Oxidative DNA damage induced by iron chloride in the larvae of the lace coral 
*Pocillopora damicornis*

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Biochemical and molecular biomarkers tools are utilized as early warning signatures of contaminant exposure to target and non-target organisms. The objective of this study was to investigate the sublethal effects of iron chloride to the larvae of the lace coral *Pocillopora damicornis* by measuring a suit of oxidative-stress biomarkers. The larvae were exposed to a range of sublethal concentrations of iron chloride (0.01, 0.1, 1, 10, and 100 ppm) for seven days. With reference to oxidative stress biomarkers, the no-observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of iron chloride were observed to be 0.01 and 100 ppm respectively. At the end of the seventh day the antioxidant status of the larvae was evaluated by the levels of glutathione (GSH), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST), in both experimental and control groups. For the quantification of cellular oxidative damage, lipid peroxidation (LPO) activity was determined in the same and the extent of DNA damage was assessed by the expression of DNA apurinic/apyrimidinic (AP) sites. Iron chloride exhibited a concentration-dependent inhibition of GSH and GPX and induction of GR, GST, LPO, and DNA-AP sites in the *P. damicornis* larvae when compared to the control group. The oxidative stress biomarkers of the larvae exposed to 0.1, 1, and 10 ppm of iron chloride did not show any significant overall differences when compared to the control group. However the activities of LPO, GSH, GPX, GR, GST and DNA-AP in the larval group exposed to 100 ppm of iron chloride exhibited statistically significant (P=0.002, 0.003, 0.002, 0.002, 0.005 and 0.007) differences when compared to the control group. The research results indicated that iron chloride in concentrations at the 100 ppm level caused oxidative stress in the *P. damicornis* larvae.

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1. Introduction

Rapid industrial developments are threatening, impacting and stressing coral-reef ecosystems at an alarming rate. Metal contamination in coral-reef environments is often associated with contaminated effluent discharges into the ocean, near-shore and offshore mining activities, vessel groundings, and industrial wastewater effluents (Reichelt-Brushett and McOrist, 2003). The scleractinian coral *Pocillopora damicornis* is a simultaneous hermaphroditic coral which broods its larvae. Populations of *P. damicornis* depend on the settlement and recruitment of planula larvae during the juvenile polyp stage and these juvenile polyps later develop into mature corals (Permuta et al., 2000). Recognizing these readily observable sensitivities, Goh (1991) and Richmond (2005) both suggest that scleractinian coral larvae make good test subjects for monitoring stress on coral reefs and are useful candidates for coral-reef ecotoxicology studies.

The ecotoxicological effects of iron and other ubiquitous metals are of interest due to their frequent presence and impacts to marine ecosystems. Iron entering coastal waters via direct discharges from iron-ore mining, smelters, steel plants, and other land-based sources is usually in the form of iron chloride, iron oxide, or iron sulfate (Hamoutene et al., 2000; Payne et al., 2001; Sotero-Santos et al., 2007). The redox properties and coordination chemistries of iron are needed for various catalytic functions of living cells such as electron transport, cellular respiration, cell proliferation, cell differentiation, and gene regulation (Harris, 2002; Gurzau et al., 2003). However excessive concentrations of iron will lead to generation of free radical species, by way of the Fenton/Haber–Weiss reaction resulting in the oxidative stress and peroxidation of membrane lipids (Winterboum, 1995; Liu et al., 2003).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + [\text{H}_2\text{O}_2^-] \rightarrow \text{OH}^- + \text{OH}^-
\]

“Reactive oxygen species” (ROS) are harmful byproducts of oxidative metabolism and cause molecular damage in living cells (Baker...
et al., 1997). Iron is capable of generating ROS by undergoing cyclic oxidation and reduction where the reaction with hydrogen peroxide results in more highly reactive and toxic species causing a wide range of biological injury. Under normal physiological circumstances, equilibrium exists between the amounts of generated ROS and anti-oxidants system, protecting the organism against oxidative stress. Glutathione (L-γ-glutamyl-cysteinyl-glycine) is a non-protein thiol present in cells in its reduced form, which basically acts as an intracellular reductant, nucleophile and modulator of cellular homeostasis, including detoxification of metals. The protective effect of GSH and its associated enzymes has also been attributed to its ability to stabilize metal in its neutralized state, preventing redox cycling and free radicals generation (Oliveira et al., 2004). Lipid peroxidation (LPO), an important process in cellular damage is mediated through the free radical metabolites, leading to the degradation of polyunsaturated fatty acids and causing cross-linking in lipids, proteins and nucleic acids. One of the more prevalent forms of cellular oxidative DNA damage is the formation of apurinic/apyrimidinic (AP or abasic) lesions (Sczepanski et al., 2010). Oxidative attack by ROS on the deoxyribose moiety leads to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites. AP sites are also produced by the spontaneous hydrolysis of N-glycoside bonds as a result of excessive exposure to chemicals and/or irradiation (Nakamura et al., 2000).

Our ability to evaluate the health of corals has improved over the past few years largely through recent developments in cellular diagnostics and in the recognition of biomarkers capable of identifying sublethal responses to stressors at the cellular level (Downs et al., 2005). While others have investigated the effects of metals on the fertilization success, motility, and settlement success of coral larvae (Goh, 1991; Reichelt-Brushett and Harrison, 1999; Negri and Heyward, 2001; Reichelt-Brushett and Harrison, 2004; Victor and Richmond, 2005), few have studied metal toxicity and the oxidative-stress biomarkers in corals (Downs et al., 2002; Mitchemore et al., 2007; Farina et al., 2008). In view of the above we investigated the effect of iron chloride on the P. damicornis larvae with reference to oxidative-stress biomarkers and DNA damage. The larval stage of P. damicornis was chosen as the test subject because availability of the larvae which undergo major morphological and physiological changes throughout their development during which they are vulnerable to both environmental and pollutant stresses that will be reflected in the larval developmental process.

2. Materials and methods

2.1. Collection and maintenance of corals

Colonies of P. damicornis were collected from the reefs of Kaneohe Bay, Hawaii, USA. Selected coral colonies were detached from the loose substrate (as gently as was possible using a hammer and chisel), placed in buckets filled with seawater, and transported to the laboratory. Coral colonies were acclimated to the laboratory environment in aquarium tanks (50 L) providing a continuous flow of both seawater and air maintained at 25 ± 2 °C. Tanks were examined daily for proper water flow and air circulation. Large motile organisms such as anemones, fan worms, slugs, andurchins were removed from the corals as, during the onset of spawning, such organisms hinder coral-gamete collection.

2.2. Collection of larvae

A polyvinyl-chloride piping grid was established to provide continuous seawater supplies for eight adult coral colonies each measuring 15–20 cm in diameter. Each colony was contained in a separate vessel designed so that the continuous seawater-supply overflow would run into a container constructed from the base of a plastic beaker. The vessels with their overflow containers were housed in a deep water-table basin. Each vessel was fitted with a Nytex plankton mesh (80 μm) filter bag to gently filter naturally brooded planula contained in the seawater overflow and each filter bag was kept partially submerged in the basin. The corals were maintained under continuous flow conditions and, as this coral species releases planula at night, each filter bag was inspected every morning. Planula larvae of brooding P. damicornis are approximately 1 mm in diameter. Using Pasteur pipettes any larvae found in the filter bags were transferred into one-liter beakers containing seawater.

2.3. Bioassay test for biomarkers

A stock solution of iron chloride (FeCl3) was prepared using ultra-pure Millipore water. The test concentrations of iron chloride (0.01, 0.1, 1, 10, and 100 ppm) were prepared by diluting the stock solution with filtered seawater. The larval specimens were scored for the presence of intact edges, motility (spinning motion), and response to stimuli. To assess the biomarkers, healthy P. damicornis larvae were randomly divided into 6 groups of 25 larvae each. Exposure to iron chloride was carried out under static renewal conditions in clean test tubes. The control group larvae (Group I) were reared in seawater without iron chloride. The experimental group larvae (Groups II, III, IV, V, and VI) were reared in seawater containing, 0.01, 0.1, 1, 10, and 100 ppm of iron chloride. For each group three test tubes were setup to triplicate the experiment and continued for seven days. The bioassay was performed in a controlled temperature (25 ± 2 °C) and photoperiod (12L: 12D) under lab conditions. Seawater and the various concentrations of test solutions were renewed daily to expose each group of larvae to constant levels of iron chloride.

2.4. Assessment of biomarkers

At the end of seventh day the experiment was terminated and 25 larvae from each of the 3 chambers were pooled for each of the 6 groups. Each group was then separately homogenized in an all-glass homogenizer, individually using 1 ml of ice-cold 0.02 M Tris–HCl buffer. Homogenates were centrifuged at 15,000g for 10 min. The supernatant was collected from each sample and used for the assessment of oxidative-stress biomarkers. The LPO level was assayed by the method of Ohkawa et al. (1979) in which the amount of malondialdehyde (MDA) released serves as the index. The GSH content was determined by the enzymatic recycling method of Griffith (1980), where GSH gets successively oxidized and reduced by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The GPX (EC 1.11.1.9) level was assayed by the method of Rotruck et al. (1973) based on the reaction of GPX and DTNB to form a colored complex. The GR (EC 1.8.1.7) activity was estimated by the reduction in NADPH method of Staal et al. (1969). The GST (EC 2.5.1.18) activity was measured according to the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The total protein content was estimated according to the method of Ghosh et al. (1988) as described by Downs (2005a) using bovine serum albumin as the standard. For the determination of DNA-AP sites, genomic DNA was isolated from the control and the iron chloride-exposed larvae using the “Get PureDNA Kit” (Dojindo Molecular Technologies, Inc., MD, USA). The DNA abasic quantitative lesion assay was performed by the method described by Downs (2005b) which is based on the Dojindo AP assay kit system (Dojindo Molecular Technologies, Inc., MD, USA).

2.5. Statistical analysis

All data were checked for normality and homogeneity of variances prior to statistical analysis. The data were normally distributed and hence parametric tests were used. Mean values and standard error (X ± SE) were calculated for each test. One-way analysis of variance
(ANOVA) was performed with all pairwise multiple comparison procedures (Holm–Sidak method) to test for significant differences among the treatments. The differences were considered as statistically significant at $P<0.05$.

3. Results

The survivorship of *P. damicornis* larvae exposed to iron chloride concentrations of 0.01, 0.1, 1.0, 10, and 100 ppm was similar to that of larvae in the control treatment. Therefore larval exposure to these levels of iron chloride for seven days had no significant lethal effect. In all test chambers the behavior of larvae was observed to be normal and no mortality was observed through the seven-day experiment. For the iron chloride, no-observed effect concentration (NOEC) having no significant effect and the lowest observed effect concentration (LOEC) having significant effect on the oxidative stress biomarkers were observed to be 0.01 and 100 ppm respectively. The exposure of *P. damicornis* larvae to 100 ppm of iron chloride showed significant difference ($P<0.05$) for all the oxidative-stress biomarkers when compared to the control group. Iron chloride exhibited a concentration-dependent inhibition of GSH and GPX and a concentration-dependent induction of GR, GST, LPO, and DNA-AP sites in the *P. damicornis* larvae when compared to the control group. The lowest test concentration of iron chloride, 0.01 ppm, did not elicit any significant response from the biochemical biomarkers for any of the tested parameters.

No significant difference was observed in the LPO levels (22.8, 23.7, 25.36, and 27.46 n moles of MDA released/mg protein) of larvae exposed to 0.01, 0.1, 1, and 10 ppm of iron chloride when compared to the control group (22.83 n moles of MDA released/mg protein), while the LPO level (31.36 n moles of MDA released/mg protein) increased significantly ($P=0.002$) in the larvae exposed to 100 ppm of iron chloride (Fig. 1). The average GSH activity of the control larvae was 2.36 μg/mg protein and this reduced significantly ($P=0.003$) to 1.6 μg/mg protein in the larvae exposed to 100 ppm of iron chloride (Fig. 2).

A significant ($P=0.002$) inhibition of GPX activity (1.05 μ moles of GSH oxidized/min/mg protein) was observed in the larvae exposed to 100 ppm of iron chloride when compared to the control group of larvae (1.65 μ moles of GSH oxidized/min/mg protein) (Fig. 3). The GR (0.42 n moles of NADPH oxidized/min/mg protein) and GST activity (0.29 μ moles of CDNB conjugated/min/mg protein) of the control larval group was induced significantly ($P=0.002$; $P=0.007$) to 0.66 n moles of NADPH oxidized/min/mg protein and 0.48 μ moles of CDNB conjugated/min/mg protein respectively in the larvae exposed to 100 ppm of iron chloride. No significant difference was observed in the larvae exposed to 0.01, 0.1, 1, and 10 ppm of iron chloride when compared to the control group of larvae (Figs. 4, 5).

The expression of DNA-AP sites (13.56 ARP/10,000 base pairs) in coral larvae exposed to 100 ppm of iron chloride was significantly ($P=0.005$) different when compared to the control (4.6 ARP/10,000 base pairs). In contrast, no significant difference was observed in
the expression of DNA-AP (5.06, 7.70, 9.58, and 10.81 ARP/10,000 base pairs) in the larvae exposed to 0.01, 0.1, 1.0, and 10 ppm of iron chloride when compared to the control (Fig. 6).

4. Discussion

The primary objective of the present study is to investigate the sublethal effect of iron chloride on oxidative-stress biomarkers in the larvae of *P. damicornis*. The activities of antioxidant systems (GSH, GPX, GST, and GR), peroxidation of lipid membrane, and DNA damage were determined as endpoints of oxidative stress. Living organisms are continuously exposed to a number of exogenous and endogenous ROS radicals that can cause damage to protein, lipid, and nucleic acids. In this study a significant increase in the LPO activity was observed as an indication of oxidative stress in the larvae exposed to 100 ppm of iron chloride for seven days.

The mechanism of iron toxicity involves the Fenton/Haber–Weiss reaction characterized by the generation of hydroxyl radicals from hydrogen peroxides and superoxides resulting in the development of oxidative stress through the attack on proteins, DNA, and membranes by these hydroxyl radicals (Winterbourn, 1995; Lesser, 2006). The observed increase in LPO may be caused by the formation of hydroxyl radicals capable of abstracting a hydrogen atom from a methylene group of polyunsaturated fatty acids, thereby increasing the LPO.

Cells possess antioxidant defense systems which work to maintain the cellular redox status and to mitigate the damage caused by oxidative stress. When the capacity of the antioxidant defense system is reduced or fails under conditions of excessive pollutant stress, deleterious toxic events such as lipid membrane peroxidation and DNA damage ensue. During the defense reaction GSH and its associated enzymes may be induced or inhibited as these are adaptive responses permitting the organism to partially or totally overcome the toxicant stress (Livingstone, 2001; Svend, 2003). GSH is a ubiquitous non-protein thiol which basically acts as an intracellular reductant and used as a cofactor by multiple peroxidase enzymes for the detoxification of free radicals (Pastore et al., 2003).

The activity of GSH and its related enzymes in the *P. damicornis* larvae exposed to iron chloride was examined to explore the function of GSH as an antioxidant system. The observed reduction in the level of GSH in *P. damicornis* larvae exposed to iron chloride may be generated by increased oxidative damage resulting from exposure to a greater number of free radicals or it may be an adaptive response to oxidative stress. GSH-related enzymes play a crucial role in GSH-mediated antioxidant activity. GPX catalyzes the reduction of hydroperoxides to water using GSH as a reductant, thereby protecting lipid membranes against oxidation (Aniagu et al., 2006). The lowered GPX activity in the larvae exposed to 100 ppm of iron chloride when compared to control is attributed to the decline in glutathione concentration. The neutralization of ROS and hydroperoxides implies the oxidation of GSH to GSSG by GPX which is then reduced to GSH by GR for oxidizing NADPH to NADP⁺. GR catalyzes the reduction of GSSG to GSH using NADPH as a cofactor and is primarily responsible for maintenance of the GSH/GSSG ratio in cells.

![Fig. 3](image3.png)

**Fig. 3.** Effect of iron chloride on glutathione peroxidase activity of *Pocillopora damicornis* larvae. The GPX activity of *P. damicornis* larvae after seven days exposure to iron chloride are expressed as μ moles of GSH oxidized/min/mg protein. The bars show mean and standard error for three observations (n = 3). * indicates the significant difference between the control and the experimental groups at P = 0.002 by one-way ANOVA.

![Fig. 4](image4.png)

**Fig. 4.** Effect of iron chloride on glutathione reductase activity of *Pocillopora damicornis* larvae. The GR activity of *P. damicornis* larvae after seven days exposure to iron chloride are expressed as n moles of NADPH oxidized/min/mg protein. The bars show mean and standard error for three observations (n = 3). * indicates the significant difference between the control and the experimental groups at P = 0.002 by one-way ANOVA.
Both GR and GPX are important components of the ascorbate/GSH cycle which also involves H$_2$O$_2$ removal (Llopis et al., 2001). GR activity exhibited a concentration-based induction in the larvae exposed to iron chloride. The excessive generation of ROS during iron metabolism might be a possible reason for the observed GR induction. GST facilitates the nucleophilic attack of the sulfhydryl group of compounds resulting in the formation of less toxic products (Peña-Llopis et al., 2003). The altered values of GST in *P. damicornis* larvae exposed to iron chloride suggest a perturbation of the redox status induced by toxicant exposure. The reduction of GSH levels also directly reflects the induction of the GR and GST activity utilizing GSH during the conjugating process of the iron chloride.

Oxidative damage produced by intracellular ROS results in modifications in the DNA base, single and double strand breaks, and the formation of AP lesions many of which are toxic and/or mutagenic. The generation of AP sites is one of the most frequent changes occurring in DNA due to the cleavage of the N-glycosylic bond between the base and the deoxyribose moiety as a result of oxidative stress induced by chemical agents (Khodyreva et al., 2010). The expression of DNA-AP sites showed a clear iron chloride concentration-related increase. When compared to the control, the greatest numbers of DNA-AP sites were observed in the larval group exposed to 100 ppm iron chloride. DNA is not reactive to iron itself; however the cellular metabolizing of iron leads to the generation of reactive intermediates that subsequently attack macromolecules and lead to multiform DNA damage such as strand breakage, DNA-protein cross-links, DNA-DNA cross-links, iron-DNA adducts, and modifications in the bases of cells.

5. Conclusion

In summary the present study demonstrates that concentrations of 100 ppm of iron chloride caused oxidative DNA damage to the larvae of *P. damicornis*. Sublethal concentrations of iron chloride caused oxidative stress in the coral larvae by affecting the glutathione homeostasis which influenced the functions of GSH-associated enzymes thereby resulting in the expression of DNA-AP sites. The observed results support the current understanding that the larval stage of *P. damicornis* corals possesses antioxidant defense mechanisms to respond to iron chloride stress. Thus the assessment of oxidative-stress biomarkers can aid in understanding the mechanisms of iron chloride-induced toxicity in *P. damicornis* corals.

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**Fig. 5.** Effect of iron chloride on glutathione-s-transferase activity of *Pocillopora damicornis* larvae. The GST activity of *P. damicornis* larvae after seven days exposure to iron chloride are expressed as μ moles of CDNB conjugated/min/mg protein. The bars show mean and standard error for three observations (*n* = 3). * indicates the significant difference between the control and the experimental groups at *P* = 0.007 by one-way ANOVA.

**Fig. 6.** Effect of iron chloride on DNA-AP sites of *Pocillopora damicornis* larvae. The expression of DNA-AP of *P. damicornis* larvae after seven days exposure to iron chloride are expressed as ARP/100,000 base pairs. The bars show mean and standard error for three observations (*n* = 3). * indicates the significant difference between the control and the experimental groups at *P* = 0.005 by one-way ANOVA.
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