

Oxidative stress biomarkers in different tissues of rainbow trout (*Oncorhynchus mykiss*) exposed to Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide

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Abstract. The aim of study was to determine the effects of exposure to the product DEZYNFEKTANT-CIP (Eng. – Disinfectant-CIP), which is formulated with peracetic acid and hydrogen peroxide, on oxidative stress biomarkers (lipid peroxidation (LPO) levels and the carbonyl content of oxidatively modified proteins) and antioxidant defenses (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), total antioxidant capacity) in muscle, gill, hepatic, and cardiac tissues of rainbow trout, *Oncorhynchus mykiss* (Walbaum). LPO and carbonyl contents changed with tissue type. Exposure to Disinfectant-CIP led to a significant decrease in LPO in muscle tissues and carbonyl content in muscle and gill tissues. The inhibition of SOD and CAT activity in muscle, hepatic, and cardiac tissues was observed probably because of increased oxidative stress during disinfection; however, hepatic and cardiac GPx activity increased in an attempt to counteract oxidative stress. We suggest that oxidative stress during the oxidation of peracetic acid and hydrogen peroxide could be counteracted by the antioxidant system in trout

tissues. Correlative analysis between oxidative stress biomarkers and antioxidant defense confirms the pivotal role of SOD and CAT against CIP-induced oxidative stress.

Keywords: rainbow trout, *Oncorhynchus mykiss*, peracetic acid, hydrogen peroxide, oxidative stress, antioxidant defense

Introduction

Efficient prophylactic approaches against fish disease are essential for the continued development of aquaculture. Recent research on substances such as acidic disinfectants, e.g., CIP, Oxim, Steridial, and other disinfecting agents formulated with peracetic acid (PAA), show they have prophylactic effects against bacterial, viral, and fungal infections when administered in a bath (Grudniewska and Dobosz 2007, Grudniewska et al. 2007, 2008).

PAA is used in many industries for its bactericidal, virucidal, fungicidal, and sporicidal effectiveness. It is a strong disinfectant with a wide spectrum of antimicrobial activity even in the presence of heterogeneous organic matter. PAA has no persistent toxic or mutagenic residuals or by-products, is only slightly dependent on pH, and requires brief contact time (Kitis 2004). PAA demonstrated the highest

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disinfectant rate at the lowest concentration when compared in tests with a broad range of other available disinfectants (Baldry 1983). Straus et al. (2012) report the effectiveness of PAA for fungus control on channel catfish, *Ictalurus punctatus* (Rafinesque), eggs. PAA has recently been suggested for use as a means to control infestations of *Ichthyophthirius multifiliis* even at low concentrations (Straus and Meinelt 2009). Picon-Camacho et al. (2012) also tested the efficacy of a H₂O₂, peracetic, acetic and peroctanoic acid-based formulation in the treatment of free-living stages of *Ichthyophthirius multifiliis* (tomonts, cysts, and theronts), which is a protozoan parasite of freshwater fish. They showed that the *in vitro* efficacy of the PAA-based product has great potential to control *I. multifiliis* infections in commercial aquacultural systems (Picon-Camacho et al. 2012). The results of Elia et al. (2006) showed that antioxidant parameters could be used as biomarkers of oxidative stress during exposure to disinfectants. Antonelli et al. (2006) evaluated the long-term disinfectant efficacy of PAA against fecal coliforms, *Escherichia coli*, and total heterotrophic bacteria. Treatments with PAA can also be used in freshwater crayfish aquaculture to prevent *Aphanomyces astaci* infections through effective disinfection. Jussila et al. (2011) also tested the efficacy of PAA on the disinfection or removal of crayfish plague, *A. astaci*, spores in the aquatic environment. They noted that a minimum lag time of 4 h was sufficient for the breakdown of PAA under practical conditions since there was a 100% crayfish survival in test groups over two weeks when exposed to PAA-treated water after a treatment lag time of more than 2 h (Jussila et al. 2011). Hebert et al. (2008) evaluated the impacts of various wastewater disinfection processes on the immune system of juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum). T lymphocyte proliferation was stimulated in the PAA treatment group (Hebert et al. 2008).

In general, the disinfection efficiency of PAA for microorganisms is ranked as follows: bacteria > viruses > bacterial spores > protozoan cysts (Liberti and Notarnicola 1999, Rudd and Hopkinson 1989, Kitis 2004). Baldry (1983) compared the

antimicrobial properties of aqueous solutions of PAA and hydrogen peroxide (H₂O₂) against vegetative bacteria, bacterial spores, and yeasts. H₂O₂ is more effective as a sporicide than as a bactericide (Baldry 1983). The combination of PAA and H₂O₂ was found to be synergistic (Alasri et al. 1992, 1993).

H₂O₂ is commonly used in the aquaculture industry against a range of skin and gill infections. Preliminary success using H₂O₂ to treat Atlantic salmon, *Salmo salar* L., affected with experimentally-induced amoebic gill disease was demonstrated by Adams et al. (2012). H₂O₂ is also effective for treating certain external bacterial infections and flagellate infestations in some species of ornamental fish (serpae tetra, *Hyphessobrycon eques*; tiger barb, *Puntius tetrazona*; blue gourami, *Trichogaster trichopterus*; suckermouth catfish, *Hypostomus plecostomus*; green swordtail, *Xiphophorus hellerii*; Russo et al. 2007). The effects of treatment with H₂O₂ against progressive infection with flatworm parasite, *Zeuxapta seriola*, were determined by Mansell et al. (2005). McAndrew et al. (1998) also demonstrated the effects of H₂O₂ treatment on different life-cycle stages of the salmon louse, *Lepeophtheirus salmonis*. The mobile adult and pre-adult stages of *L. salmonis* readily reattached to Atlantic salmon after H₂O₂ treatment. Adult female lice reattached in significantly lower numbers than they did in untreated controls (McAndrew et al. 1998).

A number of studies have illustrated that H₂O₂ at concentrations in the high physiological range induce adaptive changes increasing the resistance of biological systems not only to oxidative stress but also to many other stimuli (Stone and Yang 2006, Santos et al. 2011). The capability of H₂O₂ to induce a large number of protein syntheses and to provide cross-resistance implies that living systems might “intentionally” produce H₂O₂ as a component of adaptation in response to different fluctuations and perturbations shifting the system away from homeostasis (Yin et al. 2012).

Recent research on substances such as acidic disinfectants, e.g. disinfecting agents formulated with peracetic acid and hydrogen peroxide, show

prophylactic effects against bacterial, viral, and fungal infections when administered by bathing. Such disinfectants, although important and effective as disinfecting agents, could also have damaging effects caused by the generation of reactive oxygen species (ROS) and the formation of oxidation products in fish tissues. The following suite of fish biomarkers can be used to assess exposure to or the effects of xenobiotics on aquatic ecosystems: oxidative stress parameters, biotransformation products, stress proteins, metallothioneins, biotransformation enzymes (phases I and II), hematological, immunological, reproductive, endocrine, genotoxic, neuromuscular, physiological, histological, and morphological parameters (Van der Oost et al. 2003). Oxidative stress biomarkers and antioxidant defenses are good diagnostic and prognostic tools to assess the effects of a large number of individual and interactive processes in aquatic organisms (Wilhelm Filho 1996, 2007, Ross et al. 2001).

Thus, the aim of the present work was to study the effect of Disinfectant-CIP formulated with 15% PAA and 20% H₂O₂ (manufactured by Biochem-Art, Gdansk, Poland) administered as bath treatments on oxidative stress biomarkers and antioxidant defenses in the muscle, gill, hepatic, and cardiac tissues of rainbow trout (*O. mykiss*).

Materials and methods

Experimental Fish

Juvenile rainbow trout aged 0+ (first year of life) came from spawning performed at the Department of Salmonid Research, Inland Fisheries Institute (Rutki, Żukowo, Poland). The fish were fed daily with feed delivered by band feeders. The daily feed ration was calculated using the applicable feed table, depending on water temperature. Feed was withheld one day prior to experiment. Forty-four clinically healthy rainbow trout (*O. mykiss*) with a mean body mass of 45.8 ± 1.2 g were used in the experiments, which

were performed at a water temperature of 16°C (± 2) and a pH of 7.5–7.6. The dissolved oxygen level was about 12 ppm with additional oxygen supply. Water temperature and oxygen content were measured daily in the morning with a thermometer and oxygen meter. All enzymatic assays were carried out at the Department of Animal Physiology, Institute of Biology and Environmental Protection, Pomeranian University (Slupsk, Poland).

Experimental Procedure

The fish were divided into two groups and held in square 250 l tanks (70 fish per tank). The disinfectant agent used was Disinfectant-CIP, a solution formulated with 15% PAA and 20% H₂O₂ (Biochem-Art, Gdansk, Poland). It is marketed under license No. 0508/04 from the Minister of Health in Poland (27.01.2004) as a biocidal product under the name DEZYNFEKTANT-CIP (Grudniewska et al. 2008). The fish were exposed to the disinfectant by bathing them in a Disinfectant-CIP solution at a final concentration 16 ml m⁻³ (Group II). The fish were bathed for 20 min three times daily every three days in the mornings before feeding. Control fish (Group I) were handled in the same manner as Group II, but without the Disinfectant-CIP treatment. Two days after the last bath, 22 individuals from each group were sampled. The fish were not anesthetized before tissue sampling.

Tissue isolation

Tissues were removed from the trout after decapitation. One trout was used for each homogenate preparation. Briefly, muscle, gill, hepatic, and cardiac tissues were excised, weighed, and washed in ice-cold buffer. The minced tissues were rinsed clear of blood with cold isolation buffer and homogenized on ice in a glass Potter-Elvehjem homogenizing vessel with a motor-driven pestle. The isolation buffer contained 100 mM Tris-HCl; pH of 7.20 was adjusted with HCl.

Analytical methods

Thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), NADPH, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), quercetin, hydrogen peroxide, ammonium molybdate, sodium aside, t-butylhydroperoxide, Tween 80, urea acid, and 2,4-dinitrophenyl hydrazine (DNFH) were obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade. All enzymatic assays were carried out at 25°C (\pm 0.5) using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by adding the homogenate suspension. The specific assay conditions are presented subsequently. Each sample was analyzed in triplicate. The protein concentration in each sample was determined according to Bradford (1976) using bovine serum albumin as a standard.

Thiobarbituric acid reactive substrates (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reactive substrates (TBARS) with the Kamyschnikov (2004) method for determining the malondialdehyde (MDA) concentration. In the TBARS assay, 2.1 ml tissue homogenate was heated together with 20% TCA and 0.8% TBA reagent in boiling water for 10 min. Then the tubes were cooled, and after centrifugation (3,000 g for 10 min) the absorbance was measured at 540 nm. TBARS values were expressed in nanomoles of MDA equivalents per milligram of protein.

Assay of carbonyl derivative content of protein oxidative modification (OMP)

As an indication of oxidative damage to proteins, carbonyl groups were measured according to the method of Levine et al. (1990) as modified by Dubinina et al. (1995). Samples were incubated at

room temperature for 1 h with 10 mM DNTP in 2M HCl. Blanks were run without DNTP. Afterward, proteins were precipitated with TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol:ethylacetate (1:1) and incubated at 37°C until complete resuspension. The carbonyl content was measured spectrophotometrically at 370 nm (aldehyde derivatives, OMP₃₇₀) and at 430 nm (ketonic derivatives, OMP₄₃₀) (molar extinction coefficient 22,000 M⁻¹ cm⁻¹) and expressed as nmol per mg protein.

Superoxide dismutase activity assay

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity in supernatant was determined according to Kostjuk et al. (1990). SOD activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0). Briefly, 1.0 ml of C reagent was mixed with 0.1 ml of homogenate (1:1000). C reagent was made *ex tempore* (mixture of equal volumes of 0.1 M K/Na-phosphate buffer, pH 7.8, and 0.08M EDTA); the pH of the C reagent was adjusted to 10.0 by adding tetramethylenediamine. Distilled water (0.1 ml) was added to the blank instead of homogenate. The total volume was then made up to 2.4 ml with distilled water. The reaction was initiated by adding 0.1 ml of quercetin (1.4 μ M). Absorbance at 406 nm was measured immediately and again after 20 min. Activity was expressed in units of SOD per mg of tissue protein.

Catalase activity assay

Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of H₂O₂ concentration at 410 nm according to Koroliuk et al. (1988). The rate of decrease in H₂O₂ content is directly proportional to the CAT activity in the sample. Assays were performed in a reaction mixture containing 2 ml of 0.03% H₂O₂ solution and 0.1 ml of tissue homogenate. The duration of this reaction was 10 min at room temperature. The reaction was terminated by

rapidly adding 1.0 ml of 4% ammonium molybdate dissolved in 12.5 mM H₂SO₄ and 1 ml of 125 mM H₂SO₄. Instead of the homogenate, the blank assay included 0.1 ml of distilled water. All samples were centrifuged at 3,000 g for 10 min. One unit of CAT activity was defined as the decrease of 1 μ mol of H₂O₂ per minute per milligram protein.

Glutathione reductase activity assay

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed as described by Glatzle et al. (1974) with some modifications. The enzymatic activity was assayed spectrophotometrically by measuring NADPH consumption. In the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH, resulting in a decrease of absorbance at 340 nm. Quantification was based on the molar extinction coefficient of 6.22 mM⁻¹ cm⁻¹ of NADPH. One unit of GR was defined as the amount of enzyme that reduced 1 μ mol of NADPH with GSSG per minute per mg protein.

Glutathione peroxidase activity assay

Glutathione peroxidase (GPx, EC 1.11.1.9) activities were determined by detecting the nonenzymatic utilization of GSH as the reacting substrate at an absorbance of 412 nm after incubation with DTNB according to the method of Moin (1986). Briefly, the reaction mixture contained 0.8 ml of 0.1M Tris-HCl buffer (pH 8.9) with 12 mM sodium azide, 6 mM EDTA, 0.2 ml of homogenate, 0.1 ml of 4.8 mM glutathione, and 0.1 ml of 20 mM t-butylhydroperoxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.2 ml of 20% TCA, and centrifuged. The supernatant was assayed for glutathione content with Ellman's reagent (39.6 mg of DTNB in 10 ml of 1% sodium citrate). A unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μ mol of GSH per min, and the activity of GPx was calculated based on tissue protein concentration.

Total antioxidant capacity (TAC) assay

The TAC level was estimated spectrophotometrically at 532 nm following the method with Tween 80 oxidation (Galaktionova et al. 1998). Briefly, 0.2 ml of tissue homogenate was added to 2 ml of 1% Tween 80. Instead of the sample, the blank assay included 0.1 ml of distilled water. The mixture was incubated for 48 hours at 37°C. After cooling, 1 ml of 40% TCA was added. The mixture was centrifuged at 3,000 g for 10 min. After centrifugation, 2 ml of supernatant and 2 ml of 0.25% TBA reagent were mixed in. The mixture was heated in a boiling water bath at 100°C for 15 minutes. The absorbance of the solution obtained was measured at 532 nm and was compared with the blank. The TAC level was expressed in %.

Statistical analysis

The results obtained were analyzed statistically using the Statistica 10.0 software package (StatSoft, Poland). The results are expressed as means \pm S.E.M. The Kolmogorov-Smirnov test was applied to evaluate normality, while the Levene test was used to test the homogeneity of variance. As most of the distributions deviated from the normal distribution, non-parametric tests were used for further analyses. To assess the differences between the studied group and untreated control, the non-parametric Mann-Whitney *U*-test was used, and P-values of less than 0.05 were considered significant (Zar 1999).

Results

TBARS as biomarkers of lipid peroxidation as well as protein carbonyl contents as biomarkers of protein oxidation in muscle, gill, hepatic, and cardiac tissues of rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP are presented in Figures 1 and 2. Muscle TBARS content was lower by 24% ($P = 0.047$), and no significant difference was found in the TBARS content of gill, hepatic, or cardiac tissues after Disinfectant-CIP treatment (Fig. 1).

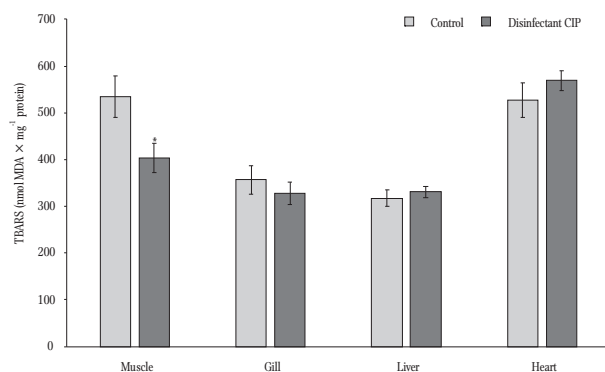


Figure 1. Level of lipid peroxidation (nmol MDA per mg protein) in muscle, gill, liver, and heart tissues of rainbow trout (*O. mykiss*) treated with Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide. *indicates significant difference from the untreated group ($P < 0.05$) (mean \pm S.E.M.)

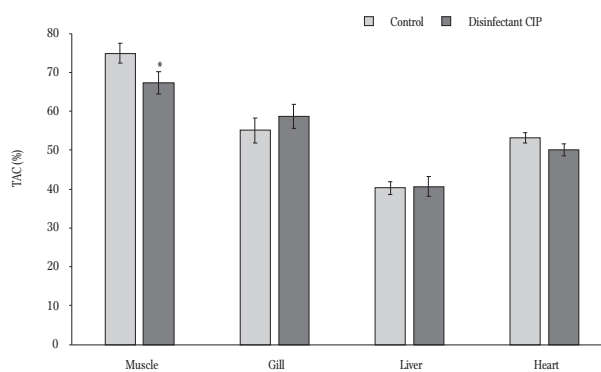


Figure 3. Total antioxidant capacity in the muscle, gill, liver, and heart tissues of rainbow trout (*O. mykiss*) treated with Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide. *indicates significant difference from the untreated group ($P < 0.05$) (mean \pm S.E.M.)

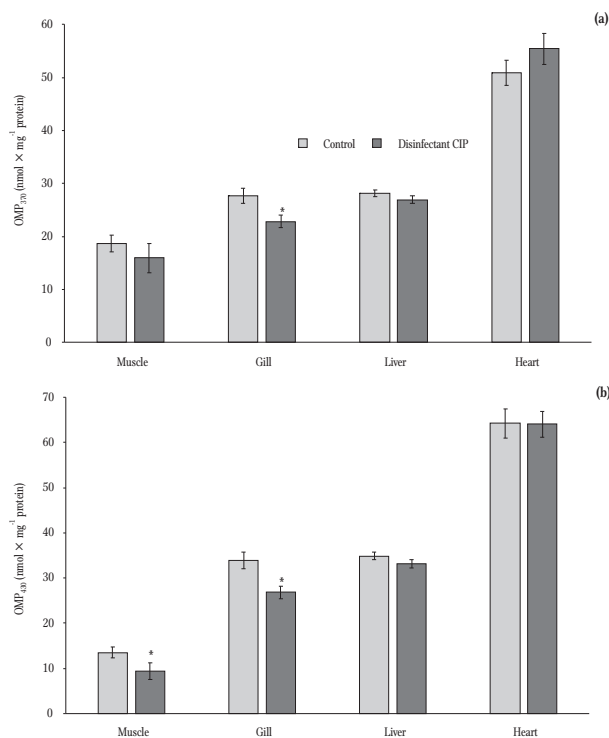


Figure 2. Aldehyde (a) and ketonic derivatives (b) of carbonyl content in the muscle, gill, liver, and heart tissues of rainbow trout (*O. mykiss*) treated with Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide. *indicates significant difference from the untreated group ($P < 0.05$) (mean \pm S.E.M.)

As biomarkers of oxidative stress, carbonyl content showed non-significant changes between the untreated and CIP-exposed groups except in muscle and gill tissues (Fig. 2). The ketonic derivatives of

protein carbonyl content in muscle and gill tissues of CIP-exposed trout were lower by 31% ($P = 0.003$) and by 21% ($P = 0.003$), respectively, compared to the control group (Fig. 2). Aldehyde derivatives of oxidatively modified proteins in gill tissue of CIP-exposed trout were also lower by 17.5% ($P = 0.007$) (Fig. 2).

Antioxidant enzyme activities in muscle, gill, hepatic, and cardiac tissues of rainbow trout exposed to Disinfectant-CIP are shown in Tables 1 and 2. After Disinfectant-CIP exposure, muscle, hepatic, and cardiac SOD activities were lower by 25% ($P = 0.000$), 38% ($P = 0.005$), and 43% ($P = 0.000$), respectively, compared to the control group. CAT activity in the muscle and cardiac tissues was lower by 31% ($P = 0.007$) and 26% ($P = 0.008$), respectively. Statistically significant differences in muscle and hepatic GR activity was observed between CIP-treated and control trout. Cardiac and hepatic GPx activity of the CIP-exposed group was higher by 28% ($P = 0.047$) and 26% ($P = 0.008$), respectively, compared to the control group. Gill GPx activity was lower by 25% ($P = 0.004$) in CIP-treated group.

Regarding total antioxidative capacity (Fig. 3), Disinfectant-CIP significantly decreased muscle TAC by 10% ($P = 0.030$). No significant differences of TAC in gill, hepatic, or cardiac tissues for CIP-treated trout were observed. Data from correlation analysis

Table 1

Antioxidant enzyme activities in muscle and gill tissues of rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide. SOD – superoxide dismutase, CAT – catalase, GR – glutathione reductase, GPx – glutathione peroxidase

Antioxidant enzymes	Muscle		Gill	
	Control	Disinfectant-CIP	Control	Disinfectant-CIP
SOD (U mg ⁻¹ protein)	801.75±19.10	604.21±29.24*	309.17±43.97	327.77±32.69
CAT (μmol H ₂ O ₂ min ⁻¹ mg ⁻¹ protein)	20.48±1.41	14.09±1.68*	8.71±1.18	9.87±0.74
GR (μmol NADPH ₂ min ⁻¹ mg ⁻¹ protein)	8.18±0.95	3.92±0.50*	5.26±0.53	5.56±0.66
GPx (μmol GSH min ⁻¹ mg ⁻¹ protein)	207.87±15.74	253.66±19.90	203.98±10.41	152.67±13.92*

*indicates significant difference from the control group (P < 0.05) (mean ± S.E.M.)

Table 2

Antioxidant enzyme activities in liver and heart tissues of rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP formulated with peracetic acid and hydrogen peroxide. SOD – superoxide dismutase, CAT – catalase, GR – glutathione reductase, GPx – glutathione peroxidase

Antioxidant enzymes	Liver		Heart	
	Control	Disinfectant-CIP	Control	Disinfectant-CIP
SOD (U mg ⁻¹ protein)	395.40±41.52	245.45±20.67*	753.58±27.99	425.87±26.74*
CAT (μmol H ₂ O ₂ min ⁻¹ mg ⁻¹ protein)	20.31±0.47	18.98±0.45	39.84±2.38	29.58±2.48*
GR (μmol NADPH ₂ min ⁻¹ mg ⁻¹ protein)	6.44±0.83	2.87±0.35*	2.66±0.26	2.75±0.28
GPx (μmol GSH min ⁻¹ mg ⁻¹ protein)	14.40±1.39	18.41±1.13*	223.38±19.59	281.47±13.97*

*indicates significant difference from the control group (P < 0.05) (mean ± S.E.M.)

among lipid peroxidation, oxidative modified protein levels, and antioxidant enzyme activity in different tissues of rainbow trout from the control group and the group exposed to Disinfectant-CIP are shown in Figures 4-7. In the untreated control group, muscle TAC was determined by GR ($r = 0.697$, $P = 0.000$) and GPx activity ($r = 0.493$, $P = 0.020$), while the decrease of ketonic derivatives of protein carbonyl content depended on CAT and SOD activity in the CIP-treated group (Fig. 4). In gill tissue, the TBARS level was correlated with SOD ($r = 0.491$, $P = 0.020$) and GPx activity ($r = 0.471$, $P = 0.027$) in the untreated group (Fig. 5a). The decreases of aldehyde and the ketonic derivatives of protein carbonyl content resulted from lower GPx activity in the gill tissues of the CIP-exposed group (Fig. 5b). Hepatic SOD activity correlated with oxidative stress biomarkers in the control group (Fig. 6a). In the CIP-exposed group, the hepatic TBARS level was correlated with SOD activity ($r = -0.524$, $P = 0.018$) and the aldehyde level of oxidatively modified proteins ($r = 0.796$, $P = 0.000$)

(Fig. 6b). The relationship between oxidative stress parameters in the cardiac tissue of the control group and the group exposed to Disinfectant-CIP was positive (Fig. 7a). The cardiac TBARS level was correlated with GR activity ($r = 0.547$, $P = 0.013$) in the CIP-exposed group (Fig. 7b).

Discussion

The aim of this study was to evaluate the effect of Disinfectant-CIP, formulaed with PAA and H₂O₂, on oxidative stress biomarkers and antioxidant defenses in the muscle, gill, hepatic, and cardiac tissues of rainbow trout. The findings of this study showed that lipid peroxidation in muscle tissues decreased in the CIP-exposed group when compared with the control group (Fig. 1). TBARS content as biomarker of lipid peroxidation in the gill tissue showed a downward trend after the disinfectant treatment. Similarly, the oxidative modification of protein also showed

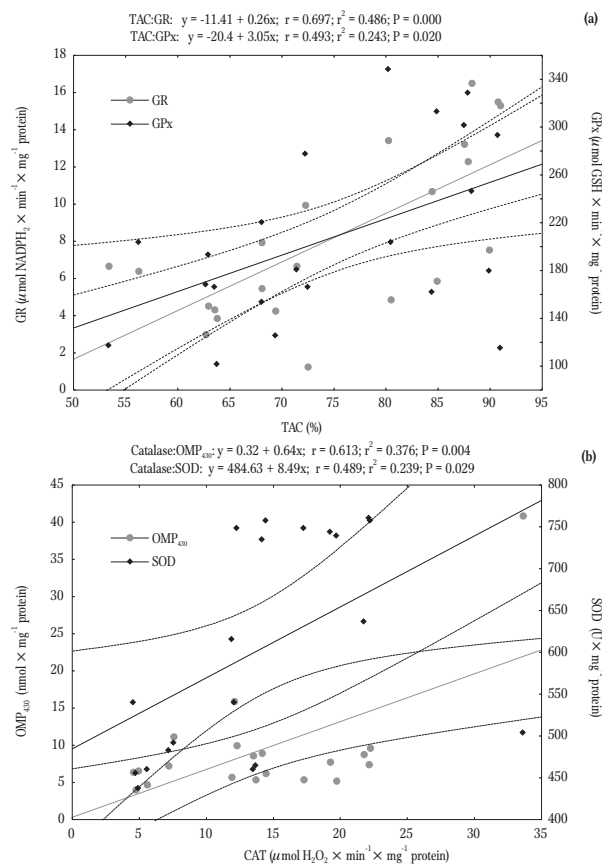


Figure 4. Correlation between TAC, GR, and GPx activity (a), and CAT and SOD activity (b), and ketonic derivatives of oxidatively modified protein levels in the muscle tissues of the control group and rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP.

a downward trend after disinfection treatment in muscle and gill tissues. No significant changes were noted in this study in the oxidative stress biomarkers (TBARS content as a biomarker of lipid peroxidation and carbonyl content as the oxidative damage of proteins) of hepatic and cardiac tissues in the CIP-treated group compared with the control group (Figs. 1 and 2).

In our study, the CIP-treated group showed decreased SOD activity in muscle, hepatic, and cardiac tissues, while higher activity was noted in the control group. CAT activity in muscle and cardiac tissues also showed similar trends. We assume that the excessive release of superoxide radicals after Disinfectant-CIP exposure can cause a decrease of SOD and CAT activity. Moreover, it is noted that SOD and CAT play a pivotal role in decreasing the oxidative stress

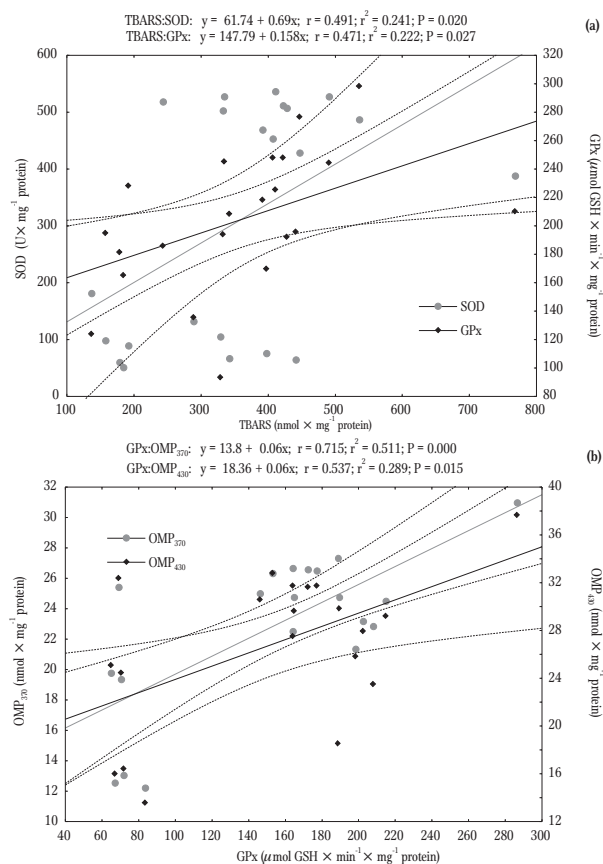


Figure 5. Correlation between TBARS level, SOD, and GPx activity (a), and GPx activity (b), aldehyde and ketonic derivatives of oxidatively modified protein levels in the gill tissue of the control group and rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP.

biomarker level in the muscle, gill, and hepatic tissues of the CIP-treated group (Figs. 4-6, Table 1). TAC decreased only in the muscle tissue of trout treated with Disinfectant-CIP (Fig. 3). In contrast, the GPx activity trend in both cardiac and hepatic tissues was increasing, while it was decreasing in gill tissue after disinfectant treatment. The correlation between the carbonyl level of protein damage and GPx activity in gill tissue confirms the assumption about the role of GPx in oxidative stress reduction. Similarly, correlative analysis among TBARS, aldehyde derivatives of oxidatively modified protein levels, and GR activity showed the role of glutathione-dependent antioxidant defense in cardiac tissue of the CIP-treated group (Fig. 7).

Differences in tissue response to the influence of the Disinfectant-CIP could be explained by the

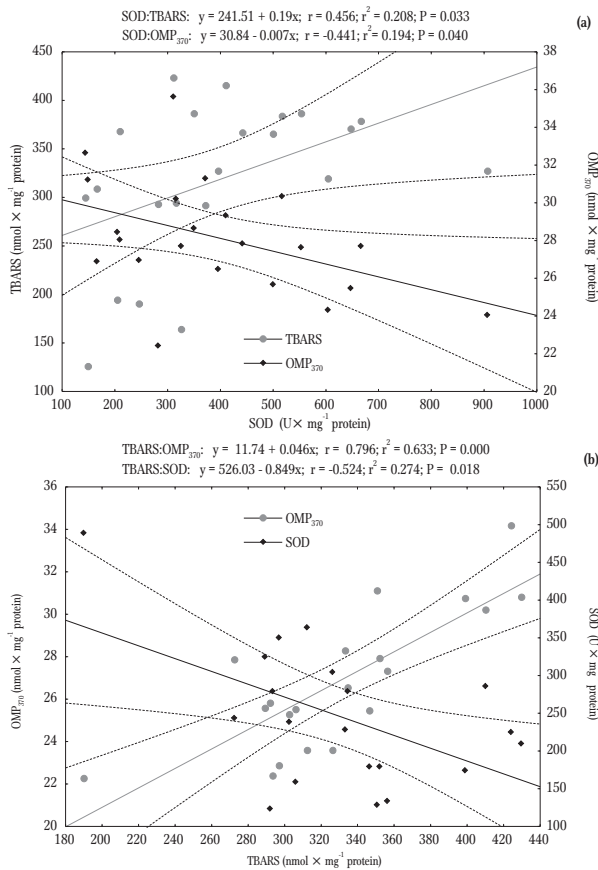


Figure 6. Correlation between SOD activity (a), TBARS (b) and aldehyde derivatives of oxidatively modified protein level in the hepatic tissue of the control group (A) and rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP.

different mechanisms of PAA and H₂O₂ action, as well as different distributions of antioxidants among cell and tissues. PAA combines the active oxygen characteristics of peroxide within an acetic acid molecule and belongs to the class of organic peroxides, which are synthetic chemicals. Organic peroxides can contain peroxide radicals (oxygen-oxygen bond) that are a source of oxygen (Kitis 2004). PAA was found to be an efficient disinfecting agent, and was not only bacteriostatic, but it was also a bactericide (Antonelli et al. 2006). PAA action oxidizes the outer cell membrane of cells. The mechanism of oxidation is the transfer of electrons, therefore the stronger the oxidizer, the faster the electrons are transferred to cells and the faster the cells are inactivated or killed. Therefore, PAA has a higher oxidation potential

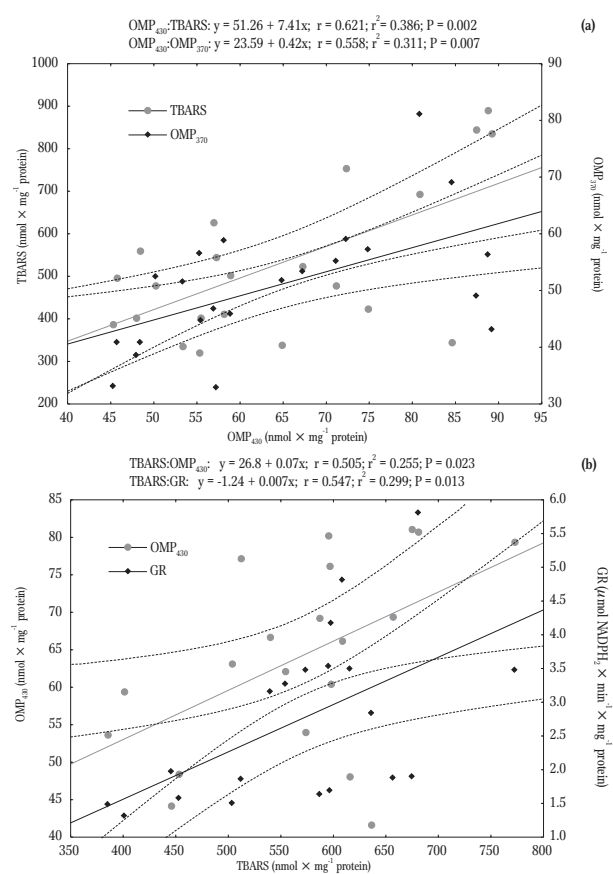


Figure 7. Correlation between TBARS, aldehyde and ketonic derivatives of oxidatively modified protein level (a), and TBARS, ketonic derivatives of oxidatively modified protein level, and GR activity (b) in the cardiac tissue of the control group and rainbow trout (*O. mykiss*) exposed to Disinfectant-CIP.

(Marjani et al. 2010). PAA can damage all types of macromolecules associated with microorganisms carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids because the denaturation of proteins and enzymes and increased cell wall permeability by the breaking of sulfhydryl and disulfide bonds results in cell lysis and true microbial death (Baldry 1983, Marjani et al. 2010). Thus, the primary mode of PAA action is oxidative stress through the oxidation of cell macromolecules.

Oxidative stress is a condition characterized by the elevation in the cellular steady-state concentration of reactive oxygen species (ROS) from an imbalance between the production of ROS and the destruction by antioxidant enzymes (Halliwell and Gutteridge 2007). Manipulations that increase ROS

generation or decrease the antioxidant capacity of cells lead to a state of oxidative stress. A number of antioxidant enzymes are expressed at the same time to protect the cells from the cytotoxic effects of ROS directed against engulfed microorganisms (Ebert-Dümig et al. 1999). On the other hand, in living organisms, besides its well-known cytotoxic effects, ROS play an essential role in regulating diverse biological processes such as immune cell activation (macrophage activation and phagocytosis) and apoptosis (Yin et al. 2012). Macrophage activation and the phagocytosis of foreign particles are regularly accompanied by a so called “respiratory burst”, an increase in the production of ROS, exerted by the enzyme complex NADPH oxidase.

Hydrogen peroxide induces production of $O_2^{\cdot-}$ by activating NADPH oxidase (Coyle et al. 2006). A number of studies have illustrated that, at concentrations in the high physiological range, H_2O_2 induces adaptive changes, increasing protein synthesis and provides cross-resistance (Stone and Yang 2006, Santos et al. 2011). Moreover, living systems may “intentionally” produce H_2O_2 as a component of adaptation in response to different fluctuations and perturbations shifting the system away from homeostasis (Yin et al. 2012).

Some investigators have studied the effects of PAA and H_2O_2 on various organs. The study by Ferraris et al. (2005) showed the effects of disinfection on the formation of toxic compounds in lake water treated with sodium hypochlorite, chlorine dioxide, and PAA and its potential toxic effects on rainbow trout hepatocytes. None of the water concentrates showed any cytotoxic effect or variations in GSH content in rainbow trout hepatocytes, but significant increases in ROS production were detected in fall water concentrates from treatments with sodium hypochlorite and chlorine dioxide. Cytochrome P450 expression was not modified in the hepatocytes exposed to the water concentrates. PAA caused a decrease in GSH and a statistically significant increase in oxygen radicals in hepatocyte cells exposed for 2 h, while prolonged treatment (24 h) caused a GSH increase, without variations in the ROS content. This phenomenon could be interpreted as the cellular

adaptation response to initial oxidative stress (Marabini et al. 2006).

Since the primary mode of PAA and H_2O_2 action is the oxidation of cell membranes, we assumed that CIP-induced oxidative stress is limited by antioxidant defenses in the tissues of rainbow trout. In liver and heart tissues of the CIP-treated group, oxidative stress could have been restricted by intracellular GPx, while SOD and CAT activity were inhibited (Table 2).

Different subcellular sites and different cell types can contain varying amounts of antioxidant enzymes (Sies 1997). All cells in eukaryotic organisms contain powerful antioxidant enzymes (Halliwell and Gutteridge 2007). Antioxidant systems prevent the uncontrolled formation of ROS, or inhibit their reactions with biological structures. Hydrophilic scavengers are found in cytosolic, mitochondrial, and nuclear compartments (Chaudière and Ferrari-Iliou 1999). The three major classes of antioxidant enzymes are SOD, CAT, and GPx. In addition, numerous specialized antioxidant enzymes react with and detoxify oxidant compounds. Indirect antioxidant functions carried out by enzymes are: (a) the back-up function, e.g., the replenishment of GSH from glutathione disulphide (GSSG) by the GR; (b) the transport and elimination of reactive compounds, e.g., glutathione S-transferases and the transport systems for glutathione S-conjugates. SOD can catalyze the dismutation of the superoxide radical to H_2O_2 and molecular oxygen. Then it can be converted to H_2O by CAT and GPx (Yin et al. 2012). As an important component of antioxidant defense system in fish, tripeptide glutathione (GSH) directly or indirectly regulates the scavenging of ROS and their reaction products. Additionally, several GSH-associated enzymes such as GR, GPx, and glutathione-S-transferase cumulatively protect fish against ROS, and their reaction products accrue anomalies under oxidative stress (Srikanth et al. 2013). There are synergistic interactions between GSH and other components of the antioxidant defense system such as vitamin C, vitamin E, and SOD (Meister 1994, Gerard-Monnier and Chaudière 1996). Synergistic and co-operative interactions of antioxidants rely on

the sequential degradation of peroxides and free radicals as well as on the mutual protection of enzymes. The antioxidant network plays an important role in the regulation of protein expression and/or activity at the transcriptional or post-translational levels (Chaudière and Ferrari-Iliou 1999). Elia et al. (2006) assessed the effects of sodium hypochlorite, chlorine dioxide, and PAA treatment for 10 and 20 days on antioxidant response (enzymatic activities of glutathione S-transferases, glyoxalase I, glyoxalase II, GPx, GR, CAT, and total glutathione content) of carp, *Cyprinus carpio* L. Differences in biochemical parameters were observed in specimens following exposure to these disinfectants, and mainly chlorine compounds induced marked biochemical variations of carp liver compared to those induced by PAA treatment (Elia et al. 2006).

In our study, the CIP-treated group showed decreased SOD and CAT activity in muscle, hepatic, and cardiac tissues (Table 1). GPx activity increased in both the hepatic and cardiac tissues of trout treated with Disinfectant-CIP (Table 2). This could be an adaptive response to the disinfecting procedure which neutralizes the impact of ROS and could be of importance in preventing membrane lipid peroxidation when the latter is initiated by a combination of Fe^{3+} and O_2^- generating system. GPx acts on lipid hydroperoxide substrates that are released from membrane phospholipids (Van Kuijk et al. 1987).

The gill and cardiac tissues of CIP-treated rainbow trout displayed positive correlations between oxidative stress biomarkers and glutathione-dependent antioxidant enzymes, while SOD and CAT activity play a pivotal role against CIP-induced oxidative stress in hepatic and muscle tissues. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide and molecular oxygen, and plays a critical role in the defense of cells against the toxic effects of oxygen radicals (Yim et al. 1990). Catalase is a ubiquitous antioxidant enzyme that is involved in the detoxification of H_2O_2 to molecular oxygen and two molecules of water (Halliwell and Gutteridge 2007).

Changes in CAT activity in cardiac and muscle tissues were more pronounced than those in gill and liver tissues. The robust protective effect of CAT activity can be exerted under synergetic effects with SOD activity (Fig. 4b). The inhibition in SOD and CAT activity in the muscle, hepatic, and cardiac tissues was probably observed because of increased ROS generation during CIP-induced oxidative stress; however, hepatic and cardiac GPx activity was increased in an attempt to counteract oxidative stress. The results show that Disinfectant-CIP induced increased GPx activity in liver and heart tissues for attenuating oxidative stress. We suggested that ROS generation under the oxidation of peracetic acid and hydrogen peroxide could be counteracted by the antioxidant defences in tissues of Disinfectant-CIP treatment. In the present study, we found that the level of oxidative stress biomarkers decreased in muscle and gill tissues, while it was not altered in the cardiac and hepatic tissues of rainbow trout after exposure to the Disinfectant-CIP. Thus, Disinfectant-CIP in the final concentration of 16 ml per m^3 can be used as a safe disinfecting agent in rainbow trout aquaculture. The data provide important information for future research of disinfectant effects on oxidative stress and antioxidant defenses biomarkers in different tissues of rainbow trout.

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