EFFECT OF INORGANIC NUTRIENT POLLUTANTS ON OVIPOSITION PREFERENCE, EGG HATCHING RATE AND LARVAL DEVELOPMENT OF *CULEX QUINQUEFASCIATUS*

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Abstract. The present research aims to evaluate the effects of ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{-3}) on oviposition preference, hatching rate, time to pupation, pupation rate, time to adult emergence, adult emergence rate and female to male ratio of *Culex quinquefasciatus* mosquito, under laboratory conditions. The mosquito preferred to oviposit in containers containing 5 ppm NH_4^+, 45 ppm NO_3^- or 30 ppm PO_4^{-3} solution. No significant effect of these polutants on egg hatching rate was observed. The larvae that existed in containers containing 5 ppm NH_4^+, 45 ppm NO_3^- or 30 ppm PO_4^{-3} solution reached earlier to pupal and adult stages. The presence of NH_4^+ (5 ppm), NO_3^- (45 ppm) or PO_4^{-3} (30 ppm) in aqueous medium, resulted in decreased time to pupation or adult emergence and increased pupation and adult emergence rates. The adults emerged from these larvae showed significantly higher female to male ratio as compared to control adults ($P<0.05$). From the findings of the present research, it is concluded that the levels of NH_4^+, NO_3^- or PO_4^{-3} set in effluents for wastewater reuse in agricultural irrigation by WHO (2006) are favorable for the survival and development of *Cx. quinquefasciatus* mosquito, however higher levels of NH_4^+, NO_3^- or PO_4^{-3} are not favorable for the survival and development of this mosquito. These results help in identification of concentration ranges of the ammonium, nitrate and phosphate that make the larval habitat favorable for survival and reproduction of *Culex* mosquitoes. This could be helpful in effective control of mosquito borne diseases.
Keywords: *oviposition preference, hatching rate, pupation rate, time to pupation, emergence rate, time to adult emergence, female to male ratio*

Introduction

Mosquito larval habitats are influenced by many physical, chemical and biological factors. The results of several studies have shown the relationship between habitat characteristics and mosquito larval abundance and distribution (Kenea et al., 2011). Ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{-3}) are the well-known inorganic nutrient pollutants of water bodies. The NH_4^+, NO_3^- and other ionic forms of inorganic nitrogen enter the surface water bodies both from natural and anthropogenic sources (Wetzel, 2001; Rabalais, 2002). Phosphate is also an important inorganic nutrient in aquatic ecosystem and surface water receives it from various

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sources such as agricultural use of fertilizers, domestic and industrial wastewater, and atmospheric deposition (Tchobanoglous and Burton, 1991; Grubb et al., 2000).

The inorganic nutrient pollutants such as NH_4^+, NO_3^- and PO_4^{-3} in mosquito breeding sites influences the development of mosquito larvae (Noori et al., 2015). Breeding sites with higher NH_4^+, NO_3^- and PO_4^{-3} concentration provide better microbial food source for mosquito larvae (Dowling et al., 2013). Knowledge of mosquito breeding habitats is useful for identifying the most productive water bodies in a given area and developing efficient control approaches (Noori et al., 2015). Noori et al. (2015) designed a mesocosm experiment for investigating the relationships between inorganic nutrient pollutants (i.e., NH_4^+, NO_3^- and PO_4^{-3}) concentrations and larval development of *Cx. quinquefasciatus*. High concentrations of nitrate and phosphate favored the larval development and survival rates. High NO_3^- concentration suppressed the development of female mosquito but favored the development of male mosquitoes. The adult mosquitoes which emerged in high NO_3^- concentration solution were found to be faster in development. It was further observed that in the absence of NO_3^-, the addition of PO_4^{-3} slow the larval development. Schletzbaum (2013), evaluated the influence of water nutrient pollutant on the sequence of larval hatching of two mosquito species, *Culex pipiens* and *Anopheles albopictus* in container habitats. Higher NO_3^- concentration in the larval environment resulted in larger *Anopheles albopictus* females and higher emergence rate and shorter time to emergence for *Culex pipiens*.

Increased level of different forms of inorganic nitrogen in water is toxic to fresh water invertebrates, fishes and amphibian (Hickey and Vickers, 1994; Camargo et al., 2005). Phosphate (PO_4^{-3}) toxicity with aquatic invertebrates has also been reported (Kim et al., 2013). According to Williams et al. (1986), the elevated levels of nitrates, ammonia and phosphorus in the surface waters (in both marine and fresh water), can affect invertebrate diversity in feeding grounds. According to the compendium of standards for wastewater reuse in the Eastern Mediterranean region, the maximum permissible concentrations of NH_4^+, NO_3^- and PO_4^{-3} in effluents for reuse in agricultural irrigation are 5, 45 and 30 ppm, respectively (WHO, 2006). Irrigation water for agriculture has the potential to create mosquito breeding habitats within the storage system for water along the water supplying channels or in the field that receive water.

Monitoring of pollutants in aquatic environment is necessary for investigating the status of pollution in such environment for protection of human life and wildlife. From environmental, ecological and economic point of view, regular assessment of the pollutant levels and their destiny in different media of environment is essential. Regulation and re-evaluation of the threshold levels of different pollutants set by different international and national organizations is very important. The present research aims to evaluate the effects of threshold levels of three inorganic nutrient pollutants (NH_4^+, NO_3^- and PO_4^{-3}) set by WHO (2006) compendium of standards for wastewater reuse in agricultural irrigation in the Eastern Mediterranean region on the life-history of a common and economically important insect (*Culex quinquefasciatus* Say, 182) in water under laboratory conditions. The study contributes to the science of the effects of increasing level of inorganic nutrient pollutants on insect populations.

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Materials and methods

During the present research, the effects of NH_4^+, NO_3^- and PO_4^{-3} on oviposition preference, hatching rate and larval development of *Culex quinquefasciatus* (Say, 182) were studied in laboratory condition. During larval development, the effects of above inorganic nutrient pollutants on time to pupation, pupation rate, time till first appearance of adult, adult emergence rate, and female to male ratio were studied.

Required chemicals

The chemicals used included ammonium chloride (NH_4Cl) of Merck Co. (Darmstadt, Germany) for NH_4^+, sodium nitrate ($NaNO_3$) of Sigma-Aldrich (USA) for NO_3^- and sodium phosphate dibasic (Na_2HPO_4) of Sigma-Aldrich (USA) for PO_4^{-3}. The following are the detail of molar mass of the compounds and the related ions used during the present research:

- Ammonium chloride (NH_4Cl) molar mass is 53.5 g and contains 18 g of NH_4^+.
- Sodium nitrate ($NaNO_3$) molar mass is 85.5 g and contains 62 g of NO_3^{-1}.
- Sodium phosphate dibasic (Na_2HPO_4) molar mass is 144 g and contains 97 g of PO_4^{-3}.

Preparation of solutions

Initially 1000 ppm stock solution of each pollutant was prepared. For preparation of stock solution of 1000 ppm (1000 mg/L) of NH_4^+, 2.9 gram of NH_4Cl was required. For preparation of stock solution of 1000 ppm (1000 mg/L) of NO_3^-, 1.4 gram of $NaNO_3$ was required. For preparation of stock solution of 1000 ppm (1000 mg/L) of PO_4^{-3}, 1.5 gram of Na_2HPO_4 was required.

Laboratory rearing of Cx. quinquefasciatus

Experiments were conducted on the effects of NH_4^+, NO_3^{-1} and PO_4^{-3} on larval mortality, egg laying preference, egg hatching rate and larval development of *Cx. quinquefasciatus*. Laboratory colonies of *Cx. quinquefasciatus* were maintained during April and May 2016.

Laboratory conditions

The experiments were conducted during April and May 2016. Inside the laboratory, minimum temperature was ≥ 20°C and maximum temperature was ≤ 33°C. The laboratory was wide, well ventilated and receiving sun light at the day time through glass windows.

Bioassay for effect of inorganic nutrient pollutants on oviposition preference

According to the standards for wastewater reuse in the Eastern Mediterranean Region, the maximum permissible concentrations of NH_4^+, NO_3^{-1} and PO_4^{-3} in effluents for reuse in agricultural irrigation are 5, 45 and 30 ppm, respectively (WHO, 2006). Irrigation water for agriculture has the potential to create mosquito breeding habitats within the storage system for water along the water supplying channels or in the field that receive water. The present study aimed to investigate the effect of NH_4^+, NO_3^{-1} and PO_4^{-3} on oviposition preference of *Cx. quinquefasciatus* at the concentrations which are permissible in effluents for reuse in agricultural irrigation (WHO, 2006). The effect of

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NH_4^+, NO_3^{-1} and PO_4^{-3} on oviposition preference of *Cx. quinquefasciatus* was also studied at concentrations higher than WHO recommended concentrations of these nutrient pollutants in effluents for reuse in agricultural irrigation (WHO, 2006). Thus, two concentrations of NH_4^+ (5 and 10 ppm), NO_3^{-1} (45 and 90 ppm) and PO_4^{-3} (30 and 60 ppm) were tested for their effect on oviposition preference of *Cx. quinquefasciatus* gravid female adult. The main purpose was to investigate the effect of NH_4^+, NO_3^{-1} and PO_4^{-3} on oviposition preference of *Cx. quinquefasciatus* at concentrations which are permissible in effluents for reuse in agricultural irrigation (WHO, 2006). Three mosquito cages (cage 1-3) were used during this experiment (*Figure 1*).

Figure 1. *Picture showing mosquito cages used during the present research*

During the study of effect of NH_4^+ on oviposition preference of *Cx. quinquefasciatus* gravid female adult, two NH_4^+ solutions of 5 and 10 ppm (300 ml each) in two 400 ml polyethylene containers were placed in mosquito cage (45 cm × 45 cm × 45 cm). In addition, a control polyethylene container containing 300 ml non-chlorinated tap water was also placed inside the mosquito cage along with NH_4^+ solution. For studying the effect of NO_3^{-1} on oviposition preference, two NO_3^{-1} solutions of 45 and 90 ppm (300 ml each) in two 400 ml polyethylene containers were placed in mosquito cage. In addition, a control polyethylene container containing 300 ml non-chlorinated tap water was also placed inside the mosquito cage along with NO_3^{-1} solution. For studying the effect of PO_4^{-3} on oviposition preference, two PO_4^{-3} solutions of 30 and 60 ppm (300 ml each) in two 400 ml polyethylene containers were placed in a mosquito cage. In addition, a control polyethylene container containing 300 ml non-chlorinated tap water was also placed inside the mosquito cage along with PO_4^{-3} solution. The experiment was run in triplicate.

For each mosquito cage, 100 blood fed and gravid female adult mosquitoes (5 days of age after emergence from pupae) were caught from the existing laboratory colony of *Cx. quinquefasciatus* through mouth aspirator and then introduced into the cage. The gravid female mosquitoes laid egg into the containers after 2 or 3 days of introduction. The number of egg rafts laid by female mosquitoes in each jar was counted under stereo binocular microscope with digital camera (Labomed Inc, USA) so that to determine the effect of inorganic nutrient pollutants on the egg laying preference of *Cx. quinquefasciatus* female adult mosquitoes. Schematic of experiment on the effect of inorganic nutrient pollutants on oviposition preference and egg hatching rate is shown in *Figure 2*. Egg rafts and eggs within rafts have been shown in *Figures 3-4*.

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Bioassay for effect of inorganic nutrient pollutants on egg hatching rate

The egg rafts laid by the gravid female mosquitoes during oviposition assays for NH_4^+, NO_3^- and PO_4^{-3}, hatched into first instar larvae. After two days of hatching, all the containers were brought out of the mosquito cages and the total number of eggs and the number of hatched out larvae were counted.

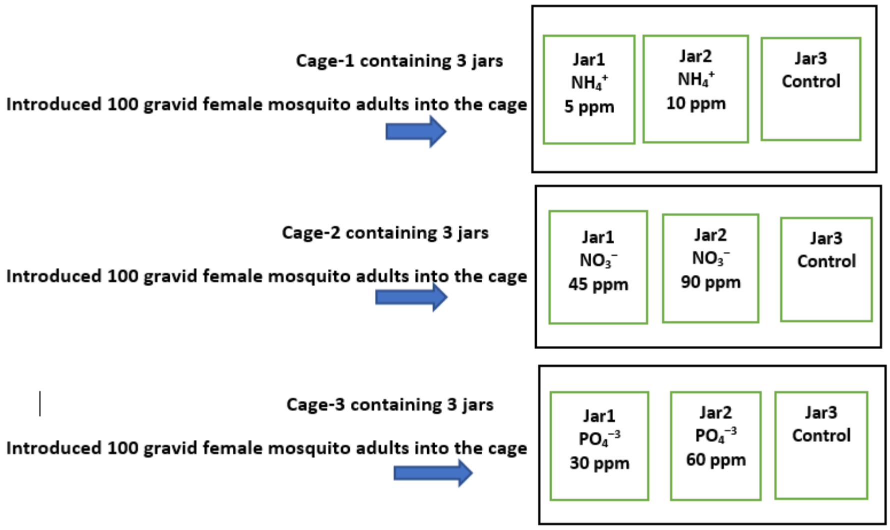

Figure 2. *Schematic of experiment on the effect of inorganic nutrient pollutants on oviposition preference and egg hatching rate*

Figure 3. *Egg rafts deposited by Culex quinquefasciatus during the present experiments*

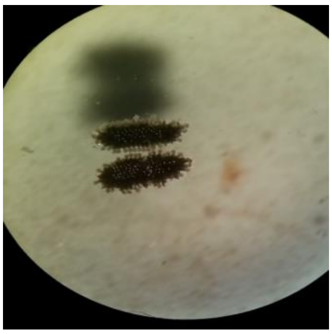

Figure 4. *Microscopic picture of Culex quinquefasciatus eggs in rafts taken during the present experiments*

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Bioassay for effect of inorganic nutrient pollutant on larval development

The effect of high concentration of NH_4^+ (5 ppm), NO_3^- (45 ppm) and of PO_4^{-3} (30 ppm) in water on larval development of *Cx. quinquefasciatus* were also studied as these concentrations are allowable according to a compendium of standards for wastewater reuse in the Eastern Mediterranean Region (WHO, 2006). The schematic of experiment on the effect of inorganic nutrient pollutants on larval development of *Cx. quinquefasciatus* is shown in *Figure 5*. Thus 5 ppm concentration of NH_4^+, 45 ppm concentration of NO_3^- and 30 ppm concentration of PO_4^{-3} were prepared in three 1000 ml polyethylene jars. The solutions were prepared from stock solutions in sieved pond water for ensuring the presence of natural communities of microflora (Noori et al., 2015). The volume of each testing solution was 500 ml. In addition, a control 1000 ml polyethylene jar containing only 500 ml sieved river water was also arranged. Dried leaf litter of *Chenopodium album* was collected along the boundary wall of University of Malakand as there was no history of pesticides application. The leaf litter was ground into powder. During this experiment, 100 mg of leaf litter powder was added to each jar including control jar for providing source of carbon (Schletzbaum, 2013; Noori et al., 2015).

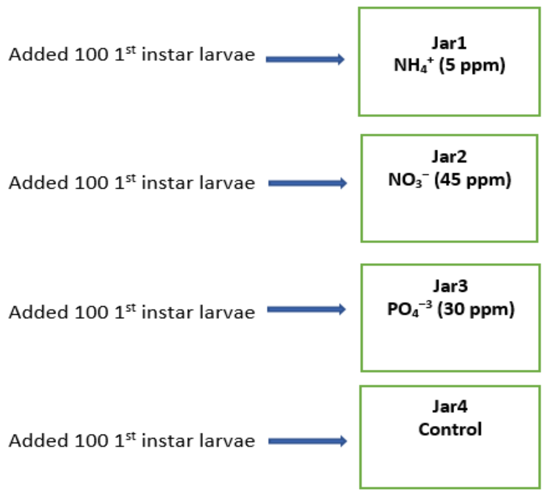

Figure 5. *Schematic of experiment on the effect of inorganic nutrient pollutants on larval development of Cx. quinquefasciatus*

One hundred newly hatched 1st instar larvae (a day after hatching) larvae of *Cx. quinquefasciatus* from laboratory colony were transferred to each polyethylene jar. This experiment was run in four replicates. The jars were capped with gauze to prevent the escape of emerging adult mosquitoes. The jars were daily checked for the appearance of pupae and adults. The observations were continued till all the larvae or pupae in all the jars have died or emerged as adults. The effect of NH_4^+, NO_3^- and PO_4^{-3} on time to pupation, pupation rate, time to adult emergence, adult emergence rate and female to male ratios were studied. All the mosquito adults were counted and the female to male ratio was determined by dividing the total number of female adults by the total number of male adults (Neira et al., 2014). Time to pupation or adult emergence was calculated by the following method (Kosalwat and Knight, 1987) (*Eq.1*):

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$$T = \sum (D \times N) / \sum N \quad \text{(Eq.1)}$$

where T represents the average time to pupation or adult emergence (in days), D represents the number of days from day zero of exposure of first instar larvae to pupation or adult emergence and N represents the number of pupae or adults produced. *Figure 6* shows the pictures of egg, larval, pupal and adult stages of life history of *Culex quinquefasciatus* mosquito. The pictures have been taken in the laboratory during the present experiments.

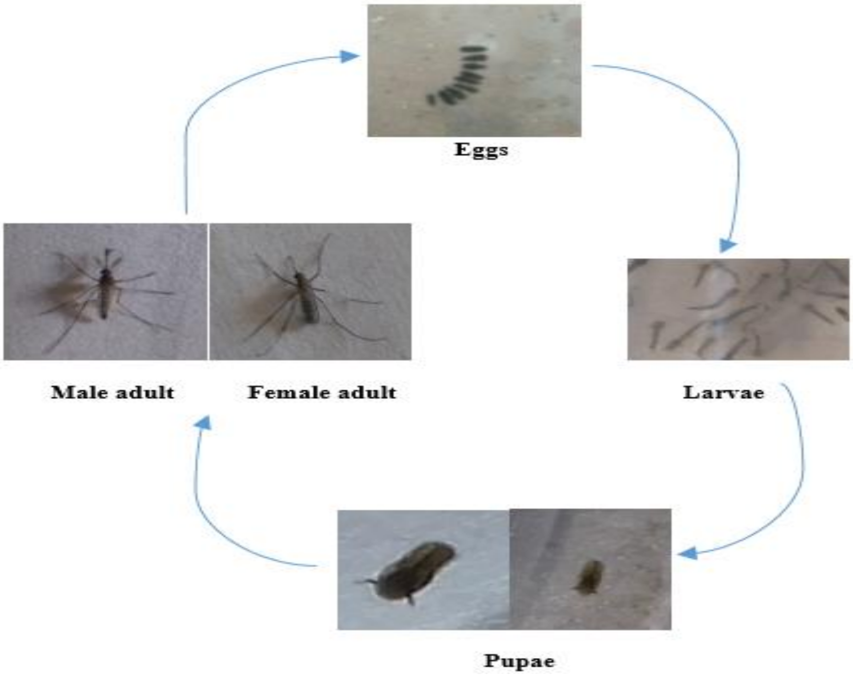

Figure 6. *Picture showing the egg, larval, pupal and adult stages of life history of Culex quinquefasciatus mosquito. The figure has been produced from the pictures taken in the laboratory during the present experiments*

Statistical analysis

The data was initially analyzed by normality test of Shapiro-Wilk. If the P value was less than 0.05 then the data was analyzed by Kruskal-Wallis test for comparison (Schlotzhauer and Little, 1987). When the P value in Kruskal-Wallis test was less than 0.05 then the Kruskal-Wallis test was followed by Bonferroni's post hoc test for pairwise comparison. The data was presented as the mean rank. If the value of P in normality test was greater than 0.05 then the data was analyzed by Dunnett's Test at P>0.05 significance level for assessing significant difference between control and each of the treatment solution. In this case the data was presented as mean with standard error. The LC_{50} values were compared by 95 % confidence limits overlap method (Wheeler et al., 2006).

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Results

Oviposition preference of Cx. quinquefasciatus

The effect of NH_4^+ (5 ppm and 10 ppm), NO_3^- (45 ppm and 90 ppm) and PO_4^{-3} (30 ppm and 60 ppm) on oviposition preference of *Cx. quinquefasciatus* is shown in *Table 1*. Data is presented as means (with standard error of means) and mean ranks of egg rafts. Based on normality test of Shapiro-Wilk, the data was not normally distributed, therefore the data was analyzed by Kruskal-Wallis test at $P<0.05$ significance level for determining significant difference.

During the study of effect of NH_4^+ on oviposition preference, the Kruskal Wallis χ^2 value was 6.0 and the P value was 0.05. The overall test showed significant difference in the mean ranks of egg rafts across the control and treatment containers (5 ppm and 10 ppm NH_4^+ solutions). The mean rank of egg rafts in 5 ppm NH_4^+ solution container (mean rank = 8.0) was significantly higher when compared to control container ($P<0.05$). The control container and 10 ppm NH_4^+ solution container showed similar mean rank of egg rafts (mean rank = 3.5 each).

Table 1. *Effect of inorganic nutrient pollutants in water on oviposition preference of Cx. quinquefasciatus (n=3)*

Pollutants	Concentration	N. of egg rafts Mean± SE	Mean rank of egg rafts
Ammonium (NH_4^+)	Control	1.6±0.3	3.5
	5 ppm	7.3±0.7*	8
	10 ppm	1.6±0.6	3
			KW, χ^2=6.0 P=0.05
Nitrate (NO_3^-)	Control	1.3±0.3	2
	45 ppm	8.0±1.2*	8
	90 ppm	3.6±0.3	5
			KW, χ^2=7.3 P=0.03
Phosphate (PO_4^{-3})	Control	2.6±0.3	4
	30 ppm	12.7±1.8*	8
	90 ppm	2.3±0.3	3
			KW, χ^2=6 P=0.049

KW - Kruskal-Wallis, *-.significantly different from the control at $P<0.05$ significance level in Kruskal-Wallis test

During the study of effect of NO_3^- on oviposition preference, the Kruskal Wallis χ^2 value was 7.3 and P value was 0.03. The overall test showed significant difference in mean ranks of egg rafts across the control and treatment containers (45 ppm and 90 ppm NO_3^- solutions). The mean rank of egg rafts in 45 ppm NO_3^- solution container (mean rank = 8.0) was significantly higher when compared to control container (mean rank = 2) ($P<0.05$). There was no significant difference ($P>0.05$) in the mean rank of 90 ppm NO_3^- solution container (mean rank = 5) and control container (mean rank = 2).

During the study of effect of PO_4^{-3} on oviposition preference, the Kruskal Wallis χ^2 value was 6.0 and P value was 0.050. The overall test showed significant difference in mean ranks of egg rafts across the control and treatment containers (30 ppm and 60 ppm PO_4^{-3} solutions). The mean rank of egg rafts in 30 ppm PO_4^{-3} solution container

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(mean rank = 8.0) was significantly higher when compared to control container (mean rank = 4) (P<0.05). The mean rank of egg rafts of 60 ppm PO_4^{-3} solution container (mean rank = 3) was insignificantly lower (P>0.05) when compared to control container (mean rank = 4).

Hatching rate of Cx. quinquefasciatus

The effect of NH_4^+, NO_3^- and PO_4^{-3} on hatching rate of *Cx. quinquefasciatus* is shown in *Table 2*. Based on normality test of Shapiro-Wilk, the data was normally distributed, therefore the data was analyzed by Dunnett's Test at P<0.05 significance level for determining significant difference between control container and each of the inorganic nutrient pollutant solution containers. Data is presented as means with standard error of means. During this study, no significant difference in egg hatching rate of *Cx. quinquefasciatus* eggs was observed between the control container and any of the treatment containers (P>0.05).

Table 2. *Effect of inorganic nutrient pollutants in water on egg hatching rate of Cx. quinquefasciatus (n=3)*

Pollutants	Concentration (ppm)	Number of Eggs (Mean ± SE)	Number of hatched larvae (Mean ±SE)	Hatching rate (Mean % ± SE)
Ammonium (NH_4^+)	Control	153.3 ± 31.8	134.6 ± 32.8	86.9±4.6
	5	724.7 ± 68.7	703.3 ± 69.9	97± 0.6
	10	160.0 ± 35.1	140.0 ± 27.5	88.8±3.2
				P>0.05
Nitrate (NO_3^-)	Control	136.3 ± 35.4	123.7 ± 31.9	96.7±1.9
	45	780.0 ± 118.4	758.3 ± 118.7	97.03±1.3
	90	353.3 ± 34.4	337.7 ± 31.2	96.6.6±1.6
				P>0.05
Phosphate (PO_4^{-3})	Control	262.3 ± 41.2	239.3 ± 40.01	90.7±1.7
	30	1417.3 ± 123.2	1320.7 ± 115.8	93.2±0.6
	60	223.3 ± 26.2	212.0 ± 29.1	93.8±2.8
				P>0.05

There was no significant difference (P>0.05) in egg hatching rate between control and treatments

Larval development of Cx. quinquefasciatus

During the present research, the effect of 5 ppm NH_4^+, 45 ppm NO_3^- and 30 ppm PO_4^{-3} on larval development of *Cx. quinquefasciatus* were studied in the laboratory conditions. In addition, each container (including control) was also containing 100 grams leaf litter powder. The larval development study included the effect of above inorganic nutrient pollutants on time to pupation, pupation rate, time to adult emergence, adult emergence rate and female to male ratios. Following are the details:

Time to pupation of Cx. quinquefasciatus

The effect of 5 ppm NH_4^+, 45 ppm NO_3^- and 30 ppm PO_4^{-3} on time to pupation in *Cx. quinquefasciatus* is shown in *Table 3*. During this study, a total of one hundred first instar larvae were exposed to each nutrient pollutant. The value of significance in normality test of Shapiro-Wilk showed that the data is normally distributed, therefore the data was analyzed by Dunnett's Test (significance level=0.05) for determining significant difference between control and each of the inorganic nutrient pollutants. Data is presented as the mean and standard error of mean of four replicates. Each

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pollutant caused a decrease in time to pupation. The minimum number of days to pupation was observed for PO_4^{-3} solution (8.5±0.6 days) followed by NO_3^- (12.5±0.6 days), NH_4^+ (13.5±0.6) and control (14.5±0.6). The difference in time to pupation between the control and PO_4^{-3} solution container was significant ($P<0.05$). However, the difference between the control and the rest of the pollutants i.e., NH_4^+ or NO_3^- was insignificant ($P>0.05$).

Table 3. *Effect of inorganic nutrient pollutants in water on time to pupation in Cx. quinquefasciatus (n=4)*

Treatment	Days (Means ± SE)	95% Confidence Intervals		Minimum	Maximum
		Lower limit	Upper limit		
Control	14.5 ± 0.6	12.4	16.6	13.0	16.0
Ammonium (NH_4^+)	13.5 ± 0.6	11.4	15.6	12.0	15.0
Nitrate (NO_3^-)	12.5 ± 0.6	10.4	14.6	11.0	14.0
Phosphate (PO_4^{-3})	8.5 ± 0.6*	6.4	10.6	7.0	10.0
Statistics	F= 16.6, DF within groups= 12, P=0.000				

*- Significantly different from the control at P<0.05 significance level

Pupation rate of Cx. quinquefasciatus

The effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) on pupation rate in *Cx. quinquefasciatus* is shown in *Table 4*. The value of significance in normality test of Shapiro-Wilk showed that the data is normally distributed, therefore the data was analyzed by Dunnett's Test (significance level≤0.05) for determining significant difference between control and each of the pollutants. Data is presented as the mean and standard error of mean of four replicates. Pupation rates in NH_4^+ solution container (52.0±9.9%) and PO_4^{-3} solution container (51.8±9.6%) were significantly higher ($P<0.05$) when compared to control containers (27.0±4.9%). Pupation rate in NO_3^- solution container (33.0±3.4%) was insignificantly higher than pupation rate in control container ($P>0.05$).

Table 4. *Effect of inorganic nutrient pollutants in water on pupation rate in Cx. quinquefasciatus (n=4)*

Treatment	Pupation rate (% Means ± SE)	95% Confidence Intervals		Minimum	Maximum
		Lower limit	Upper limit		
Control	27.0 ± 4.9	11.4	42.6	18.0	39.0
Ammonium (NH_4^+)	52.0 ± 9.9*	9.6	94.4	36.0	70.0
Nitrate (NO_3^-)	33.0 ± 3.4	22.2	43.8	25.0	40.0
Phosphate (PO_4^{-3})	51.8 ± 9.6*	21.04	82.5	30.0	77.0
Statistics	F= 3.2, DF within groups= 12, P<0.066				

*- Significantly different from the control at P<0.05 significance level

Time to adult emergence of Cx. quinquefasciatus

The effect of 5 ppm NH_4^+, 45 ppm NO_3^- and 30 ppm PO_4^{-3} in water on time to adult emergence in *Cx. quinquefasciatus* is shown in *Table 5*. The value of significance in normality test of Shapiro-Wilk showed that the data is normally distributed, therefore

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the data was analyzed by Dunnett's Test (significance level=0.05) for determining significant difference between control and each of the inorganic nutrient pollutants. Data is presented as the mean and standard error of mean of four replicates. The minimum number of days to adult emergence was observed for PO_4^{-3} solution (11.5±1.7 days) followed by NO_3^- (15.0±1.4 days), NH_4^+ (17.0±1.4 days) and control (18.5±2.08 days). The difference in time to adult emergence between the control and PO_4^{-3} or NO_3^- solution container was significant (P<0.05). However, the difference between the control and NH_4^+ was insignificant (P>0.05).

Table 5. *Effect of inorganic nutrient pollutants in water on time to adult emergence in Cx. quinquefasciatus (n=4)*

Treatment	Days (Means ± SE)	95% Confidence Intervals		Minimum	Maximum
		Lower limit	Upper limit		
Control	18.5 ± 2.08	15.2	21.8	16.0	21.0
Ammonium (NH_4^+)	17.0 ± 1.4	14.7	19.3	16.0	19.0
Nitrate (NO_3^-)	15.0 ± 1.4*	12.7	17.3	13.0	16.0
Phosphate (PO_4^{-3})	11.5 ± 1.7*	8.74	14.3	10.0	14.0
Statistics	F= 12.9, DF within groups= 12, P<0.01				

*- Significantly different from the control at P<0.05 significance level

Adult emergence rate of Cx. quinquefasciatus

The effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) in water on adult emergence rate in *Cx. quinquefasciatus* is shown in *Table 6*. Data was presented as the mean and standard error of mean of emergence rates (n=4). The value of significance in normality test of Shapiro-Wilk showed that the data is normally distributed, therefore the data was analyzed by Dunnett's Test (significance level≤0.05) for determining significant difference between control and each of the pollutant solution.

Table 6. *Effect of inorganic nutrient pollutants in water on adult emergence rate of Cx. quinquefasciatus (n=4)*

Treatment	Emergence rate (%) (Mean ± SE)	95% Confidence Intervals		Minimum	Maximum
		Lower limit	Upper limit		
Ammonium (NH_4^+)	31.5 ± 4.4	17.5	45.6	22.3	40.01
Nitrate (NO_3^-)	61.9 ± 8.7*	34.3	89.5	44.3	83.3
Phosphate (PO_4^{-3})	61.5 ± 1.9*	55.4	67.4	56.8	65.0
Ammonium (NH_4^+)	57.1 ± 11.5*	20.5	93.8	32.5	86.6
Statistics	DF=12, F=3.6, P<0.05				

*- Significantly different from the control at P<0.05 significance level

Significantly higher number of adults emerged in the NH_4^+, NO_3^- or PO_4^{-3} solution container when compared to control container (P<0.05). Maximum adult emergence rates were observed in NH_4^+ (61.9±8.7%) and NO_3^- solution container (61.5±1.9%).

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Female to male ratio of Cx. quinquefasciatus

All the adults were counted and the percentage of female and male mosquitoes to the total number of adults produced were determined for each jar. Female to male ratio was obtained through dividing the number of female adult mosquitoes by the number of male adult mosquitoes. Data was presented as the mean of percentage of female and male *Cx. quinquefasciatus* mosquito and mean female to male ratio (*Table 7*). The value of significance in normality test of Shapiro-Wilk showed that the data is distributed normally, therefore the data was analyzed by Dunnett's Test (significance level≤0.05) for determining significant difference between control and each of the pollutant solutions.

Table 7. *Effect of inorganic nutrient pollutants in water on female to male ratio of Cx. quinquefasciatus (n=4)*

Treatment	% Female (Mean ± SE)	% Male (Mean ± SE)	Female to male ratio (Mean ± SE)
Control	42.9 ± 2.3	57.1 ± 2.3	0.7 ± 0.07
Ammonium (NH_4^+)	52.4 ± 0.9*	47.7 ± 0.9*	1.1. ± 0.94*
Nitrate (NO_3^-)	62.7 ± 2.2*	37.3 ± 2.1*	1.7 ± 0.13*
Phosphate (PO_4^{-3})	60.8 ± 1.2*	39.2 ± 1.2*	1.6 ± 0.08*

*- Significantly different from the control at P<0.05 significance level

The female to male adult ratio for NH_4^+, NO_3^- and or PO_4^{-3} solution container was significantly higher than control container (P<0.05). Maximum female to male ratio was observed for NO_3^- solution container that was 1.7±0.13 (62.7±2.2% females, 37.3±2.1% males). After NO_3^-, maximum female to male ratio was noted for PO_4^{-3} that was 1.6±0.08 (60.8±1.2% females, 39.2±1.2% males). The lowest female to male ratio was observed for control container that was 0.7±0.07 (42.9±2.3% females, 57±2.3% males).

Discussion

During the present study, the effects of NH_4^+, NO_3^- and PO_4^{-3} on oviposition preference, hatching rate and larval development parameters of *Cx. quinquefasciatus* were studied under laboratory conditions. During the study of effect of these pollutants on oviposition preference, the effect of two environmentally realistic concentrations of NH_4^+ (5 ppm and 10 ppm) on oviposition preference of gravid female adult *Cx. quinquefasciatus* was studied. Gravid female *Cx. quinquefasciatus* adults preferred the container containing 5 ppm NH_4^+ solution but did not prefer the container containing 10 ppm NH_4^+ solution or control container (containing only non-chlorinated tap water) (*Table 1*). For example, the number of egg rafts in the container containing 5 ppm NH_4^+ was significantly higher than control container where as the number of egg rafts in the container containing 10 ppm NH_4^+ was insignificantly lower than control container (P>0.05). Nguyen et al. (2012) observed higher number of mosquito egg rafts in water containing NH_4^+ above 2 ppm when temperature was high (> 32°C). They observed lower number of egg rafts at the same concentration of NH_4^+ when temperature was lower. During the present research, minimum temperature was ≥ 20°C and maximum temperature was ≤ 33°C in which NH_4^+ at 5 ppm concentration acted as oviposition attractant for *Cx. quinquefasciatus*. During the study of effect of NO_3^- (at 45 ppm and 90 ppm) on oviposition preference of *Cx. quinquefasciatus,* the results

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show that gravid female *Cx. quinquefasciatus* mosquito preferred water bodies with lower NO_3^- concentration (45 ppm) for oviposition. Water without NO_3^- and water with higher NO_3^- concentration (90 ppm) were less preferred for oviposition. These results suggest that NO_3^- in water attract the gravid female *Cx. quinquefasciatus* mosquito for oviposition, however water with higher NO_3^- concentration is not highly preferred for oviposition by *Cx. quinquefasciatus* mosquito (*Table 1*). To the author knowledge, limited studies have been conducted on the effect of NO_3^- on the oviposition preference of mosquitoes. For example, Nguyen et al. (2012) studied seasonal, weather, nutrients, and conspecific presence impacts on *Cx. quinquefasciatus* mosquito in combined sewage overflows. It was observed that high NO_3^- concentration i.e. >1 ppm is associated with decrease in the number of oviposited egg rafts. During the present research, the same trend of association of decreased number of egg rafts with high NO_3^- concentration was observed. During the experiment of Grech and Juliano (2017), the female *Cx. restuans* mosquito laid more eggs in containers having low amount of plant detritus but high concentration of total dissolved nitrogen, however after some days the gravid female *Cx. restuans* preferred those containers for oviposition which were containing high amount of plant detritus but low concentration of total dissolved nitrogen and high phosphate concentration. They concluded that the concentrations of total dissolved nitrogen and phosphorus have differential effect on the oviposition preference of the gravid female *Cx. restuans*, and the mosquitoes favor different nutrients in different circumstances and do not always oviposit preferentially in containers rich in plant detritus. During the study of effect of PO_4^{-3} (at 30 ppm and 60 ppm) on oviposition preference of *Cx. quinquefasciatus*, the gravid female *Cx. quinquefasciatus* preferred the container for oviposition having lower PO_4^{-3} concentration (30 ppm), however they oviposited less number of egg rafts in containers containing no PO_4^{-3} (control) or containing higher concentration of PO_4^{-3} (60 ppm). A breeding site rich in PO_4^{-3} and NH_4^+ is an excellent larval habitat for *Cx. quinquefasciatus* (Nguyen et al., 2012). The NH_4^+ and PO_4^{-3} promote the growth of bacteria in breeding site; the bacteria serve as food source for mosquito larvae (Beehler and Mulla, 1995; Sunish and Reuben, 2001) and attract adult gravid female mosquito for oviposition (Beehler et al., 1994). Nguyen et al. (2012) studied the effect of seasons, weather, nutrients, and conspecific presence impacts on *Cx. quinquefasciatus* mosquito in combined sewage overflows. At lower PO_4^{-3} concentration, *Cx. quinquefasciatus* oviposited more eggs rafts, however at higher PO_4^{-3} concentration (>10 ppm), a decrease in the number of oviposited egg rafts was observed. Similar trend was observed during the present research. Agricultural fertilizers also influence the oviposition preference of adult gravid female mosquitoes. For example, Kibuthu et al. (2016) studied the effect of sub lethal concentrations of cypermethrin, glyphosate, ammonium sulfate and diammonium phosphate on oviposition preference of *Anopheles arabiensis* and *Culex quinquefasciatus* mosquitoes. Highest number of egg rafts were observed in diammonium phosphate and ammonium sulfate treatments.

During the study of effect of inorganic nutrient pollutants on egg hatching rate, no significant difference in egg hatching rate of *Cx. quinquefasciatus* in NH_4^+, NO_3^- or PO_4^{-3} solution container and control container was observed (P>0.05) (*Table 2*). To the author knowledge, there is no reported study about the effect of inorganic nutrient pollutants i.e., ammonium, nitrate and phosphate on the egg hatching rate in mosquitoes. The effect of temperature on hatching rate of mosquito eggs has been reported. For example, Oda et al. (1999) studied the effect of temperature on hatching

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rate and adult survival of *Cx. pipiens molestus* and *Cx. quinquefasciatus*. The egg hatching rate of *Cx. pipiens molestus* became very low with rise in temperature, however no effect of temperature rises on egg hatching rate of *Cx. quinquefasciatus* was observed. Yang (2008) studied the effect of site deprivation on oviposition performance and egg hatching rate of naturally blood–fed gravid *Culex quinquefasciatus* in the laboratory. Gravid female adults failed to form egg rafts, and egg hatching rate decreased significantly. Vitek and Livdahl (2006) compared the hatching rates of *Ae. Albopictus* eggs in both field and laboratory settings. The hatching rates were compared for mosquitoes exposed to regular, periodic hatch stimulation and random hatch stimulation. The hatching rate in laboratory treatments was not significantly different from the field treatments. Ezeakacha (2015) reported increase in egg hatch rate of *Aedes albopictus*, *Aedes aegypti*, *Aedes triseriatus* and *Culex quinquefasciatus* with increase in relative humidity and egg storage period.

The present study also aimed to investigate the effect of NH_4^+, NO_3^-, and of PO_4^{-3} in water at concentration of 5 ppm, 45 ppm and 30 ppm, respectively, on larval development parameters (such as time to pupation, pupation rate, time to adult emergence, adult emergence rate and female to male ratios) of *Cx. quinquefasciatus*. During this experiment, 100 mg of leaf litter powder was added to each jar including control jar for providing source of carbon (Schletzbaum, 2013; Noori et al., 2015). During the study of the effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) on time to pupation, larvae of control container larvae took more days to reach pupation, however the larvae in the containers that were containing NH_4^+, NO_3^- or PO_4^{-3} reached earlier to pupation (*Table 3*). Among the inorganic nutrient pollutants, PO_4^{-3} caused a significant decrease in time to pupation when compared to control ($P<0.05$). To the author knowledge, very limited studies have been conducted on the effect of pollutants on time to pupation in mosquitoes. For example, Noori et al. (2015) studied the effect of NH_4^+, NO_3^- and PO_4^{-3} in water on larval development of *Cx. quinquefasciatus*. During their study, larvae that were exposed to phosphate at concentration range of 1 ppm to 12 ppm without leaf litter, took more time in reaching to pupation. During the present study, the phosphate solution which was also containing 100 gram of leaf litter powder, caused a decrease in time to pupation. This indicated that larvae exposed to higher phosphate concentration (30 ppm) in presence of leaf litter reach early to pupation.

During the study of effect of NH_4^+ (5 ppm), NO_3^- (45 ppm), and PO_4^{-3} (30 ppm) on pupation rate of *Cx. quinquefasciatus*, significantly higher number ($P<0.05$) of larvae reached to pupation in NH_4^+ and PO_4^{-3} solution containers when compared to control container (*Table 4*). These results agree with the findings of Sunish and Reuben (2001) as they reported a positive influence of ammonia nitrogen and PO_4^{-3} on aquatic stages of mosquitoes. Similarly, in another study, increase in the concentration of NH_4^+ or PO_4^{-3} was associated with increase in the number of *Cx. quinquefasciatus* larvae that reached to pupation (Noori et al., 2015). There was observed no significant effect of NO_3^- (at 45 ppm) on pupation rate when compared to control ($P>0.05$). Noori et al. (2015) also found no significant correlation between increase in NO_3^- concentration and pupation rate. The presence of NH_4^+, NO_3^- and PO_4^{-3} accelerate the multiplication of microorganisms (Sunish and Reuben, 2001) that in turn favors the survival of *Culex* larvae. Paul et al. (2006) suggested that higher concentration of phosphorus in agricultural stream is associated with faster breakdown rate of leaf litter. The faster breakdown rate of leaf litter in water bodies results in multiplication of microorganisms.

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Microorganisms in mosquito breeding sites constitute the major food source of mosquito larvae.

During the study of effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) in water on time to adult emergence of *Cx. quinquefasciatus*, in each of the NH_4^+, NO_3^- and PO_4^{-3} solution containers, the larvae took less number of days to reach to adulthood than in the control container (*Table 5*). The first instar larvae exposed to 45 ppm NO_3^- or 30 ppm PO_4^{-3} solution took significantly lower ($P<0.05$) number of days in reaching to adulthood when compared to the control. The larvae exposed to PO_4^{-3} took longer in reaching to adulthood. The larvae exposed to 5 ppm NH_4^+ solution took insignificantly higher number of days in reaching to adulthood when compared to control ($P>0.05$). The early emergence of adults in NH_4^+, NO_3^- or PO_4^{-3} solution containers (each containing 100 g leaf litter powder) can be attributed to increasing microbial activities (major food source of *Culex* larvae) (Sunish and Reuben, 2001; Paul et al., 2006).

During the study of effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) in water on adult emergence rate of *Cx. quinquefasciatus*, significantly higher number of adults emerged in NH_4^+, NO_3^- and PO_4^{-3} solution containers when compared to control container ($P<0.05$) (*Table 6*). Noori et al. (2015) reported the boosting effect of NH_4^+, NO_3^- and PO_4^{-3}on adult emergence rate of *quinquefasciatus*. Schletzbaum (2013) also reported the positive effect of NO_3^- concentration on adult emergence rate of *Cx. pipiens*. Agricultural fertilizers have also been reported for their effect on adult emergence rate of mosquitoes. For example, Kibuthu et al. (2016) observed significantly higher emergence rate of *Cx. quinquefasciatus* in diammonium phosphate treatment. The high emergence rate in NH_4^+, NO_3^- or PO_4^{-3} solution container can be attributed to increasing microbial activities (Sunish and Reuben, 2001; Paul et al., 2006).

During the study of effect of NH_4^+ (5 ppm), NO_3^- (45 ppm) and PO_4^{-3} (30 ppm) in water on female to male ratio of *Cx. quinquefasciatus*, the female to male adult ratio for each nutrient solution container was significantly higher than control container ($P<0.05$) (*Table 7*). Maximum female to male ratio was observed for NO_3^- and PO_4^{-3} solution container. To the author knowledge, very limited studies have been conducted on the effect of inorganic nutrient pollutants on female to male ratio in mosquitoes. For example, Noori et al. (2015) reported that high NO_3^- levels in water without leaf litter favored the development of male mosquitoes and suppresses the development of female mosquitoes. They suggested that the development of females might need more PO_4^{-3} than male development. But the findings of the present study suggest that high concentration of NH_4^+, NO_3^- or PO_4^{-3} favors the development of females. For the control container (containing only 100 grams leaf litter), the percentage of males was higher than the percentage of females (42.9±2.3% females, 57±2.3% males).

Conclusion

It is concluded that the levels of NH_4^+, NO_3^- and PO_4^{-3} recommended in effluents for wastewater reuse in agricultural irrigation by WHO (2006) are favorable for the survival and development of *Cx. quinquefasciatus* mosquito, however higher levels of NH_4^+, NO_3^- and PO_4^{-3} are not favorable for the survival and development of this mosquito. These results help in identification of concentration ranges of the NH_4^+, NO_3^- and PO_4^{-3} that make the larval habitat favorable for survival and reproduction of *Culex* mosquitoes. This could be helpful in effective control of mosquito borne diseases.

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