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# Toxicity to Lead, Cadmium and Copper in nymphs of three Odonate species

**Ikram Ilahi[1*], Ali Muhammad Yousafzai[2], Taqweem Ul Haq[3], Abdur Rahim[1], Muhammad Attaullah[1] and Dil Naz[1]**

[1]Department of Zoology, University of Malakand, Chakdara, Dir Lower, Khyber Pakhtunkhwa, **Pakistan**
[2]Department of Zoology, Islamia College Peshawar, Peshawar, Khyber Pakhtunkhwa, **Pakistan**
[3]Department of Biotechnology, University of Malakand, Chakdara, Dir Lower, Khyber Pakhtunkhwa, **Pakistan**

***Correspondence:** ikramilahi@uom.edu.pk **Received 25 -07-2020, Revised: 03-11-2020, Accepted: 13-11-2020 e-Published: 15-11-2020**

The present research aimed to investigate the effect of seven days exposure to lead (Pb), cadmium (Cd) and copper (Cu) on survival and feeding rate of nymphs of three odonate species i.e., *Ischnura elegans*, *Trithemis aurora* and *Pantala flavescens*. During this study, the nymphs of all the three odonate species survived when exposed separately to 40 ppm each of Pb and Cd, and 10 ppm of Cu. Cu appeared most toxic. The $LC_{50}$ values of Cu against *I. elegans, T. aurora* and *P. flavescens* was 148.2, 101.8 and 173.6 ppm, respectively. The feeding rate of *I. elegans* and *T. aurora* nymphs when separately exposed to sublethal concentration of Pb and Cd (40 ppm each) was not different significantly ($P>0.05$) from control nymphs. However, the feeding rate of *I. elegans* and *T. aurora* nymphs exposed to the sublethal concentration of Cu (10 ppm) was significantly lower ($P<0.05$) from the control nymphs. The feeding rate of *P. flaviscans* nymphs when exposure to the sublethal concentration of any of the three metals was significant lower ($P<0.05$) than control nymphs. It is concluded that among Pb, Cd and Cu, the Cu is most toxic to *I. elegans*, *T. aurora* and *P. flavescens* nymphs. It is further concluded that among Pb, Cd and Cu, the sublethal concentration of Cu significantly reduces the feeding rate of *I. elegans*, *T. aurora* and *P. flavescens* nymphs.

**Keywords: heavy metals, water contamination, toxicity, lethal concentration, damselfly nymphs, dragonfly nymphs**

## INTRODUCTION

Damselflies belong to the order Odonata and sub order Zygoptera, while dragonflies belong to the sub order Anisoptera of the order Odonata. They are medium to large size flying insects (Rehen, 2001). They do not undergo complete metamorphosis. Their larvae are aquatic and are called naiads or nymphs. Their nymphs occur both in stagnant as well as running water as well as in stagnant water (Zia, 2010). Damselfly larvae are mostly long and slender with three long respiratory gills attached to the posterior (Nair, 2011). Dragonfly nymphs breathe through gills in their rectum and can rapidly propel themselves by suddenly expelling water through the anus (Mill and Pickard, 1975). The nymphs of both damselflies and dragonflies are voracious predators and they capture their prey such as mosquito larvae and other smaller aquatic invertebrates and even larvae of fish and amphibians with the help of specialized protractible labium (Boyd, 2005).

Odonate nymphs play important role in the regulation of mosquito population (Din et al. 2013). Due to predatory role against mosquito larvae, these naiads have gained attention for their use in ecofriendly control of vectors of malaria, dengue and yellow fever (Mitra, 2006).

Odonate nymphs are considered good biological control agent against mosquitoes (Chatterjee et al. 2007). Odonate nymphs face serious threats including urbanization and water pollution. The major sources of water pollution arise from anthropogenic activities, industrial effluents and agricultural practices (Hunt et al. 2004). Environmental pollutants adversely affect the damselfly and dragonfly nymphs (Clark and Samways, 1996).

There is increasing concern about the wide distribution of heavy metals in the environment due to their constant application in agriculture, industries, domestic activities and medical technologies. Their wide distribution in the environment affect human health and the survival of other organisms (Tchounwou et al. 2014). Some heavy metals such as cadmium (Cd), mercury (Hg) and lead (Pb) have got attention due their potential adverse effect on environment and human health (Ilyin et al. 2004; Pacyna et al. 2009). Heavy metals exist either in chemical compounds or in elemental form (Ilyin et al. 2004). Heavy metals are responsible for damaging cellular membranes, deoxyribonucleic acids (DNA) and important enzymes involved in repair, detoxification and metabolism (Wang and Shi, 2001; Beyersmann et al. 2008). Surface water receives heavy metals from industries in effluent, agricultural activities, mining and natural weathering of rocks (Shazili et al. 2006; Dragun et al. 2009). Heavy metals accumulate in animal tissues and their increasing level is toxic to animals (Villalobos-Jiménez et al. 2016). Heavy metal contamination in water has resulted in reduction of species diversity (Gunn, 1995). Metals and metalloid contamination also exert adverse impact on insect behaviors including feeding behavior (Faria et al. 2006; Mogren and Trumble, 2010; Burden et al. 2019).

To the author knowledge, few studies have been conducted on toxicity of heavy metals against odonate nymphs. For example, Tollett et al. (2009) studied the effect of heavy metals exposure on the nymphs of two odonate species such as *Pachydiplax longipennis* and *Enallagma simplicicollis*. During their study, exposure to lead caused no appreciable mortality in either species at concentrations below 185 ppm, while exposure to cadmium caused no appreciable mortality in either species at concentrations below 100 ppm. During exposure to copper, mortality of odonate nymphs occurred at concentrations above 150 µg /L. Metals and metalloid contamination also exert adverse impact on insect behaviors including feeding behavior (Faria et al. 2006; Mogren and Trumble, 2010; Burden et al. 2019).

In Pakistan, a reduction in Odonata population has been observed during a country wide surveys conducted for five consecutive years (Zia, 2010). A noticeable reduction in odonate diversity during a period of one decade has occurred (Zia et al. 2011a). Deforestation, urbanization and contamination of water bodies are the biggest threats to Odonata (McKinney, 2008; Dudgeon, 2010). Keeping in view, the adverse impact of heavy metals on aquatic organisms, a laboratory study was conducted for investigating the effect of heavy metals such as Pb, Cd and Cu on the survival and feeding rate of nymphs of three native odonate species i.e., blue tailed damselfly (*Ischnura elegans*), crimson marsh glider dragonfly (*Trithemis aurora*) and globe skimmer dragonfly (*Pantala flavescens*).

## MATERIALS AND METHODS

### Collection of Odonate Nymphs

Several puddles on the bank of River swat near the campus of University of Malakand, were visited for collection of damselfly and dragonfly nymphs. Damselfly and dragonfly nymphs of different instars were collected by using a rectangular polyethylene dipper (30 cm × 20 cm × 5 cm). The nymphs were transported in plastic jars containing water of the collection site to the laboratory at University of Malakand within one hour of capture. In the laboratory damselfly and dragonfly nymphs were separately maintained in rectangular polyethylene containers (38 cm × 28 cm × 6.5 cm) containing water brought from the collection site in large plastic bottles. Each container was receiving solar illumination through windows and oxygenated by using air pumps. Few strings of aquatic plants brought from the collection site were also added to the containers which provided clinging sites for the nymphs. Before conducting experiments, the nymphs were fed with mosquito larvae.

### Identification of damselfly and dragonfly nymphs

The specimens were identified to the species level with the help of literature (Yousuf et al. 1996, Anjum, 1997; Mitra, 2002; Din et al. 2013). During the identification of odonate nymph, help was also taken from the literature and unpublished documents provided by Dr. Ahmad Zia (personal communication), Senior Scientific Officer and Taxono

mist in the Insect National Museum at National Agricultural Research Council (NARC), Islamabad, Pakistan. One species of damselflies (order Odonata, sub order Zygoptera, family *Coenagrionidae*) namely *Ischnura elegans* (Vander Linden, 1820) and two species of dragonflies (order Odonata, sub order Anisoptera, family Libellulidae) namely *Trithemis aurora* (Burmeister, 1839) and *Pantala flavescens* (Fabricius, 1798) were identified and found in sufficient number; therefore, experiments were conducted on nymphs of these species. Before conducting experiments, the nymphs were fed with dried yeast powder and mosquito larvae.

During the present research, the toxicity of heavy metals such as Pb, Cd and Cu against *I. elegans*, *T. aurora* and *P. flavescens* were studied during seven days exposure in the laboratory. The collection of nymphs and experiments were conducted during April-May 2017. The maximum laboratory temperature was 19-25$^0$C during experiments.

**Exposure of Odonate nymphs to Lead (Pb)**

**Preparation of Pb solutions**

Lead sulphate ($PbSO_4$) was used for preparation of stock solution of Pb. The molecular weight of one mole of $PbSO_4$ is 303.2 g which contains 207.2 g of Pb. For preparation of stock solution of 1000 ppm (1000 mg/L) of Pb, 1.46 gram of $PbSO_4$ was dissolved in volumetric flask in which some non-chlorinated tap water was already added. Then further non-chlorinated tap water was added to make a volume of 1000 ml. From the stock solution, testing solutions of different concentrations were prepared by applying the dilution equation (C1V1= C2V2).

**Preliminary bioassay for determining concentration ranges of Lead (Pb)**

Odonate nymphs i.e., nymphs of *I. elegans*, *T. aurora* and *P. flavescens* were initially exposed to Pb solutions of 1, 2, 5, 10, 20, 40 and 75 ppm concentrations in 400 ml polyethylene containers for finding concentration range to be used for determining the LC50 values. The ecological effects test guidelines of Environmental Protection Agency, USA (EPA, 1996) for aquatic invertebrate acute toxicity test were followed with some modifications for determining the concentration range. Volume of testing solution in each polyethylene container was 250 ml. A 400 ml polyethylene control container containing 250 ml non-chlorinated tap water was also kept. Nymphs of each species were exposed individually in containers to avoid cannibalism. Due to individual placement, limited laboratory space and limited availability of nymphs, the number of nymphs to be exposed at a time was kept restricted. The method of Hardersen and Wratten (1996) was followed as they faced the same problem of cannibalism, limited availability of odonate nymphs and limited laboratory space. The detail is as under: eight intact 8$^{th}$ to 10$^{th}$ instar nymphs of each species were placed in eight plastic containers (seven concentrations of Pb based on molar mass of Pb component of $PbSO_4$ and one control). In short, 24 containers were arranged for the nymphs of three odonate species, eight for each species. Experiment was run in five replicates. The period of exposure was 7 days. Following standard toxicity protocols, the nymphs were not fed during the 7 days exposure (ASTM standard E47, 2008). At day 7$^{th}$ of exposure period, the numbers of dead and live nymphs were noted. The criterion for death was lack of response to prodding. During preliminary exposure, there occurred no mortality of nymphs up to 40 ppm. During preliminary experiments, no mortality of nymphs occurred up to 40 ppm Pb solution, however the Pb solutions of 75 ppm caused mortality of nymphs. Therefore, further experiments were performed using high concentrations i.e., 75, 150, 300 and 500 ppm. The schematic of this bioassay has been shown in figure 1.

**Definitive bioassay for toxicity study of Lead (Pb)**

For each odonate nymph species, four containers containing 75, 150, 300 and 500 ppm of Pb solutions were arranged. A control container was also kept. Five intact last instar nymphs of each species were placed individually in their respective five containers (four concentrations and one control). Experiment was run in triplicate. After 7 days of exposure, the number of dead and surviving nymphs were noted. Several trips were conducted for collection of nymphs and experiments were repeated continuously till the number of nymphs in each replica for each concentration reached 20. In total 20 independent experiments were conducted. The schematic of this bioassay has been shown in figure 2.

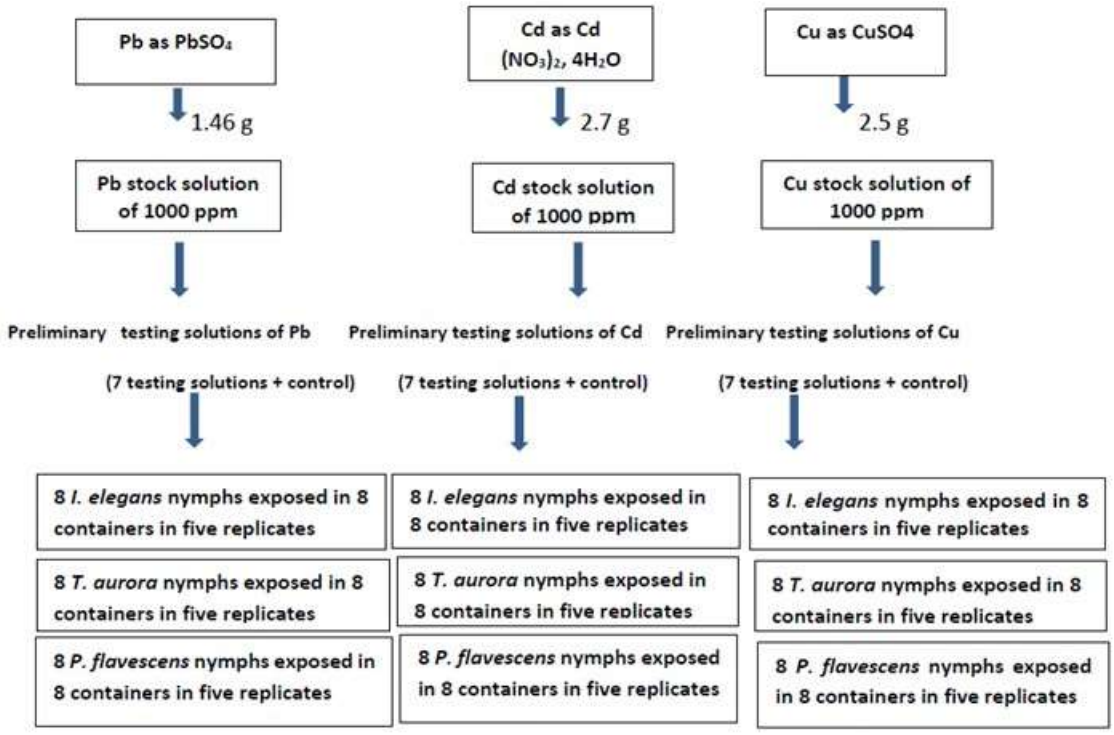


**Figure 1**: **Schematic of experiments for preliminary bioassays**.

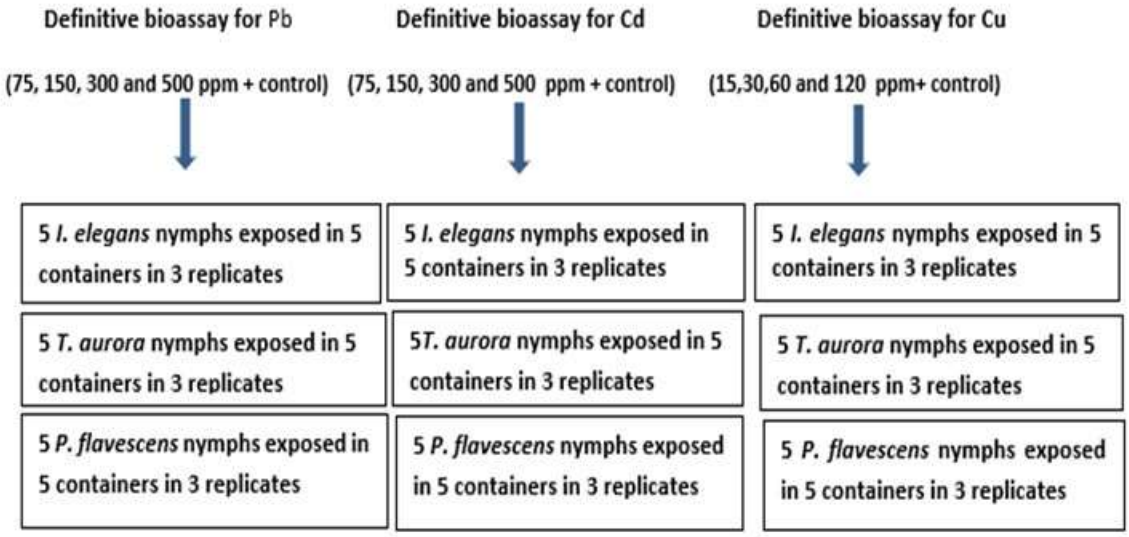


**Figure 2:Schematic of experiments for definitive bioassays**

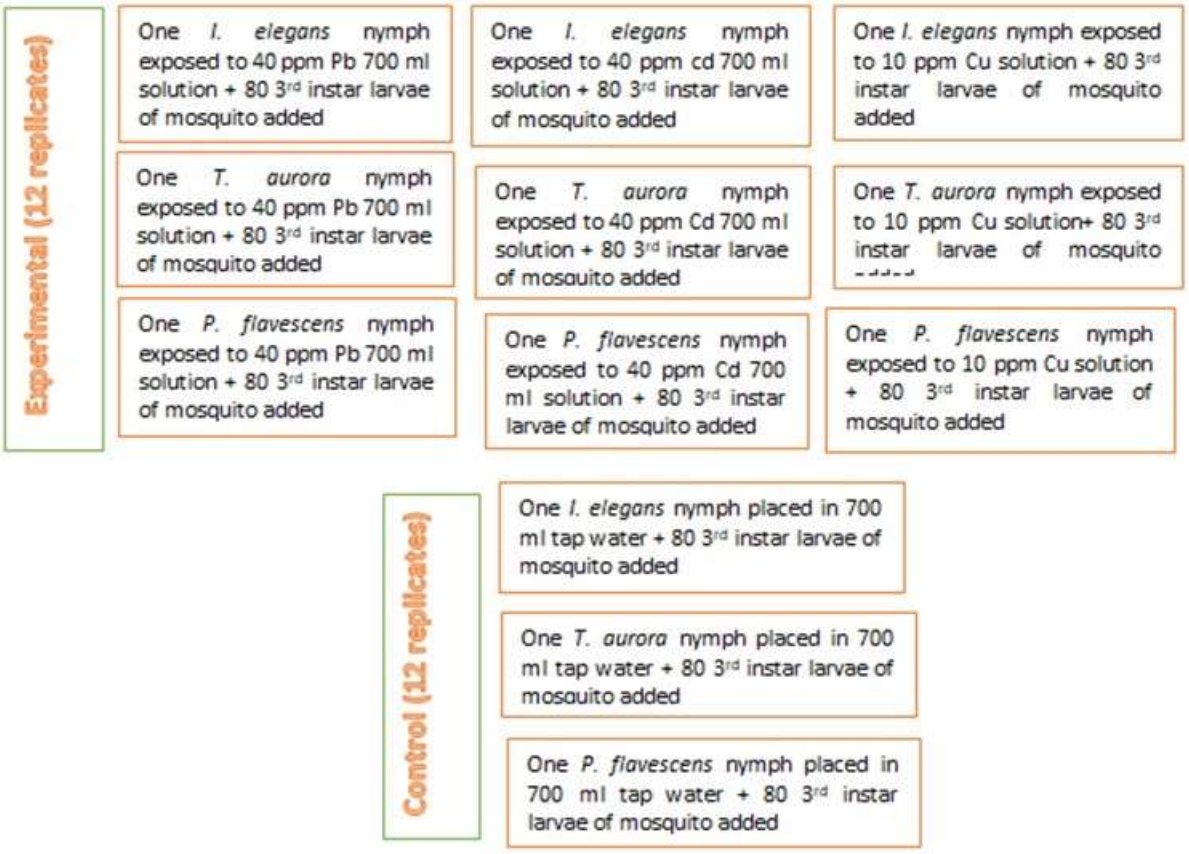


**Figure 3: The schematic of experiment for the effect of heavy metal exposure on the feeding rate of Odonate nymphs {note for editor: This figure has been corrected, please consider this figure but ignore the one in uncorrected proof because a mistake was identified and now correction has been made)**

**Exposure of Odonate nymphs to Cadmium (Cd)**

**Preparation of Cadmium (Cd) solutions**

Cadmium nitrate tetra hydrate ($Cd(NO_3)_2$, $4H_2O$) was used for making stock solution of Cd. The molecular weight of one mole of $Cd(NO_3)_2$, $4H_2O$ is 308.4 g which contains 112.4 g of Cd. For preparation of stock solution of 1000 ppm (1000 mg/L) of Cd, 2.7 gram of $Cd(NO_3)_2$, $4H_2O$ was dissolved in volumetric flask in which some non-chlorinated tap water was already added. Then further non-chlorinated tap water was added to make a volume of 1000 ml. From the stock solution, testing solutions of different concentrations were prepared.

**Preliminary bioassay for determining concentration ranges of Cadmium (Cd)**

Nymphs of *I. elegans*, *T. aurora* and *P. flavescens* were initially exposed to Cd solutions of 1, 2, 5, 10, 20, 40 and 75 ppm concentrations in 400 ml polyethylene containers for finding concentration range to be used for determining the LC50 values. During preliminary experiments, no mortality of nymphs occurred up to 40 ppm Cd solution, however the Cd solutions of 75 ppm caused mortality of nymphs as observed in case of Pb. The detail of this preliminary experiment is the same as describe for Pb. The schematic of this bioassay has been shown in figure 1.

**Definitive bioassay for toxicity study of Cadmium (Cd)**

For each Odonate nymph species, four containers containing 75, 150, 300 and 500 ppm of Cd solutions were arranged. A control container was also kept. The detail of this definitive bioassay is the same as describe for Pb. The schematic of this bioassay has been shown in figure 2.

**Exposure of Odonate nymphs to Copper (Cu)**

**Preparation of Cu solutions**

Copper sulphate anhydrous (CuSO4) was used for making stock solution of Cu. The molecular weight of one mole of CuSO4 is 159.55 g which contains 63.55 g of Cu. For preparation of stock solution of 1000 ppm (1000 mg/L) of Cu solution, 2.5 gram of CuSO4 was dissolved in volumetric flask in which some non-chlorinated tap water was already added. Then further non-chlorinated tap water was added to make a volume of 1000 ml. From the stock solution, testing solutions of different concentrations were prepared by applying the dilution equation C1V1= C2V2.

**Preliminary bioassay for determining concentration ranges of Copper (Cu)**

Nymphs of *I. elegans*, *T. aurora* and *P. flavescens* were initially exposed to Cu solutions of 1, 2, 5, 10, 20, 40 and 75 ppm concentrations in 400 ml polyethylene containers for finding concentration range to be used for determining the LC50 values. During preliminary experiments, no mortality of nymphs occurred up to 10 ppm Cu concentration, however the Cu solutions of 20 ppm caused mortality of nymphs. Therefore, further experiments were performed using high concentrations i.e., 15, 30, 60 and 120 ppm. The rest of detail of this bioassay is the same as described for Pb. The schematic of this bioassay has been shown in figure 1.

**Definitive bioassay for toxicity study of Copper (Cu)**

To calculate LC50, for each odonate nymph species, five polyethylene containers (four for Cu concentrations i.e., 15, 30, 60 and 120 ppm and one control container) were arranged. In short, 15 containers were arranged for the nymphs of three odonate species, five for each species. Five intact last instar nymphs of each species were placed individually in five containers (four concentrations and one control). The rest of detail of this bioassay is the same as described for Pb. The schematic of this bioassay has been shown in figure 2.

**Effect of exposure to heavy metals on feeding rate of donate *nymphs***

During this study, the effect of short-term exposure to heavy metals in water on the feeding rate of odonate nymphs was studied at sublethal concentrations. During the above experiments, the highest concentration of Pb and Cd that caused no mortality of any odonate nymphs was 40 ppm, each. Similarly, the highest concentration of Cu that caused no mortality of any odonate nymphs was 10 ppm. During this experiment (conducted in April 2020), three nymphs of the three odonate species (one nymph of each species) i.e., *I. elegans*, *T. aurora* and *P. flavescens*, were separately exposed (as described during the above experiments) to 40 ppm solution of Pb in three 1000 ml plastic containers, one container for each species. The volume of this metal ion solution in each plastic container was 700 ml. At the same time, 80 third instar larvae of *Culex quinquefasciatus* mosquito were added to each container. The experiment was conducted in 12 replicates. During this experiment, the nymphs of the same three species were also exposed to 40 ppm solution of Cd and 10 ppm solution of Cu and then 3rd instar larvae of *Culex quinquefasciatus* were added. The experimental details were the same as described for Pb. In control, the nymph of each species was separately placed in 1000 ml plastic container containing 700 ml tap water and then 80 3rd instar larvae were added to each container. This experiment was started at 06:00 hour of Pakistan standard time. The number of mosquito larvae consumed by each odonate nymph was noted after 24 hours i.e., next day at 06:00 hour. The larvae which were not consumed were removed from each container and again 80 3rd instar larvae of *Culex quinquefasciatus* were added to each container and the number of larvae consumed by each odonate nymph was counted after next 24 hours. This procedure was repeated for 12 consecutive days (considered 12 replicas). The schematic of this experiment has been shown in figure 3

**Statistical analysis of data**

The percent mortality of nymphs in each replica of each concentration of each heavy metal was calculated from cumulative total of 20 nymphs after 20 independent experiments (single nymph exposed to each concentration during each experiment). The average percent mortality data were subjected to log probit analysis (Finney, 1971) for calculating $LC_{50}$ values. The $LC_{50}$ values were compared by 95 % confidence limits overlap method of Wheeler et al. (2006). The mean percentages of survived nymphs were compared by Tukey test in one-way anova. Similarly, the 24-hour feeding rates of nymphs exposed to sublethal concentration of Pb, Cd and Cu ions in

aqueous medium were compared to the 24-hour feeding rate of nymphs in control by applying Tukey test in one-way anova. For these analysis, SPSS 16 software was used.

## RESULTS

### Preliminary bioassay for determining concentration ranges of Lead (Pb)

During this experiment, the *I. elegans*, *T. aurora* and *P. flavescens* nymphs were exposed to 1, 2, 5, 10, 20 and 40 ppm concentrations of Pb in aqueous medium for determining concentration ranges to be used for definitive test (Table 1). During this study, even the highest concentration of Pb i.e., 40 ppm caused no mortality of odonate nymphs.

### *Definitive bioassay for toxicity study of Lead (Pb)*

Based on the result of preliminary experiment, the odonate nymphs were exposed to higher concentrations (75,150,300 and 500 ppm) of Pb for determining the $LC_{50}$ values of Pb against odonate nymphs. Table 2 shows the mean percent mortality of odonate nymphs of each species from 20 independent experiments (each experiment conducted in triplicate). The lowest concentration of Pb that caused mortality of predators of mosquito larvae was 75 ppm. At 75 ppm, 10 % or < 10 % mortality of nymphs of three different predator species was observed. Susceptibility of different odonate species during exposure to Pb was not the same. For example, at 75 ppm concentration of Pb, minimum mortality (5.0 $\pm$ 0 %) was observed in *P. flaviscans* followed by *I. elegans* (6.6 $\pm$ 1.6 %) and *T. aurora (*10.0 $\pm$ 2.8 %). Similar trend was noted during exposure to higher concentrations. Table 3 shows the $LC_{50}$ values of Pb against odonate nymphs during definitive bioassay for toxicity study of Pb against different odonate nymphs. Maximum $LC_{50}$ value was shown observed for *P. flaviscans* ($LC_{50}$= 923.9 ppm) followed by *I. elegans* ($LC_{50}$= 762.7 ppm) and *T. aurora (*$LC_{50}$= 712.1 ppm).

The calculation of surviving odonate nymphs was made for highest concentration (500 ppm) of Pb during definitive bioassay for toxicity study of Pb (Figure 4). No significant difference in percentage of survived nymphs was observed among the three species. However, insignificantly ($P>0.05$) higher percentage (65.0±7.6 %) of nymphs that survived was belonging to *P. flaviscans* species followed by *I. elegans* (58.3±4.4 %) and *T. aurora* (56.6±8.8 %).

**Table 1:The results of preliminary bioassay for determining concentration ranges of Pb, Cd and Cu for conducting definitive bioassay**

| Metals | Highest concentration (ppm) that caused no mortality | Lowest concentration (ppm) that caused mortality |
|---|---|---|
| Pb | 40 | 75 |
| Cd | 40 | 75 |
| Cu | 10 | 15 |

**Table 2: Seven days mortality data of odonate nymphs during definitive bioassay for toxicity study of lead (Pb)**

| Lead Concentration (ppm) | Mean % Mortality ± SE | | |
|---|---|---|---|
| | I. elegan | T. aurora | P. flaviscans |
| 0 (control) | 0 | 0 | 0 |
| 75 | 6.6 ± 1.6 | 10.0 ± 2.8 | 5.0 ± 0.0 |
| 150 | 10.0 ± 2.8 | 13.3 ± 1.6 | 8.3 ± 1.6 |
| 300 | 21.6 ± 4.4 | 28.3 ± 3.3 | 20.0 ± 2.8 |
| 500 | 41.6 ± 4.4 | 43.4 ± 8.8 | 35.0 ± 7.6 |

**Table 3: Median lethal concentration ($LC_{50}$) values of lead (Pb) against different odonate nymphs**

| Lead Concentration (ppm) | Mean % Mortality ± SE | | |
|---|---|---|---|
| | ***I. elegan*** | ***T. aurora*** | ***P. flaviscans*** |
| 0 (control) | 0 | 0 | 0 |
| 75 | 6.6 ± 1.6 | 10.0 ± 2.8 | 5.0 ± 0.0 |
| 150 | 10.0 ± 2.8 | 13.3 ± 1.6 | 8.3 ± 1.6 |
| 300 | 21.6 ± 4.4 | 28.3 ± 3.3 | 20.0 ± 2.8 |
| 500 | 41.6 ± 4.4 | 43.4 ± 8.8 | 35.0 ± 7.6 |

The alphabetical order in column is according to increasing LC50 values. LC50 values sharing no letter are significantly different, SEM-standard error of mean.

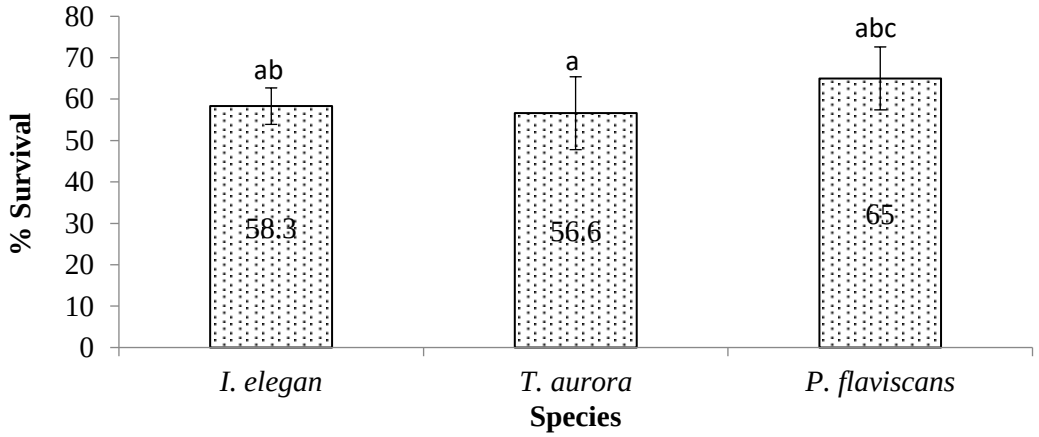


**Figure 4: The percentage of surviving Odonate nymphs during definitive bioassay for toxicity study of lead (Pb)**

**Table 4 : Seven days mortality data of odonate nymphs during definitive bioassay for toxicity study of Cadmium (Cd)**

| Cadmium Concentration (ppm) | Species | | |
|---|---|---|---|
| | **I. elegan** | **T. aurora** | **P. flaviscans** |
| 0 (control) | 0 | 0 | 0 |
| 75 | 10.0 ± 2.3 | 11.6 ± 1.6 | 6.6 ± 1.6 |
| 150 | 16.6 ± 4.4 | 26.6 ± 6.1 | 15.0 ± 2.9 |
| 300 | 30.0 ± 7.6 | 35.0 ± 2.8 | 18.3 ± 1.7 |
| 500 | 46.7 ± 12.01 | 58.3 ± 6.1 | 31.6 ± 4.4 |

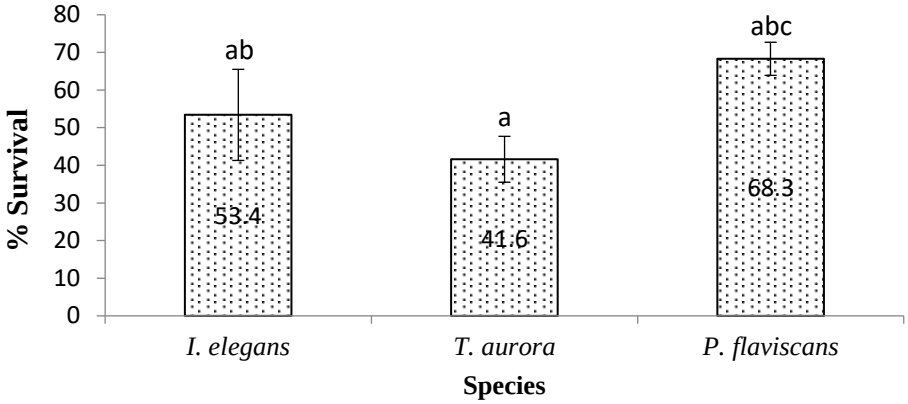


**Figure 5: The percentage of surviving Odonate nymphs during definitive bioassay for toxicity study of Cadmium (Cd)**

This calculation of surviving odonate nymphs was made for highest concentration (500 ppm) of Pb. Alphabetical order is according to increasing mean value. Columns share a letter in common therefore represent insignificant difference at P < 0.05.

**Preliminary bioassay for determining concentration ranges of Cadmium (Cd)**

During this experiment, the *I. elegans*, *T. aurora* and *P. flavescens* nymphs were exposed to 1, 2, 5, 10, 20 and 40 ppm concentrations of Cd in aqueous medium for determining concentration ranges to be used for definitive test (Table 1). During this study, even the highest concentration of Cd i.e., 40 ppm caused no mortality of odonate nymphs.

**Definitive bioassay for toxicity study of cadmium (Cd)**

Based on the result of preliminary experiment, the odonate nymphs were exposed to higher concentrations (75,150,300 and 500 ppm) of Cd for determining the $LC_{50}$ values of Cd against odonate nymphs. Table 4 shows the mean percent mortality of odonate nymphs of each species from 20 independent experiments (each experiment conducted in triplicate). The lowest concentration of Cd that caused mortality of nymphs was 75 ppm. At 75 ppm, < 12 % mortality of nymphs of three different predator species was observed. Susceptibility of different odonate species during exposure to Cd was not the same. For example, at 75 ppm concentration of Cd, minimum mortality (6.6 $\pm$ 1.6 %) was observed in *P. flaviscans* followed by *I. elegans* (10.0 $\pm$ 2.3 %) and *T. aurora (*11.6 $\pm$ 1.6 %). Similar trend was noted during exposure to higher concentrations. For example, at highest concentration (600 ppm), minimum mortality was observed in nymphs of *P. flaviscans* (31.6 $\pm$ 4.4 %) followed by *I. elegans* (46.7 $\pm$ 12.01 %) and *T. aurora (*58.3 $\pm$ 6.1 %), respectively. Table 5 shows the $LC_{50}$ values of Cd against odonate nymphs during definitive bioassay for toxicity study of Cd against different odonate nymphs. Maximum $LC_{50}$ value was shown by *P. flaviscans* (1441.7 ppm) followed by *I. elegans* (618.4 ppm) and *T. aurora (*417.6 ppm).

The calculation of surviving odonate nymphs was made for highest concentration (500 ppm) of Cd during definitive bioassay for toxicity study of Cd (Figure 5). No significant difference in mean percentage of surviving nymphs was observed among the three odonate species. Insignificantly (P>0.05) higher percentage of nymphs that survived was belonging to *P. flaviscans* (68.3±4.4 %) species followed by *I. elegans* (53.4±12.1 %) and *T. aurora* (41.3±6.1 %).

This calculation of surviving odonate nymphs was made for highest concentration (500 ppm) of Cd. Alphabetical order is according to increasing mean value. Columns share a letter in common therefore represent insignificant difference at P < 0.05.

**Preliminary bioassay for determining concentration ranges of Copper (Cu)**

During this experiment, the *I. elegans*, *T. aurora* and *P. flavescens* nymphs were exposed to 1, 2, 5, 10, 20 and 40 ppm concentrations of Cu in aqueous medium for determining concentration ranges to be used for definitive test (Table 1). Up

to 10 ppm concentration of Cu, no mortality of nymphs occurred. Exposure to higher concentrations resulted in mortality of nymphs.

**Definitive bioassay for toxicity study of Copper (Cu)**

Based on the result of preliminary experiment, the odonate nymphs were exposed to higher concentrations i.e., 15, 30, 60 and 120 ppm of Cu for determining the $LC_{50}$ values of Cu against odonate nymphs. Table 6 shows the mean percent mortality of nymphs of each odonate species from 20 independent experiments (each in triplicate). The lowest concentration of Cu that caused mortality of odonate nymphs was 15 ppm. At 15 ppm, < 7 % mortality of nymphs of different odonate species was observed. The susceptibility of different odonate species during exposure to Cu was not the same. During exposure to 15 ppm of Cu, minimum mortality (5.0±0.0 %) was observed in *P. flaviscans*. In case of *I. elegans* and *T. aurora*, similar percentage of mortality i.e., 6.6±1.6 %, was observed. At highest concentrations (120 ppm), minimum mortality was observed in *P. flaviscans* (41.6±4.4) followed by *I. elegans* (48.3±7.3) and *T. aurora* (56.7±8.8). Table 7 shows the $LC_{50}$ values of Cd against odonate nymphs during definitive bioassay for toxicity study of Cu against different odonate nymphs. Maximum $LC_{50}$ value was shown by *P. flaviscans* ($LC_{50}$= 173.6 ppm) followed by *I. elegans* ($LC_{50}$= 148.2 ppm) and *T. aurora* ($LC_{50}$= 101.8 ppm). The calculation of surviving odonate nymphs was made for highest concentration (120 ppm) of Cu during definitive bioassay for toxicity study of Cu (Figure 6). No significant difference in mean percentage of survived nymphs was observed among the three odonate species. Insignificantly (P>0.05) higher percentage of survived nymphs was belonging to *P. flaviscans* species (58.3±4.4 %) followed by *I. elegans* (51.7±7.3 %) and *T. aurora* (43.3±8.8 %).

**Comparison of median lethal concentration ($LC_{50}$) values of Pb, Cd and Cu**

Tables 8 shows the comparison of $LC_{50}$ values of Pb, Cd and Cu that were determined during definitive bioassay for toxicity study of Pb, Cd and Cu against three different odonate nymph species i.e., *I. elegans*, *T. aurora* and *P. flavescens*. In case of *I. elegans*, significantly lowest $LC_{50}$ value was observed for Cu ($LC_{50}$= 148.2) followed by Cd (LC50= 618.4) and Pb (LC50= 762.7). Similarly, in case of *T. aurora,* significantly lowest $LC_{50}$ value was observed for Cu ($LC_{50}$= 101.8 ppm) followed by Cd ($LC_{50}$= 417.6 ppm) and Pb ($LC_{50}$= 712.1 ppm). Similarly, during the study of susceptibility of *P. flavescens* to heavy metals, significantly lowest $LC_{50}$ value was observed for Cu ($LC_{50}$= 173.6) followed by Pb ($LC_{50}$= 923.9 ppm) and Cd ($LC_{50}$= 1441.7 ppm).

**Effect of exposure to heavy metals on feeding rate of donate nymphs**

Table 9 shows the effect of short-term exposure of odonate nymphs to sublethal concentration of heavy metal ions i.e., 40 ppm of Pb, 40 ppm of Cd and 10 ppm of Cu on feeding rate. The 24-hour feeding rates of nymphs exposed to sublethal concentration of Pb, Cd and Cu ions in aqueous medium were compared to the 24-hour feeding rate of nymphs in control. The feeding rate of *I. elegans* nymphs exposed to Pb and Cd (at 40 ppm each) was not different significantly (P>0.05) from control *I. elegans* nymphs. However, the feeding rate of *I. elegans* nymphs (consumed 7.2 ± 2.1 larvae/24-hour) exposed to Cu (at 10 ppm) was significantly lower (P<0.05) when compared to control nymphs (consumed 12.2 ± 2.3 3rd instar mosquito larvae/24-hour).

**Table 5: Median lethal concentration ($LC_{50}$) values of Cadmium (Cd) against different odonate nymphs**

| Odonate Species exposed to Cadmium | $LC_{50}$ (ppm) | 95 % confidence limits | Chi-square |
|---|---|---|---|
| *I. elegan* | $618.4^{ab}$ | 459.0-1032.3 | 0.7 |
| *T. aurora* | $417.6^{a}$ | 335.04-577.8 | 2.3 |
| *P. flaviscans* | $1441.7^{abc}$ | 792.7-6160.5 | 1.2 |

The alphabetical order in column is according to increasing LC50 values. LC50 values sharing no letter are significantly different, SEM-standard error of mean

**Table 6: Seven days mortality data of odonate nymphs during definitive bioassay for toxicity study of Copper (Cu)**

| Copper Concentration (ppm) | Species | | |
|---|---|---|---|
| | ***I. elegan*** | ***T. aurora*** | ***P. flaviscans*** |
| **0 (control)** | 0 | 0 | 0 |
| **15** | 6.6 ± 1.6 | 6.6 ± 1.6 | 5.0 ± 0.0 |
| **30** | 15.0 ± 2.8 | 15.0 ± 2.8 | 10.0 ± 2.8 |
| **60** | 20.0 ± 5.8 | 31.7 ± 7.3 | 20.0 ± 5.8 |
| **120** | 48.3 ± 7.3 | 56.7 ± 8.8 | 41.6 ± 4.4 |

**Table 7: Median lethal concentration ($LC_{50}$) values of Copper (Cu) against different odonate nymphs**

| ***Odonate Species exposed to Copper*** | **$LC_{50}$ (ppm)** | **95 % confidence limits** | **Chi-square** |
|---|---|---|---|
| ***I. elegan*** | $148.2^{ab}$ | 110.3-239.4 | 3.2 |
| ***T. aurora*** | $101.8^{a}$ | 83.1-134.9 | 0.34 |
| ***P. flaviscans*** | $173.6^{abc}$ | 126.2-296.8 | 0.7 |

The alphabetical order in column is according to increasing $LC_{50}$ values. $LC_{50}$ values sharing no letter are significantly different, SEM-standard error of mean.

**Table 8: Comparison of median lethal concentration (LC50) values of Pb, Cd and Cu against odonate nymphs**

| **Metals** | ***I. elegans*** | ***T. aurora*** | ***P. flavescens*** |
|---|---|---|---|
| Lead | 762.7 (555.5-1330.48) b | 712.1 (509.8-1301.8) c | 923.9 (638.9-1848.8) b |
| Cadmium | 618.4 (459.0-1032.3) b | 417.6 (335.04-505.8) b | 1441.7 (792.7-6160.5) b |
| Copper | 148.2 (110.3-239.4) a | 101.8 (83.1-134.9) a | 173.6 (126.2-296.8) a |

The alphabetical order in columns is according to increasing $LC_{50}$ values. $LC_{50}$ values in column sharing no letter are significantly different.

**Table 9: The effect of short-term exposure of odonate nymphs to sublethal concentrations of heavy metal ions on 24 hour feeding rate**

| **Odonate species** | **Control** | **Concentration (in ppm) of heavy metal ions 24 hour feeding rate (number of larvae consumed per 24 hours)** | | | **F value** |
|---|---|---|---|---|---|
| | | Pb (40 ppm) | Cd (40 ppm) | Cu (10 ppm) | |
| ***I. elegan*** | 12.2 ± 2.3 | 11.6 ± 2.1 | 10. 8 ± 2.1 | 7.2 ± 2.1* | 17.3 |
| ***T. aurora*** | 15.7 ± 3.2 | 16.2 ± 3.3 | 15.1 ± 2.3 | 11.7 ± 4.1* | 20.4 |
| ***P. flaviscans*** | 28.0 ± 3.1 | 21.1 ± 4.2* | 22.3 ± 3.1* | 18.2 ± 5.2* | 10.5 |

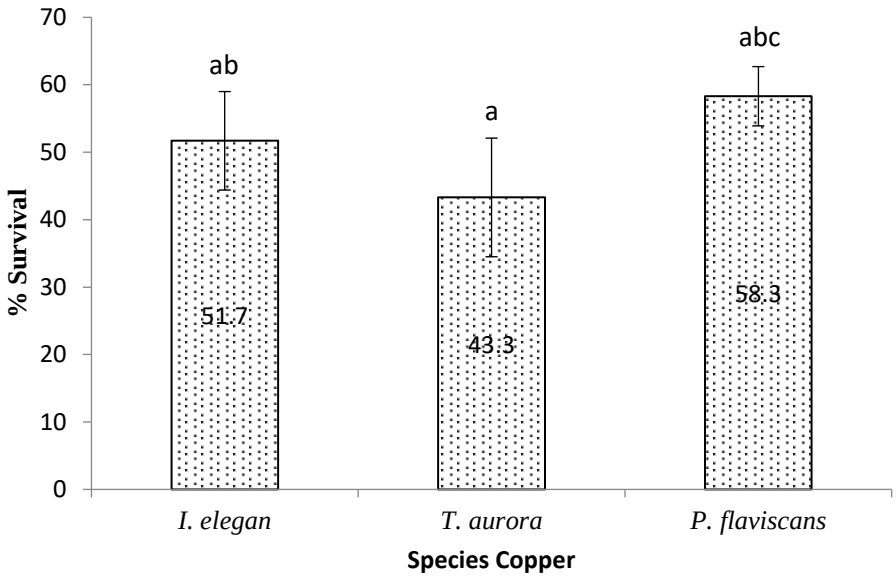


**Figure 6 : The percentage of surviving Odonate nymphs during definitive bioassay for toxicity study of Copper (Cu)**

This calculation of surviving odonate nymphs was made for highest concentration (500 ppm) of Cu. Alphabetical order is according to increasing mean value. Columns share a letter in common therefore represents insignificant difference at $P < 0.05$.

Similar trend was observed in case of *T. aurora* nymphs. On the other hand, in case of *P. flaviscans* nymphs, exposure to each of the heavy metal ions resulted in significant decreased ($P<0.05$) of feeding rate of these nymphs when compared to the control nymphs. The number of 3rd instar mosquito larvae ingested by *P. flaviscans* nymphs per 24 hours in control, 40 ppm Pb solution, 40 ppm Cd solution and 10 ppm Cu solution was 28.0 ± 3.1, 21.1 ± 4.2, 22.3 ± 3.1 and 18.2 ± 5.2, respectively.

## DISCUSSION

Heavy metals are the most important water contaminants that are harmful in minor concentration for the animals and are xenobiotics. Heavy metals i.e., Pb, Cd and Cu are highly toxic to aquatic organisms and they also bio accumulate in the animal tissues (Demirak et al. 2006; Villalobos-Jiménez et al. 2016). To the author knowledge, few studies have been conducted on the response of odonate nymphs to environmental contaminants. During the present research, the effect of shorte-term (seven days) exposure to lead (Pb), cadmium (Cd) and copper (Cu) in laboratory on survival and feeding rate of nymphs of three odonate species i.e., *Ischnura elegans*, *Trithemis aurora* and *Pantala flavescens* was investigated. The toxicity of heavy metals with *I. elegans* has been reported (Tollett et al. 2009). However, there is no report about the toxicity of heavy metals with *T. aurora* and *P. flavescens*. The present research is the first study of heavy metals toxicity with *T. aurora* and *P. flavescens*. During the present study,

During preliminary exposure to Pb, nymphs of all the three odonate species survived up to 40 ppm concentration (Table 1). Exposure to higher Pb concentrations (75 ppm and above) during definitive bioassay resulted in mortality (Table 2). At 75 ppm, there occurred less than 10 % mortality of Odonate nymphs. Even at 150 ppm, no appreciable mortality (< 14 % mortality) of odonate nymphs was noted. Similar trend was observed by Tollett et al. (2009) who exposed nymphs of two odonate species i.e., *Pachydiplax longipennis* and *Enallagma simplicicollis* to equimolar concentrations of heavy metals for seven days. They observed that exposure to Pb at concentrations below 185 ppm caused no

appreciable mortality of Odonate nymphs. The lowest concentration of Pb that caused mortality of odonate nymphs during the present research is far above the concentration to which odonate nymphs would be exposed in the field. According to Pakistan EPA (1997), the permissible level of Pb in municipal and liquid industrial effluents is 0.5 ppm. The US EPA (2005) recommended Criterion Continuous Concentration of Pb to protect aquatic life at water hardness of 100 mg/L CaCO3 is 2.50 µg/L. The Pb concentrations recommended by Pakistan EPA (1997) and US EPA (2005) are far below the minimum concentration of Pb that caused mortality of Odonate nymphs during the present research. Among the nymphs of three odonate species, nymphs of *P. flavescens* were found least susceptible to Pb ($LC_{50}$= 923.9 ppm) (Table 3). The maximum percentage of nymphs that survived at highest concentration (500 ppm) of Pb was belonging to *P. flavescens* species (65.0±7.6 %) (Figure 4). The difference in susceptibility of nymphs of *I. elegans, T. aurora* and *P. flavescens* species during exposure to Pb may be explained by differences in how species uptakes, metabolizes, detoxifies, and effluxes a specific metal which can lead to different levels of accumulation and different degrees of impact (Rainbow, 1997; Rainbow, 2002; Luoma and Rainbow, 2005).

During preliminary exposure of odonate nymphs to Cd (Table 1), nymphs of each species survived up to 40 ppm concentration as was in the case of Pb. Exposure of odonate nymphs to higher Cd concentrations (75 ppm and above) during definitive bioassay resulted in mortality (Table 4). At 75 ppm, < 12 % mortality of nymphs was observed. Even at 150 ppm, no appreciable mortality (< 27 % mortality) of odonate nymphs was noted. Similar trend was observed by Tollett et al. (2009) when they exposed nymphs of two odonate species, *Pachydiplax longipennis* and *Enallagma simplicicollis* to equimolar concentrations of heavy metals for seven days. During their study, exposure to Cd caused no appreciable mortality of odonate nymphs at concentrations below 100 ppm. The minimum concentration of Cd that caused mortality of odonate nymphs during the present research is far above the concentration to which odonate nymphs would be exposed in the field. According to Pakistan EPA (1997), the permissible level of Cd in municipal and liquid industrial effluents is 0.1 ppm. The US EPA (2005) recommended Criterion Continuous Concentration of Cd to protect aquatic life at water hardness of 100 mg/L CaCO3 is 0.25 µg/L. The concentrations recommended by Pakistan EPA (1997) and US EPA (2005) are far below the minimum concentration of Cd that caused mortality of odonate nymphs during the present research. Among the nymphs of three odonate species, nymphs of *P. flavescens* were found least susceptible to Cd ($LC_{50}$= 1441.7ppm) followed by *I. elegans* (LC50= 618.4 ppm) and *T. aurora (*$LC_{50}$= 417.6ppm) (Table 5). The maximum percentage of nymphs that survived at highest concentration (500 ppm) of Cd was belonging to *P. flavescens* species (68.3±4.4 %) followed by *I. elegans* (53.4±12.1 %) and *T. aurora* (41.3±6.1 %) (Figure 5). The difference in susceptibility of nymphs of *I. elegans, T. aurora and P. flavescens* during exposure to Cd may be explained by differences in how species uptakes, metabolizes, detoxifies, and effluxes a specific metal which can lead to different levels of accumulation and different degrees of impact (Rainbow, 2002; Luoma and Rainbow, 2005). Larvae of different insect species show considerable variation in susceptibility to a metal (Bat and Akbulut, 2001; Danielle et al. 2003).

During preliminary exposure of odonate nymphs to Cu, nymphs of all the three species survived up to 10 ppm concentration (Table 1). Exposure of odonate nymphs to higher Cd concentrations (15 ppm and above) during definitive bioassay resulted in mortality (Table 6). At 15 ppm Cu concentration, less than 7 % mortality of different Odonate nymphs was observed. This concentration of Cu is far above the concentration to which odonate larvae would be exposed in the field. According to Pakistan EPA (1997), the permissible level of Cu in municipal and liquid industrial effluents is 1 ppm. The US EPA (2005) recommended Criterion Continuous Concentration of Cu to protect aquatic life at water hardness of 100 mg/L $CaCO_3$ is 9.0 µg/L. The Cu concentrations recommended by Pakistan EPA (1997) and US EPA (2005) are far below the minimum concentration of Cu that caused mortality of Odonate nymphs during the present research. Among the nymphs of three odonate species, the nymphs of *P. flavescens* were found least susceptible to Cu ($LC_{50}$= 173.6 ppm) (Table 7). Maximum percentage of nymphs that survived at highest concentration (120 ppm) of Cu was belonging to *P. flavescens* (Figure 6). The difference in susceptibility of nymphs of *I. elegans, T. aurora* and *P. flavescens* during exposure to Cu might be explained by differences

in how species uptakes, metabolizes, detoxifies, and effluxes a specific metal which can lead to different levels of accumulation and different degrees of impact (Luoma and Rainbow, 2005). Heavy metals toxicity varies considerably among larvae of different insect species (Danielle et al. 2003).

During the present study, nymphs of all the three Odonate species exhibited high tolerance to Pb, Cd and CU. However, Cu appeared most toxic for the nymphs. The $LC_{50}$ value of Cu for nymphs of each Odonate species was far below than the $LC_{50}$ values of Pb and Cd against the same nymphs (Table 8). The study of Tollett et al. (2009) also showed Cu more toxic for odonate nymphs than Pb and Cd. Tollett et al. (2009) exposed the larvae of two odonate species, *Pachydiplax longipennis* and *Enallagma simplicicollis* to equimolar concentrations of Pb, Cd and Cu for seven days. During exposure to Cu, mortality of odonate nymphs occurred at concentrations above 150 µg /L. The lowest concentrations of Cd and Pb that caused mortality of odonate nymphs were far above than the lowest concentration of Cu that caused mortality of odonate nymphs. According to Maroni and Watson (1985) and Suzuki et al. (1989), Cu bound more readily to metal-binding metallothionein proteins than Cd and Pb. The presence of these proteins in odonates remain unexplored, but if odonate larvae have metal-binding proteins, then they might bioaccumulate Cu more readily than Cd or Pb and then show more toxic effects (Tollett et al. 2009). During the present study, after Cu, odonate nymphs were found more sensitive to Cd than Pb. Such trend of differential metal toxicity with odonate nymphs was also observed by (Tollett et al. 2009). During their study, *Pachydiplax longipennis* larvae were able to tolerate Cu up to 150 µg/L, Cd up to 250 ppm and Pb up to 462 ppm. The differential response of other aquatic invertebrates to Pb, Cd, Cu and other metals has also been reported. For example, recently, Ilahi et la. (2020) studied the effect of Pb, Cd and Cu on the survival and development of larvae of a culicine mosquito, *Culex quinquefasciatus*. During their study, they found mosquito larvae more susceptible to Cu. After Cu, mosquito larvae were more susceptible to Cd. Similarly, Milani et al. (2003) studied the toxicity of Cd and Cu with four species of aquatic invertebrates. *Hexagenia sp*. and *Tubifex sp.* were found more susceptible to Cu compared to Cd, whereas species of *Hyalella* and *Chironomus* were found more susceptible to Cd compared to Cu. Bat and Akbulut (2001) observed Zn most toxic in *Chironomus thummi* larvae followed Pb and Cu.

The results of the present research suggested that nymphs of *I. elegans, T. aurora and P. flavescens* are tolerant to high concentrations of heavy metal contamination in aqueous medium. More studies are required for investigating heavy metals toxicity with a variety of odonate species to fully examine metal toxicity in these organisms. It has been reported that odonate nymphs and other aquatic insect sequester high levels of metals in their cuticle (Gupta, 1995). Tollett et al. (2009) reported high level of heavy metals accumulation in odonate nymphs (>1000 µg/g dry weigh). Much of this accumulation was attributed to sequestering of metals in the exoskeleton by odonate larvae. Odonate nymphs exhibit high tolerance to heavy metals as they have the ability to accumulate high concentration of heavy metals with little effect on their mortality (Tollett et al. 2009). Due to the capability of tolerating high level of water contaminations, damselfly and dragonfly (odonate) nymphs can be used as a bio-indicators of habitat quality (Oertli, 2008; Subramanian et al. 2008; Dolný et al. 2011).

During the present study, the effect of short-term exposure of odonate nymphs to sublethal concentration of heavy metal ions i.e., 40 ppm of Pb, 40 ppm of Cd and 10 ppm of Cu on feeding rate was also studied (Table 9). The feeding rate of *I. elegans* and *T. aurora* nymphs when separately exposed to sublethal concentration of Pb and Cd (40 ppm each) was not different significantly ($P>0.05$) from control nymphs. However, the feeding rate of *I. elegans* and *T. aurora* nymphs exposed to the sublethal concentration of Cu (10 ppm) was significantly lower ($P<0.05$) from the control nymphs. In case of *P. flaviscans* nymphs, the feeding rate was significant lower ($P<0.05$) than control nymphs when exposure to the sublethal concentration of any of the mentioned three metals. The present study for the first time reports the effect of exposure to sublethal concentrations of heavy metals on the feeding abilities of *I. elegans, T. aurora* and *P. flavescens* nymphs (predators of mosquito larvae). Irving et al. (2003) studied the effect of Cd contamination on the feeding rates of aquatic insect (*Baetis tricaudatus*) of Ephemeroptera order. They observed decrease in feeding rate. Riddell et al. (2005) evaluated the effect of waterborne Cd contamination on predation performance of *Kogotus nonus* (Order Plecoptera) in mesocosm. They reported a

decrease in its locomotory and foraging activities. Metals and metalloid contamination exert adverse effect on insect behaviors including feeding behavior (Mogren and Trumble, 2010; Burden et al. 2019). Exposure to heavy metals is mostly toxic to insects and is often detrimental for the fitness of insects due to adverse effect on reproduction, development, immunity and feeding behaviors (Kula et al. 2014; Ternes et al. 2014; Martinek et al. 2018).

## CONCLUSION

From the findings of the present study, it is concluded that *I. elegans*, *T. aurora* and *P. flavescens* nymphs can tolerate high concentration of Pb, Cd and Cu in aqueous medium. Among these heavy metal ions, Cu is most toxic for *I. elegans*, *T. aurora* and *P. flavescens* nymphs. Among these nymphs, the *P. flavescens* nymphs are least susceptible to heavy metals. It is further concluded that the sublethal concentration (10 ppm) of Cu significantly reduces the feeding rate of *I. elegans*, *T. aurora* and *P. flavescens* nymphs.

## CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

## AUTHOR CONTRIBUTIONS

II and AMY designed and conducted the experiments and also wrote the manuscript. TUH, AR, MA and DN participated in the experiments and data analysis. All authors read and approved the final version.



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