involved in xenobiotics detoxification which was showed in our study through significant changes of antioxidant enzymatic activities.

Biochemical analysis revealed the installation of oxidative stress and lipid peroxidation in the fish organs intoxicated with benzenthonium chloride and cocamidopropil betaine. These changes indicate that studied cationic and amphoteric surfactants determine an acute sub-lethal toxic effect with tendencies for toxic impact on long-term.

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