Stress response and toxicity studies on zebrafish exposed to endosulfan and imidacloprid present in water

Barzah Muazzam, Kashif Munawar, Imtiaz Ahmad Khan, Sarwat Jahan, Mazhar Iqbal, Muhammad Rafique Asi, Abida Farooqi, Adila Nazli, Ijaz Hussain and Mazhar Iqbal Zafar

ABSTRACT

Fish and other aquatic biota are hampered by mixtures of pesticides which pollute natural water through agricultural runoff and other sources. Toxicity of combined exposures of endosulfan and imidacloprid on zebrafish in terms of oxidative stress and deoxyribonucleic acid (DNA) damage in liver and histological alterations in gills and muscles was investigated. Zebrafish were exposed to three different sub-lethal concentrations of endosulfan and imidacloprid along with control selected for each treatment for 21 days: control treatment (CT), treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3). T1, T2 and T3 groups were exposed to 0.1, 0.5 and 1 μg/L of endosulfan, respectively, while imidacloprid concentration was maintained at 1 ppm in all three treatments. Oxidative stress was evaluated by measuring levels of catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA). Comet assay was applied to measure degree of DNA damage. Dose- and time-dependent decrease in SOD and CAT activity was observed after 21 days of exposure while low concentrations of pesticides induced SOD and CAT activities after early exposure to reduce the oxidative stress. MDA content was found to be increased in T3 having high concentrations of pesticides. Substantial increase in DNA damage was noticed after 21 days' exposure to pesticides. Significant morphological changes were observed in gills relative to muscles.

Key words | antioxidant response, combined toxicity, *Denio rerio*, endosulfan, histopathological changes, imidacloprid

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INTRODUCTION

Water is an indispensable and precious component of human environments because it is used for many purposes such as drinking, irrigation, breeding of fish and other freshwater ecosystems and also as the main source of energy. Pesticide usage has become a necessary evil in developing countries and has increased several-fold where agriculture is anticipated to be the backbone of the economy. During the past few decades, agrochemicals have been widely used in most agricultural sectors for enhancing crop yield and improving the quality of the product (Doruchowski *et al.* 2017). Consequently, extensive application of pesticides poses potential risks to the biodiversity of freshwater aquatic

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environments because of their bioaccumulation and intrinsic toxicity (Cui *et al.* 2015). Agricultural runoff, leaching to surface and groundwater and spray drift are assumed as the major entry routes which contribute to the addition of pesticides in water bodies (Zhang *et al.* 2018) that provide support to the biological integrity of these waters. Consumption of such water sources which are contaminated with pathogens and impermissible limits of chemical toxins cause acute and chronic diseases and are also a major cause of death (Elimelech 2006; Banihashemi *et al.* 2014). In developing countries, deterioration of water quality through different sources remains an important challenge (Sohel *et al.* 2003; Sadiq *et al.* 2010).

When pesticides are applied with the prevailing application methods used for crop protection, however, it is inevitable that a proportion of sprayed pesticides will reach such untargeted edge-of-field surface waters. Since aquatic ecosystems contain species (e.g., fish) related to the target organisms of pesticides, unintended repercussions are to be expected when these ecosystems become contaminated (Liess *et al.* 2005). Recently, evaluation of the potential adverse effects of pesticide stress on sensitive non-target aquatic organisms in aquatic ecosystems has been the subject of worldwide concern (Matozzo *et al.* 2018). One of the major dilemmas in environmental risk assessment is that pesticide contamination is frequently detected as a mixture of different substances rather than individual chemicals in the aquatic environment (Schreiner *et al.* 2016). The worldwide usage of pesticides is about 2 million tonnes/year; about 45% of pesticides are used in Europe, USA 25% and the rest of the world consumes 25% (De *et al.* 2014). Pakistan is the second largest consumer of pesticides among the south Asian countries.

Xenobiotics like pesticides can induce reactive oxygen species (ROS) in organisms. These excessive ROS may cause oxidative damage to deoxyribonucleic acid (DNA), i.e., breakage of DNA strands. Oxidative stress may cause modifications in antioxidants' enzyme systems. These enzymes include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Oruc *et al.* 2004). Excessive production of ROS causes lipid peroxidation that ultimately leads to malondialdehyde (MDA) formation, resulting in toxic stress to cells (Ge *et al.* 2015). Genotoxicity is investigated by measuring the alterations in DNA or mRNA that may be in the form of genome damage or genome mutations. For assessing genotoxicity, the most promising technique applied to examine DNA damage in a single cell is single-cell gel electrophoresis (SCGE) assay (known as comet assay) (Ritter & Knebel 2009).

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo-dioxathiepin-3-oxide) is a non-systemic organochlorine insecticide, prevalently used as a broad-spectrum throughout the world. It is the most persistent and hazardous agent of all halogenated hydrocarbons, being highly resistant to microbial bioremediation and persisting as a xenobiotic. Oxidative stress is an important indicator of toxicity that is induced by endosulfan exposure to fish. Endosulfan can also affect many aquatic non-target organisms, particularly fish, for instance, modulation of antioxidant systems in fish liver can be induced (Shao *et al.* 2012). Owing to its toxic effects and persistence, endosulfan was considered as a new member of the persistent organic pollutants (POPs) group during the Stockholm Convention (POPRC 2010). Moreover, endosulfan is known as an endocrine disruptor and has the ability to bioaccumulate and biomagnify in food chains (US EPA 2002). Generally, it is found to be very toxic to fish as it is reported that concentrations of 0.50 to 0.75 μg/L have negative impacts on fish species (Alamdar *et al.* 2014) and cause chronic effects including genotoxic and developmental effects.

The insecticide imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneaimine] is a chloronicotinyl nitroguanidine and due to its systemic nature, it is ranked as the second most widely used pesticide globally (c. 70 crops over 100 countries) (Bonmatin *et al.* 2003). Environmental studies carried out on imidacloprid have indicated that it can be detected in the soil and can be carried by storm rain and runoff through which it can move into irrigation canals, streams, rivers, lakes and can leach into the groundwater, thus becoming a potential risk to aquatic life. US EPA (United States Environmental Protection Agency) has classified imidacloprid as highly toxic to aquatic invertebrates. Imidacloprid is considered as a potential groundwater and surface water contaminant, because of its leaching potential (PAN Pesticides database 2006). Acute exposure of imidacloprid and curzate on *Labeo rohita*

induced increased mortality, quick opercular and fin movement, loss of equilibrium and lethargy (Desai & Parikh 2014). Pesticides may cause acute and chronic poisoning of fish and damage their dynamic organs, causing various biochemical alterations, reproductive impairment and morphological deformities (Velasco-Santamaría *et al.* 2011; Wu *et al.* 2018).

For the present study, zebrafish (*Denio rerio*) were chosen because they are prominent biological model organisms for ecotoxicological research and are recommended by the Organization for Economic Co-operation and Development (OECD 2013) and International Organization for Standardization (ISO) as a standard test organism (ISO 1996). Zebrafish are well-accepted aquatic vertebrate models for investigating the toxicity of chemicals in aquatic environments and associated health risks. Hence, they are preferred for acute and chronic toxicity tests in the laboratory owing to many favourable characteristics, such as small body size, easy husbandry, morphology, short test cycle and rapid development. Due to these unique advantages, zebrafish can be used as a bioindicator to assess the pesticidal pollution in aquatic environments.

The liver is the organ mainly affected by contaminants as it is concerned with detoxification and biotransformation (Camargo & Martinez 2007). The gills are important organs for fish and also the first target organ to be in contact with pollutants after exposure. Therefore, pesticides accumulate in gills and their concentrations reflect the pesticide level in water where fish species reside (Napit 2013). Gills are respiration sites that are involved in osmoregulation (Fernandez & Mazon 2003). Due to their large surface area they are susceptible to pollutants in water, and are therefore considered as an appropriate water contamination indicator. Histopathology provides knowledge about the functionality of different organs. For this purpose, histological examination is a sensitive parameter and is critical for describing alterations in cells of target organs, like the liver and gills (Dutta 1996). The zebrafish is an important bioindicator organism for monitoring the contamination of freshwater resources since it has a genetic similarity to humans. As a result of illegal use of endosulfan and excessive use of imidacloprid in agricultural fields entering aquatic systems above maximum permissible limits, it is presumed that the zebrafish population is declining in developing countries (e.g., Pakistan) and may result in its extinction.

Literature shows that, according to the OECD test guidelines, zebrafish is found to be one of the most widely used model species. Therefore, the purpose of the present study is to assess the potential sublethal effects of endosulfan and imidacloprid on levels of antioxidant enzymes, MDA, and DNA damage and histopathological alterations in gills and muscle tissues of zebrafish.

MATERIALS AND METHODS

Materials and reagents

Two pesticides were evaluated in the present study, one a neonicotinoid insecticide (imidacloprid) and the other an organochlorine insecticide (endosulfan), as they are usually detected in surface waters worldwide, and known to be highly toxic to aquatic fauna. Imidacloprid (IMI; CAS: 138261-41-3; purity: 96.40% technical product) was purchased from the Chem Service, USA. Endosulfan (END; CAS: 115-29-7; purity: 99.0%) was purchased from the local distributor of Sigma-Aldrich, USA. All other chemicals and solvents used were of analytical purity and purchased from Sigma Chemical Co. (USA).

Procurement and maintenance of zebrafish

In the present study, about 360 mature adult zebrafish (*D. rerio*) in equal ratio of male and female were procured from a commercial local fish supplier (Fish Harbour) and transported to the Chemical Stress Ecology Laboratory, Department of Environmental Sciences, Quaid-i-Azam University. The fish were transferred from the polyethylene bag to a glass aquarium with continuous aeration. The size (dimensions) of the aquarium was $30 \times 30 \times 30$ cm (L × W × H) with a capacity of 25 L of water. Prior to the experiment, the fish were acclimatized to laboratory conditions for 2 weeks using dechlorinated tap water . During acclimatization, the water was changed on alternate days. The fish were maintained under specified conditions according to Diekmann *et al.* (2004), with photoperiod 12-hour light, 12-hour dark, water temperature adjusted to $26 \pm 1\,^{\circ}C$,

oxygen saturation above 70% and pH fluctuating from 7.4 to 8.1. The acclimatized healthy fish of mixed sex were selected randomly for the experiment.

Experimental design and pesticide exposure concentrations

The experiment was performed in a static closed test system. Healthy and uniform sized fish (mean body weight: 0.38 ± 0.01 g; mean body length: 3.85 ± 0.03 cm) used in this study were selected and evenly distributed into 12 glass aquariums. The acclimatized fish were divided into four different experimental groups as follows: one control group and three treatment groups (30 individuals were placed in each aquarium). Three different sub-lethal concentrations of endosulfan and imidacloprid along with control were selected in triplicate for each treatment, namely, control treatment (CT), treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3). In this experiment, three different concentrations of endosulfan and imidacloprid in the following treatments, i.e., T1, T2 and T3, were administered to zebrafish over a period of 21 days. T1 was exposed to 0.1 μg/L of endosulfan, T2 to 0.5 μg/L and T3 to 1 μg/L of endosulfan while the imidacloprid concentration of 1 mg/L was maintained in all these three treatments. Control treatment (CT) was treated without any treatment of agro-chemicals (exposed to acetone only). For each tested concentration, the experiment was performed in triplicate.

Water quality parameters (pH, temperature, dissolved oxygen and ammonia) were regularly observed and maintained within a fixed range. The water in aquariums was changed every alternate day and pesticide concentration level was maintained by re-dosing. The fish were fed once per day until satiation with commercially available dry flakes (2% of their body weight). The feed was not given to fish a day before sampling in order to avoid any trusion with faecal material. The experiment was conducted for a period of 21 days and behaviour of fish monitored regularly. No mortality was observed either in control individuals or in any of the treatment groups. After 7, 14 and 21 days of the experiment, fish were sampled, underwent biochemical analysis, estimation of DNA damage in liver tissues and histopathological alterations were examined in gills and muscles of zebrafish in order to estimate the effects of pesticide on the given species. Thirty fish at each sampling occasion were sacrificed from each treatment and control group. Before dissection, the fish were anaesthetized on ice. Liver tissues were obtained by dissecting the fish on an ice box. Tissue samples were kept on ice during preparation and later kept at −80 °C until further analysis. The whole experimental trial was subject to approval by Bioethics Committee, Quaid-i-Azam University.

Preparation of stock and treatment solutions

Stock solutions were prepared from technical-grade endosulfan (analytical standard α and β isomers) and imidacloprid and then stored at 4 °C in a refrigerator prior to further analyses. For endosulfan stock solution, 25 mg endosulfan was dissolved in 50 mL of acetone analytical reagent. From stock solution, three dosing/working solutions of concentrations, 10,000 μg/L, 5,000 μg/L and 1,000 μg/L, were made. From each dosing solution, 1.5 mL was dissolved in 14,998.5 mL of water to make three treatment levels of 0.1, 0.5 and 1 μg/L. The concentration of acetone was kept at <0.05% in all pesticide solutions used. For imidacloprid stock preparation, 100 mg of imidacloprid was dissolved in 1 L of distilled water then 150 mL of stock was added in each treatment (T1, T2, T3) level to maintain the concentration of 1 ppm in the three treatments. Concentrations of both insectcides were conserved by changing the water every alternate day.

Enzyme extraction and protein determination

Liver tissues were homogenized in 50 mM ice-cold potassium phosphate buffer (pH 7.0) in a teflon tissue homogenizer. Centrifugation of homogenate was done at 10,000 rpm at 4 °C for 10 minutes. Supernatant was instantly used for analysing enzyme activity and protein determination. Protein contents of liver tissues were estimated by Sigma Bradford method using bovine serum albumin as a standard.

Measurement of enzyme activities (CAT and SOD) and MDA contents

CAT was measured according to the method described by Claiborne (1985). For estimation of SOD activity, the

protocol of Shao *et al.* (2012) was followed in this study, while MDA content was determined following the methods used by Zhang *et al.* (2013), with the help of thiobarbituric acid (TBA) assay.

Measurement of DNA damage

SCGE was used to assess the DNA damage. Comet assay was executed to investigate the DNA strand breaks in zebrafish liver. Cell suspensions were prepared using the method described by Ge *et al.* (2015). The extent of DNA damage was estimated by Comet Assay Software Project 1.2.3.b software.

Histopathological examinations

For histology analysis, gill and muscle tissues of zebrafish were dissected and fixed in 4% paraformaldehyde for 24 hr at 4 °C. Afterwards, fixation tissues were dehydrated and further rinsed and processed in an ascending sequence of alcohol (70%, 80%, 90% and 100%) and then cleared in xylene. Tissues were embedded in paraffin and mounted on wooden blocks with the help of melted wax. Thin sections of 7-μm thickness were cut using microtome. Tissue ribbons were stretched by fixation on albumenized glass slides. Slides were hydrated using a sequence of reducing ethanol and run through a series of histological stains, Mayer's haematoxylin and eosin (H&E). Afterwards, slides were examined under light microscope (Olympus-CX41) at 400× and photographs of gill and muscle tissue were obtained using a Tucsen digital camera (Model: ISH500).

Data analysis

All statistical analyses were performed using the IBM SPSS Statistics version 21 for Windows 7 (SPSS Inc., USA). All the results were expressed as mean ± SD (standard deviation), unless specified otherwise. Quantitative data were subjected to one-way analysis of variance followed by Duncan's multiple range test (post hoc test) to estimate the significance of difference among different treatments at the same sampling point. The criterion for significance that was used in all statistical tests was set as $p < 0.05$.

RESULTS AND DISCUSSION

Combined effects of endosulfan and imidacloprid on antioxidant enzymes

Catalase activity

Changes in zebrafish CAT activities after exposure to endosulfan and imidacloprid are depicted in Figure 1.

Significant increase of CAT activity was observed in the second week of exposure in T1 as compared to the control. Endosulfan and imidacloprid induced CAT synthesis to maintain the oxygen free radical equilibrium by scavenging H_2O_2 into H_2O and O_2. CAT activity was significantly decreased in T3 after 7, 14 and 21 days of exposure, which shows excessive oxidative stress, so the antioxidant enzymes were unable to eliminate that stress and were exhausted. Control treatment showed enzyme activity in the range of 20.5–22.3 U/mg Pr (protein), indicating the normal level of catalase in zebrafish in given environmental conditions, as shown in Figure 1. There was no significant difference in CAT activity of T1 on day 7, just a slight increase of 5.3% as compared to the control was observed while CAT activity significantly decreased by 30.9 and 62.1% as compared to the control on day 7 of sampling in T2 and T3. After 14 days of exposure, a 19.9% decrease in T3 was observed while a

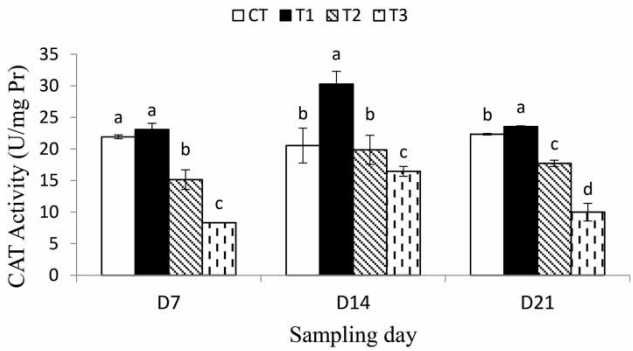

Figure 1 | Combined effects of endosulfan and imidacloprid on catalase activity in liver tissues of zebrafish: CT represents control treatment without exposure to pesticides; T1 represents treatment 1 having low concentration of endosulfan (0.1 μg/L) and 1 ppm imidacloprid; T2 represents treatment 2 having endosulfan concentration (0.5 μg/L) and 1 ppm imidacloprid; T3 represents treatment 3 having high concentration of endosulfan (1 μg/L) and 1 ppm imidacloprid. Each bar represents the mean of three replicates ± standard deviation (SD). Bars denoted with different lowercase letters indicate significant differences between treatments (Duncan's post hoc test, $p \leq 0.05$).

significant decline of 22.7 and 55.3% in T2 and T3 was observed as compared to the control after 21 days of exposure.

Superoxide dismutase activity

The effects of endosulfan and imidacloprid on SOD activity in zebrafish are illustrated in Figure 2.

A significant increase was observed in SOD activity after mild exposure to pesticides on day 7. After 21 days of exposure, a significant decrease in SOD activity was observed at the highest concentration (1 μg/L endosulfan and 1 mg/L imidacloprid) which suggests the inhibition of SOD enzyme. Fish in the control treatment (without pesticide exposure) demonstrated constant SOD activity of 12 U/mg Pr (protein) exhibiting consistent enzyme activity without toxicant, as shown in Figure 2. Compared to the control, a substantial increase of SOD activity was observed in treatments T1, T2 and T3 on day 7, i.e., 98.6, 70.2 and 22.6%, respectively. Less SOD activity was noticed in T2 and T3 as compared to T1 on day 7, but this difference was insignificant. However, SOD activity was greater in T1 and T2, by 19.5% and 9.3%, respectively, as compared to the control on day 14, but there was a insignificant difference among all three treatments in comparison to the control. On day 21, SOD activity was inhibited in all three treatments as compared to the control, but no significant

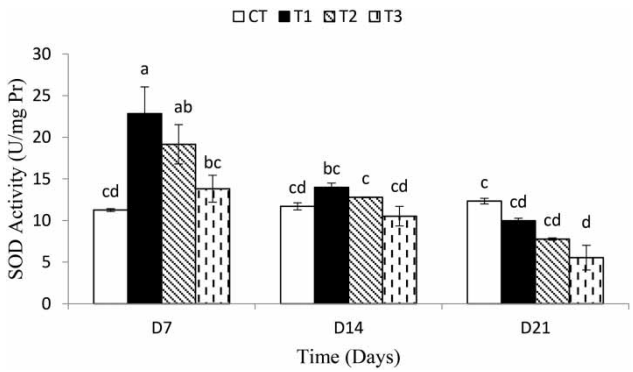

Figure 2 | Combined effects of endosulfan and imidacloprid on superoxide dismutase activity in liver tissues of zebrafish: CT represents control treatment without exposure to pesticides; T1 represents treatment 1 having low concentration of endosulfan (0.1 μg/L) and 1 ppm imidacloprid; T2 represents treatment 2 having endosulfan concentration (0.5 μg/L) and 1 ppm imidacloprid; T3 represents treatment 3 having high concentration of endosulfan (1 μg/L) and 1 ppm imidacloprid. Each bar represents the mean of three replicates ± standard deviation (SD). Bars denoted with different lowercase letters indicate significant differences between treatments (Duncan's post hoc test, $p \leq 0.05$).

decrease was observed in T1 and T2 from the control, whereas T3 activity showed a significant decline of 55.1% with respect to CT.

For monitoring the health of aquatic environments, fish can be used as an indicator to assess the quality of aquatic ecosystems. Oxidative damage is a toxicity mechanism induced by pollutants in aquatic organisms (Santos *et al.* 2004). SOD and CAT are considered as the two most important enzymes of antioxidant defensive systems. They have the ability to capture hydrogen peroxide and superoxide anions and protect organisms from oxidative stress (Han *et al.* 2016). In the present study, significant oxidative stress induced by endosulfan and imidacloprid in the livers of zebrafish was observed, which is likely to be due to the synergistic effects of both insecticides. Superoxide dismutase (SOD) activity was greatly inhibited by the increased pesticide exposure. Highest pesticide concentration and increased exposure time showed significant inhibition in SOD activity (day 21). Earlier exposures showed increased SOD activity (day 7 and 14) as compared to the control. During early exposures, SOD activity may increase to resist the oxidative stress caused by the pesticide exposure. Decreased SOD activity in all treatments after 21 days of exposure to pesticide mixtures may be explained by the fact that oxidative stress increased so much that the SOD level was not sufficient to resist that stress. These results are in agreement with the outcomes published by Ge *et al.* (2015) and Shao *et al.* (2012). A slight increase in CAT activity at low concentration (T1) of pesticide after 7 days of exposure accompanied by a significant increase after 14 days of exposure occurred. It was followed by a decrease in T1 after 21 days of exposure as compared to T1 on day 14. This may show that early exposure to pesticides induces CAT activity to reduce or balance the excessive ROS production by converting hydrogen peroxide into water and molecular oxygen. On the other hand, prolonged exposure to and higher concentration of pesticides can induce oxidative damage (Shao *et al.* 2012). Similar results were reported in a study performed by Han *et al.* (2016), in which, after exposure of azoxystrobin to zebrafish, SOD activities decreased significantly in liver as compared to control. This can happen because the increase in oxygen-free radicals due to constant exposure to pesticides for long durations reduced SOD and the active sites of this antioxidant

enzyme became inactivated, leading to enzyme dysfunction (Butterfield *et al.* 1998). Reduced SOD activity was observed in brains and kidneys of zebrafish exposed to atrazine (ATR) and chlorpyrifos (CPF). As the concentration of ATR, CPF and ATR/CPF combination increased, SOD activity reached its lowest levels. This may show the negative impact of pesticides either alone or in a mixture on zebrafish, and these observations in the form of synergistic effects are found to be in accordance with our study findings. CAT activity also followed the same pattern of decrease in activity as the pesticide concentration increased along with exposure time (Xing *et al.* 2012). Increase in CAT activity at low doses of pesticides may represent a compensatory mechanism against oxidative stress. The findings of CAT activity are consistent with the results of Rosety *et al.* (2005), where CAT activity was decreased by the exposure of insecticide malathion.

Generally, the presence of pesticide mixture is likely to be expected in aquatic ecosystems and, consequently, fish can be affected due to synergistic effects. In response to oxidative stress, highly ROS are produced that may cause oxidative damage to the cellular bodies. These highly reactive intermediates are scavenged by the natural defensive systems in the form of antioxidant enzymes, These antioxidant enzymes do not allow excessive ROS production since their basic function is to maintain a balance between production and elimination of ROS, but if the antioxidant enzymes fail to maintain this balance oxidative stress occurs (Ge *et al.* 2015; Han *et al.* 2016).

Combined effect of endosulfan and imidacloprid on MDA levels

Zebrafish MDA level was used to assess the level of lipid peroxidation exposed to endosulfan and imidacloprid, and the results are depicted in Figure 3.

For the evaluation of lipid peroxidation, MDA content was measured. No significant increase in MDA content was observed in groups having low concentrations of pesticides after day 7. On day 14, MDA level increased in T1, T2 and T3 by 55.9, 154.3 and 55.2%, respectively, as compared to similar treatments on day 7 of sampling. On day 21, a 36.8 and 66.2% increase in MDA content was observed as compared to the control. Significant increase in MDA

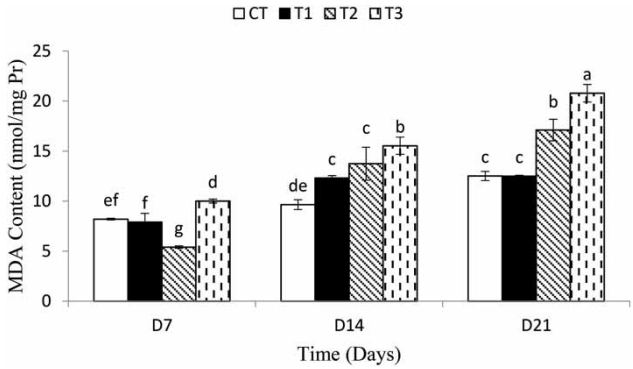

Figure 3 | Combined effects of endosulfan and imidacloprid on MDA content in liver tissues of zebrafish: CT represents control treatment without exposure to pesticides; T1 represents treatment 1 having low concentration of endosulfan (0.1 μg/L) and 1 ppm imidacloprid; T2 represents treatment 2 having endosulfan concentration (0.5 μg/L) and 1 ppm imidacloprid; T3 represents treatment 3 having high concentration of endosulfan (1 μg/L) and 1 ppm imidacloprid. Each bar represents the mean of three replicates ± standard deviation (SD). Bars denoted with different lowercase letters indicate significant differences between treatments (Duncan's post hoc test, $p \leq 0.05$).

content was observed in T3 after 7, 14 and 21 days of exposure, which shows that higher concentrations of pesticides and increased exposure durations can induce lipid peroxidation in cell membranes.

Environmental pollution can be measured by employing MDA as a biomarker of pollution. It is reported that dose-dependent increase in MDA level was observed in female zebrafish livers when exposed to atrazine (Jin *et al.* 2010) and these findings are in accordance with our results where the highest increase in MDA content was observed in groups having the highest concentration of pesticides; or, this significant increase could also be due to the synergistic effects of pesticide mixtures. Excessive ROS causes inhibition of antioxidant enzymes that leads to lipid peroxidation and MDA is the end product which is measured to evaluate the extent of oxidative stress. Similar results were observed in a study performed by Han *et al.* (2016), where increased MDA content was observed in both male and female zebrafish with increased concentration of fungicide azoxystrobin. Pesticide mixtures may also induce the same kind of changes in MDA levels as the trends observed in the case of exposure to pesticides alone. It has already been revealed that a high concentration of pesticides (atrazine and chlorpyrifos alone and in mixture) increased MDA levels of the brain and kidney in common carp (Xing *et al.* 2012). These results are in accordance with our study findings and synergistic effects are observed.

Effects on DNA damage in zebrafish

Results of DNA damage in the form of olive tail moments (OTMs) in zebrafish treated with different concentrations of endosulfan and imidacloprid are portrayed in Figure 4.

Single cell gel electrophoresis assay (comet assay) was used to evaluate DNA damage in zebrafish livers. Dose- and time-dependent increases in DNA damage were observed in zebrafish livers. Prolonged exposure increased the OTM, with the highest OTM being observed in T3 at day 21. Lipid peroxidation products and ROS produced as a result of oxidative stress can induce DNA damage. Olive tail moments in zebrafish liver tissues exposed to different treatments are shown in Figure 4. Control groups of all three sampling intervals showed the same value of per cent DNA in the head, i.e., 99.9% which shows intact DNA. DNA content in the tail increased as the pesticide concentration and exposure time period increased and this could be attributed to the factor of synergistic effects of both insecticide mixtures.

DNA damage is quite a suitable biomarker in toxicological studies assessed through comet assay. Oxidative stress and lipid peroxidation lead to the production of alkyl-free radicals which are also accountable for DNA damage. Tail length and per cent DNA in the tail gradually increased as the concentration of pesticides increased. Increase in OTM was also observed after 21 days' exposure to high concentrations of pesticides (endosulfan and imidacloprid). These results are inconsistent with the findings reported by Ge *et al.* (2015).

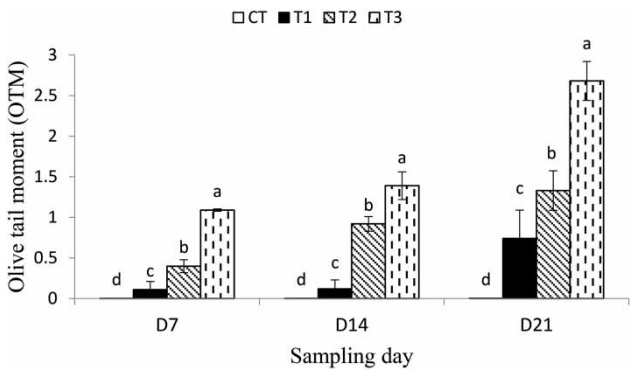

Figure 4 | Combined effects of endosulfan and imidacloprid on olive tail moments in liver tissues of zebrafish: CT represents control treatment without exposure to pesticides; T1 represents treatment 1 having low concentration of endosulfan (0.1 μg/L) and 1 ppm imidacloprid; T2 represents treatment 2 having endosulfan concentration (0.5 μg/L) and 1 ppm imidacloprid; T3 represents treatment 3 having high concentration of endosulfan (1 μg/L) and 1 ppm imidacloprid. Each bar represents the mean of three replicates ± standard deviation (SD). Bars denoted with different lowercase letters indicate significant differences between treatments (Duncan's post hoc test, $p \leq 0.05$).

DNA damage may be induced by biochemical processes, lipid peroxidation products, pesticide concentration and oxygen-free radicals. It has already been reported that significant increase in OTM was observed with increasing concentration of endosulfan in both male and female zebrafish (Shao *et al.* 2012), which is in accordance with the results of the current research. It has been revealed that endosulfan can induce genotoxicity in cultured hepatic cells by forming DNA adducts and high endosulfan-mediated genotoxicity was observed in rats and human cells (Dubois *et al.* 1996). Therefore, we may infer that at high endosulfan and imidacloprid concentrations, formation of DNA adducts could be the cause of DNA damage during the undertaken research, which can be an indication of combined toxicity showing synergistic effects. Dose-dependent response in DNA damage was observed in zebrafish hepatopancreas that was exposed to atrazine (Zhu *et al.* 2011). These results show similarity with the data obtained from the current study.

Tail length is an important parameter to investigate the magnitude of DNA damage as the damaged DNA migrates from the head towards the tail, and consequently tail length increases. In the present study, owing to synergistic effects, length increased because of increased migration of damaged DNA as the concentration of pesticide mixture and exposure time increased.

Effects on histopathological changes in gills and muscles

Hyperplasia, lifting of epithelia, fusion of secondary lamellae and narrowed water channels were observed as a result of pesticide exposure as shown in Figure 5. Moreover, marked lesions were observed in high concentration groups after prolonged exposure. After 21 days of exposure, a significant reduction in primary and secondary lamellae occurred and a higher degree of sloughing was observed, as shown in Figure 5. The findings illustrate that a constant exposure to pesticides for a longer duration leads to the deleterious effects.

Muscles of control groups at all sampling intervals showed normal morphology with the nucleus at the

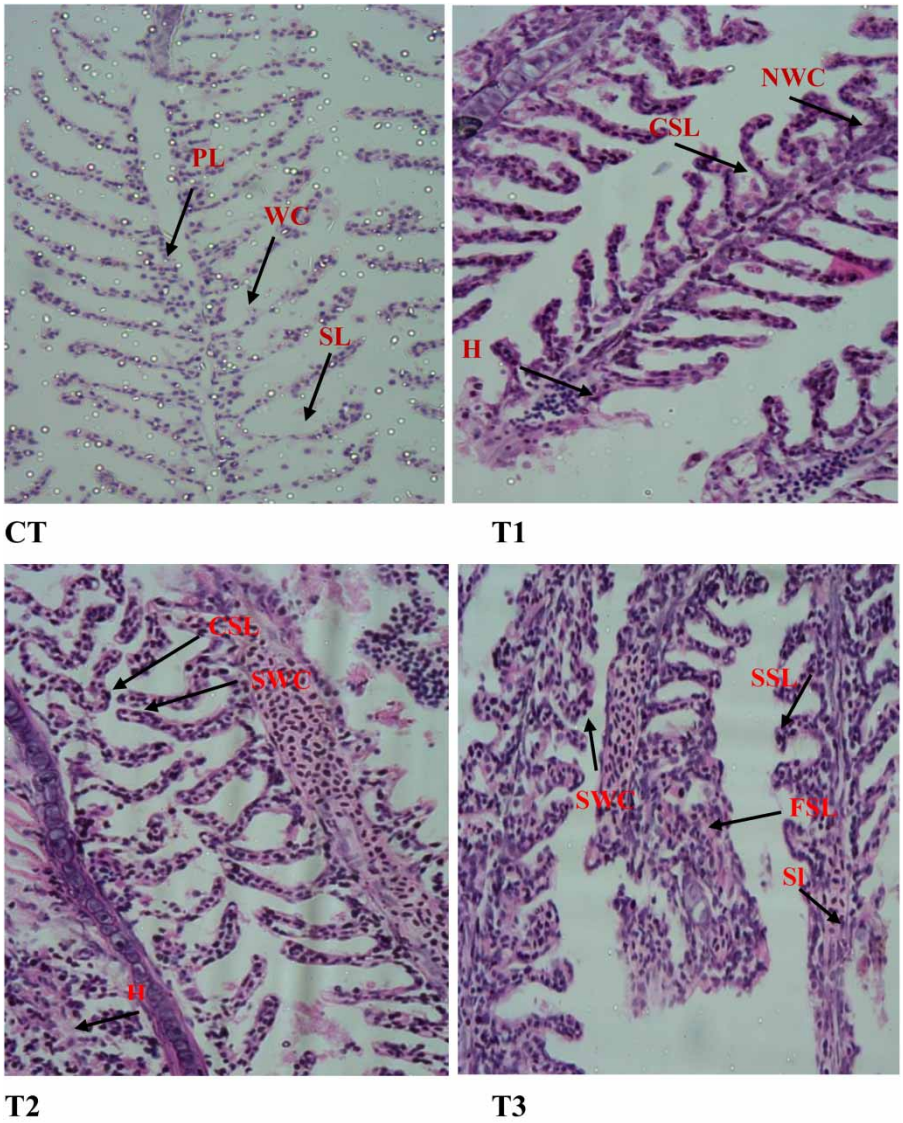

Figure 5 | Photomicrographs of gill sections of zebrafish H&E stained, (400× magnification) after 21 days of exposure. CT (control treatment): SL, secondary lamellae; PL, primary lamellae; WC, water channel. T1 (treatment group having lower concentration (endosulfan 0.1 μg/L and 1 ppm imidacloprid)): CSL, curling of secondary lamellae; NWC, narrowed water channels; H, hyperplasia. T2 (treatment group having medium concentration (endosulfan 0.5 μg/L and 1 ppm imidacloprid)): H, hyperplasia; SWC, shortening of water channels; CSL, curling of secondary lamellae. T3 (treatment group having high concentration (endosulfan 1 μg/L and 1 ppm imidacloprid)): Sl, sloughing of secondary lamellae; SSL, shortening of secondary lamellae; SWC, shortening of water channels; FSL, fusion of secondary lamellae.

periphery of fibres while mild splitting and degeneration of muscle bundles was observed in T2 and T3 after 7 days of exposure, as shown in Figure 6. After 14 and 21 days of exposure, quite significant changes were observed in muscles as can be seen from Figure 6. Severe muscle damage was noticed in the form of atrophy, necrosis, degeneration and splitting of muscle bundles.

Pathological changes in gills can be used as an indicator of water pollution. Hyperplasia, lifting of epithelia, fusion of secondary lamellae and narrowed water channels were observed as results of pesticide exposure due to synergistic effects. Sloughing was also observed in high-dose groups. This uplifting of lamellar epithelium and fusion of secondary lamellae could be the adaptations of gill tissues in order to reduce the contact between pollutants by reducing the surface area of gills. Results of the current research are in accordance with the study presented by Nowak (1992), where hyperplasia, lifting of lamellar epithelium and lamellar hypertrophy were

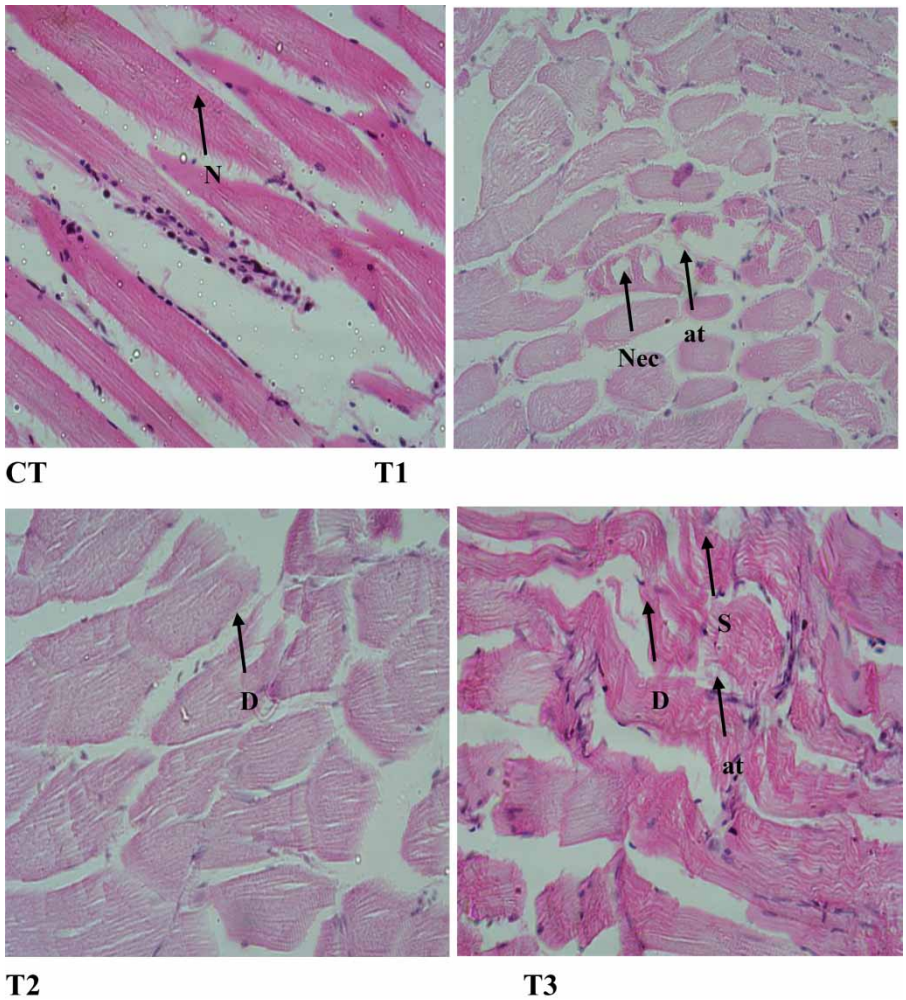

Figure 6 | Photomicrographs of zebrafish muscle sections H&E staining, (400×) after 21 days of exposure. CT (represents the muscles of zebrafish from the control group); T1 (muscles of zebrafish in treatment 1); T2 (muscles of zebrafish in treatment 2); T3 (muscles of zebrafish in treatment 3): N represents the nucleus located at the periphery of muscle fibres; S represents the splitting of muscle fibres; D represents the degeneration of muscle bundles; Nec represents necrosis and at represents atrophy.

observed in the gills of catfish having residues of endosulfan. Results presented by Bhuvaneshwari *et al.* (2015) are in accordance with our findings. Histopathological damage previously noticed in the gills of rainbow trout is in accordance with our findings and was induced by chronic exposure to diazinon at concentrations of 0.1 mg/L and 0.2 mg/L (Banaee *et al.* 2013). Low doses of pesticides did not induce any significant alterations in muscles while high doses and prolonged exposures induced splitting and degeneration of muscle bundles; slight atrophy was also observed in muscle bundles and this could be attributed to the combined effects of both insecticides in terms of synergistic impacts. Results reported by Bhuvaneshwari *et al.* (2015) are in accordance with our findings where the same alterations were produced when zebrafish were exposed to a mixture of organochlorine pesticides. Splitting of muscle fibres, disintegration of muscle bundles, focal area of necrosis, vacuolar degeneration in muscle bundles were the histopathological alterations observed in tilapia (*Orechromis mossambicus*) exposed to textile dyes (0.5, 1 and 1.5 ppm) for 21 days (Sripriya *et al.* 2014), and these findings are found to be similar to our observations.

Practical applications

Fish are an essential component of the aquatic environment and affected by pesticides which pollute natural water through agricultural runoff and various other means. The polluted water can result in the death of fish or decreasing

their numbers and other sensitive aquatic fauna in the food web resulting in an ecological imbalance. With the help of this study, we will be able to identify the oxidative stress and antioxidant levels which are supposed to be good indicators of aquatic pollution. The present work will offer valuable information related to the possible impact on biochemical parameters of zebrafish. These findings can also contribute to understanding the effect of mixture toxicity on the growth rate, feeding efficiency, survival and production of fish. Moreover, our study can also provide valuable support to fishery managers and aquaculturists in order to improve the water quality for culturing or growing fish. However, the improper use of pesticides can result in adverse harmful effects on fish; thus this study could be used for creating awareness among local farmer communities so that the indiscriminate use of these pesticides could be reduced. This information can also be useful for policy makers, the agro-chemical and fish industry, academia and the public at large.

CONCLUSIONS AND FUTURE RESEARCH PROSPECTS

Tainted water is responsible for the weak growth, disease outbreak, reproduction efficiency and also for survival and death of fish. To our knowledge, this is the first study to report the combined effects on oxidative stress and histopathology in zebrafish exposed to endosulfan and imidacloprid present in water. Successively, it is inferred that both pesticides induced oxidative stress in the liver of zebrafish which leads to lipid peroxidation resulting in DNA damage. The present study revealed that the pesticides stimulated the antioxidant enzyme activities (CAT and SOD) at low concentrations, but high concentrations of pesticides and prolonged exposure duration decreased the activity of both enzymes. As the antioxidant enzyme activity decreased, MDA content increased, which enhanced the DNA damage because of the excessive ROS. MDA content increased with increased exposure time and pesticide concentration, which demonstrated increased lipid peroxidation in cellular bodies. Similarly, a substantial increase in DNA damage was noticed after 21 days' exposure to pesticides. Based on the present study, it may be concluded that environmental pollution can be evaluated by measuring biochemical responses in organisms against stress. In summary, three biomarkers, oxidative stress, DNA damage and pathological changes may be helpful to understand the mechanism of the combined toxicity of endosulfan and imidacloprid. All the negative effects of pesticides must be taken into account while using these agrochemicals for insect-pest control in agricultural fields surrounding freshwater ecosystems. Thus, it is recommended that proper maintenance and measures are necessary in order to keep these water bodies clean and safe. Since many pesticides coexist and mixtures of pesticides are usually detected in the natural environment, additional work is required to understand the research into pesticide interaction towards non-target organisms. In the future, higher concentrations of pesticides may be expected due to their increasing use and a higher risk to aquatic organisms is anticipated. In the case that pesticides are accidentally spilled into small water bodies, their predicted environmental concentrations would affect less sensitive organisms (chronically) and more sensitive aquatic invertebrates (acutely). Henceforth, this work suggests that further (eco) toxicological studies with a broader spectrum of aquatic organisms belonging to different taxonomic groups and trophic levels need to be performed.

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