



Effects of antibiotics (Azithromycin) on different live feed species in aquaculture industry

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Abstract

Globally, intensive animal production is increasing simultaneously due to the high demand for proteinaceous nutritional sources. To increase yields and eliminate fish disease in aquaculture industry, farmers use different kinds of antibiotics for their sustainable livelihood. Azithromycin, a frequently used antibiotic, has a high potential to be mixed with surface waterbodies near agricultural fields, industrial sites, or by direct implementation into an aqua-eco-system. Fluctuations in pH may lead to changes in pharmaceuticals bioactive potential, thereby influencing its bioavailability and toxicity to exposed organisms. Therefore, we investigated the effect of Azithromycin (Antibiotic) on *Echinisca triserialis* (Littoral species), *Macrothrix rosea* (Benthic species) and *Ceriodaphnia cornuta* (Limnetic species) all are considered as important live feed species in Aquaculture industry. Firstly, we estimated tolerance level applying acute bioassay tests to elucidate 50% lethal concentration (LC50) of AZ for *Echinisca triserialis*, *Macrothrix rosea* and *Ceriodaphnia cornuta* to four different concentrations (62.5, 125, 250, 500 mg/L) of Azithromycin at three levels of pH (6.5, 7.5 and 8.5). In each replicate beaker, 10 individuals of 12h old neonates were introduced for each of the three species and mortality was recorded after 24h incubation. Secondly, for chronic bioassay testes, age at first reproduction and population growth were recorded in control and in Azithromycin (62.5 and 125 mg/L) at 6.5, 7.5 and 8.5 levels of pH for 10 days. As the results, Among the three species of cladocerans, *Macrothrix rosea* (benthic species) was the most survival and reproducing exposed to different concentration of Azithromycin at pH 8.5. *Echinisca triserialis* (littoral species) was able to survive and high reproducing at 7.5 and *Ceriodaphnia cornuta* (limnetic species) preferred to survive at pH 6.5.

Keywords: Zooplankton, antibiotic (Azithromycin), acute toxicity and population growth rates

Introduction

Pharmaceuticals are among the prime examples of contaminants that have recently been detected in water systems, with up to 90% of oral drugs that pass through the human body ending up in the water supply. These emerging pollutants, therefore, present a new global water quality challenge with potentially serious implications to human health and ecosystems. The increased consumption, disposal and presence of human pharmaceuticals in the environment, particularly in aquatic systems, has raised concerns worldwide due to their continued introduction into the environment mainly via hospital effluents, agricultural activities and waste-water treatment plants (WWTP).

India is the third largest producer of pharmaceutical chemicals after the USA and Europe, and its turnover is expected to reach US\$74 billion per year by 2020 (CCI 2012) [3]. Production and use of large quantities of pharmaceuticals for human and veterinary applications could lead to the release of more pharmaceutical substances into the environment (Kurunthachalam 2012) [7]. After administration to human and animals, up to 90 % of the antibiotics can be excreted unchanged (Hirsch *et al.* 1999) [6] and reach to the aquatic environment. Extremely high concentrations of antibiotics were found in treated sewage in India (Fick *et al.* 2009) [4].

Risk to aquatic organisms (Zhang *et al.* 2012) [18] has been correlated with pharmaceutical residues present in wastewater. Microorganisms present in the aquatic

environment are exposed to the multiple drug residues and their continual exposure may lead to mutations and development of new strains. Organisms respond to pharmaceutical exposure at trace environmental concentrations (Gullberg *et al.* 2011) [5], which indicates that pharmaceutical residuals are of significant environmental concern (Li *et al.* 2010). Antibiotic residues have now been reported in aquatic organisms (shrimps) from many southern states of India (Swapna *et al.* 2012) [15] and thus organisms of higher trophic level are exposed to such residues which may result irreversible loss to biodiversity.

Zooplankton populations, which are widespread in freshwater and marine ecosystems, are populated in aquatic ecosystems around the globe and have their density as an indispensable component of food webs in aquatic systems (Lomartire *et al.*, 2021 [10]; Midya *et al.*, 2018) [12]. According to the Millennium Ecosystem Assessment in 2005, ecosystem services are broadly classified into four categories i.e., regulating, provisioning, supporting and cultural services. It was established that the presence of zooplankton in aquatic systems affects the dynamics of the aquatic ecosystem and that the perpendicular migration patterns of the zooplankton population have too much impact on fish migration rates, as well as acting as a grazer for bacteria and algae and influencing their population communities (Bruce *et al.*, 2006 [1]; Sterner, 2009) [14]. Thus, the gradual increase in primary productivity in aquatic

systems may have key effects on the eutrophication of the waterbodies, which creates stress in aquatic communities, i.e., the zooplankton population (Dounas *et al.*, 2007). Zooplankton play an important role in nutrient recycling, they provide phytoplankton with phosphorus and nitrogen, help in the biological carbon pump, which regulates the CO₂ level in the atmosphere, (Sterner, 2009^[14]; Kwon *et al.*, 2009)^[8]. The zooplankton community has a strong effect on the biomass stocks of other various planktonic groups; it has also altered the concentration of prey and predator populations, with consequent effects on fish biomass in aquaculture (Vanni, 2002)^[16]. Zooplankton population communities are used as indicators for environmental conditions, which alter the status of ecosystems like living flora and fauna and pass on information to us about predators like fish and their abundance (Sherman *et al.*, 2002)^[13]. Zooplankton populations are used as a live feed for various farmed fish in aquatic environments; the quality of the feed has direct and indirect effects on growth and mortality, respectively, in fish and is cost-effective (Midya, 2022)^[11].

Certainly not only direct toxicity of these chemicals plays a role in studies on environmental impact assessment. One should also consider the fact that they are present in complex mixtures with other stressors, at varying pH values, irradiation conditions, etc., which make toxicity determination and its relation to a particular chemical almost impossible. The assessment of ecotoxicity of environmental compartments. Bioassays (e.g. bacterial, algal, animal and plant ones) are considered to be powerful effect-based tools for studying the combined effects of mixtures of compounds. They have the potential to elucidate the relationship between chemical contamination and ecological status (Wieczerek *et al.*, 2016a)^[17]. Additionally, they are used as a regulatory reference for deriving environmental quality standards under the Water Framework Directive (European Commission, 2011). The objective of the present study was to investigate acute toxicity and population growth rates of three species of cladocerans exposed to different concentrations of Azithromycin (antibiotics) at three levels of pH.

Materials and methods

1. Test animals

The research was conducted in Ecology and Ecosystem Laboratory, Department of Environmental Science, CUSB. Samples of zooplankton were collected through 70 µm plankton net from various lakes or ponds near CUSB

Campus. Samples were transported to the laboratory and selected zooplankton (Cladocera) were observed and identified under the stereozoom microscope. Selected species were isolated from the samples, cultured and cloned whenever feasible. To get filtered water, tap water was filtered by using a Whatman filter paper 11 µm and stored in the container for culture development. In laboratory zooplankton species were fed with microalgae *Chlorella vulgaris*.

2. Culture of Microalgae (*Chlorella vulgaris*)

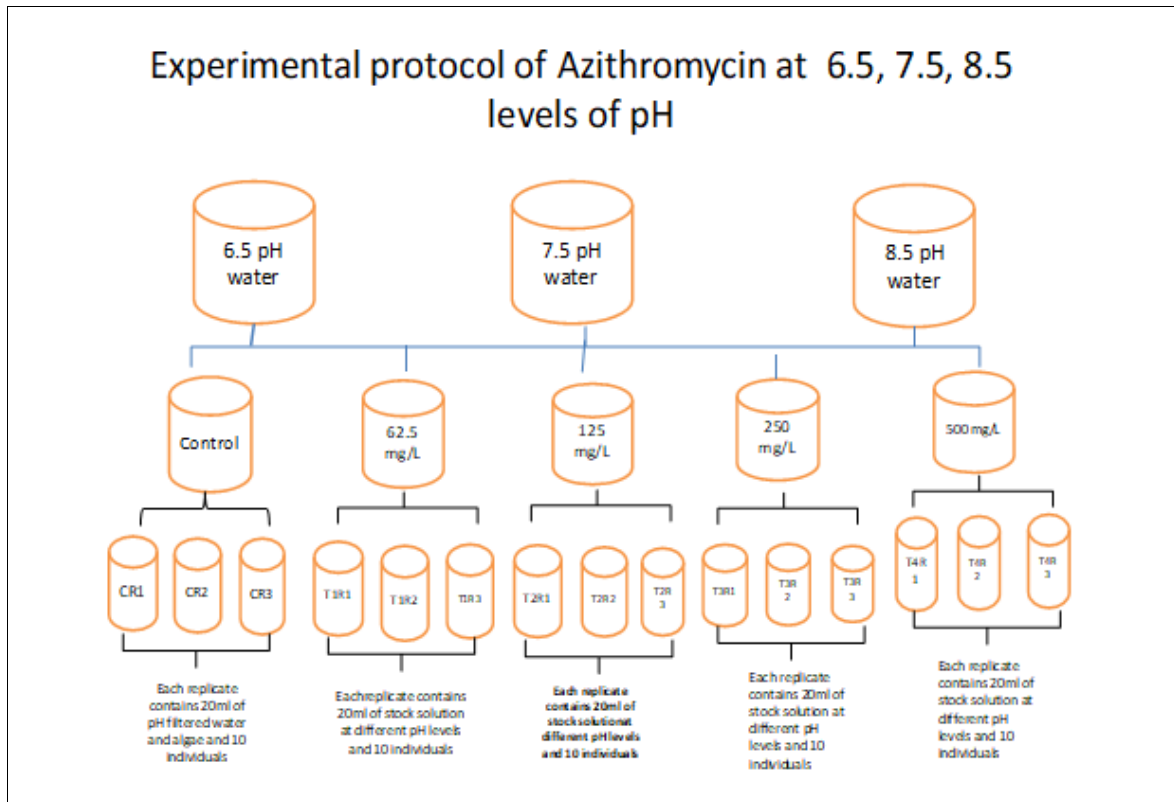
The unicellular alga, *Chlorella vulgaris* cultured in the laboratory was chosen as the test food type for all the experiments. The alga was mass cultured in borosilicate glass conical flat bottom flask with BG 11 broth powder mixing with 1 liter of distilled water. Mixed media was prepared and autoclaved, after cooling that culture media, was introduced with 5 ml of *C.vulgaris* species. The solution glass conical flask was kept in a culture room under continuous fluorescent illumination at a temperature of 22°C. Autoclaved sodium bicarbonate salt was periodically added as an additional source of carbon. For the use in experiments *Chlorella vulgaris* was always harvested in the log phase of its growth

3. Preparation of test solution

Azithromycin drug (Antibiotic) was purchased from medical shops. The drug tablets were crushed to get fine powder in the washed and dried mortar and pestle. For experiments, 10 grams of medicine powder were mixed with the autoclaved filtered tap water. That stock solution was stored in a refrigerator at 4°C. From that stock solution 5g/L, different concentrations of solution were prepared by serial dilution. Six different concentration of that solution were made 62.5 mg/L, 125 mg/, 250 mg/L, and 500 mg/L. One control solution was prepared in which filtered tap water and *C.vulgaris* algae were added, no medicine present. For each treatment three replicates were used and three replicates for control also. The treatments will be labeled as T1, T2, T3, T4, T5, T6 and their replicates as T1R1, T1R2, T1R3, T2R1, T2R2, and T2R3 respectively. Ten individuals of selected species will be transferred carefully in 50 ml beakers contained 20 ml of filtered water with the help of a fine dropper under stereozoom microscope and those 50 ml beakers will be covered to check the evaporation. Dead and live individuals of selected species will be recorded after 24±2 hr.

Table 1: Experimental protocol to be followed for acute responses, numerical responses tests on cladocerans in different concentration of Azithromycin at three levels of pH

Sr. No.	Name of Experiment conducted	Parameters to be tested	Concentration (mg/L)	Medium volume (ml)	Duration(Days/Hours)
1.	Dose response estimation (Acute test)	Survivals	62.5, 125, 250, 500	50	24 hr
	Numerical response test	Survivals, developmental states and reproduction growth rates	62.5 and 125	50	10 day



Numerical responses of three species of zooplankton

Ten cohort individuals of all three selected species zooplankton (*Echinisca triserialis*, *Ceriodaphnia cornuta* and *Macrothrix rosea*) were exposed to 62.5 and 125 mg/L of Azithromycin and control also. Their birth rate and mortality rate were recorded daily. According to Pianka (1988), the population growth rate (r) was calculated from the exponential phase of population growth using the formula;

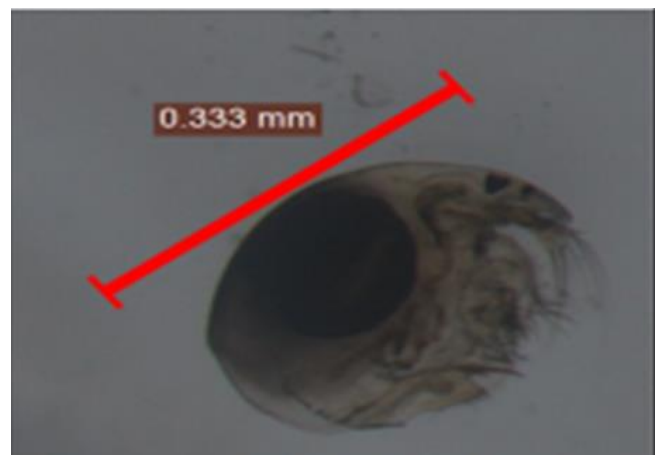
$$r = \frac{\ln N_t - \ln N_0}{t}$$

Where,

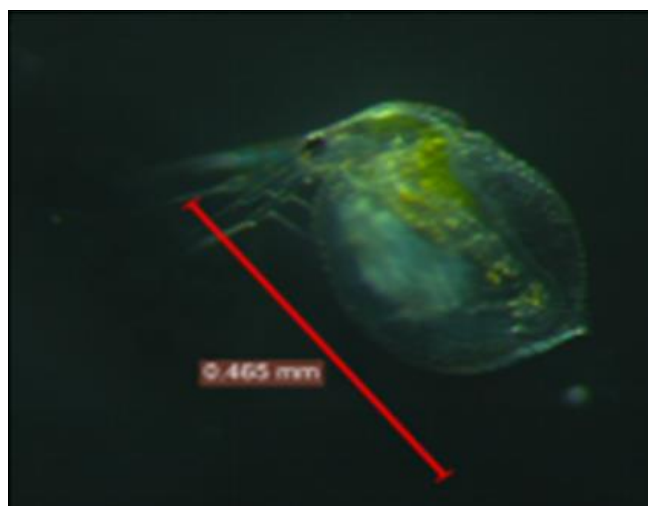
N_0 = Initial population density

N_t = Population density after time t,

r = Population growth rate



(b) *Echinisca triserialis* (Littoral species)



(a) *Ceriodaphnia cornuta* (Limnetic species)



(c) *Macrothrix rosea* (Benthic species)

Fig 1: Three species belong to Cladocera

Result

1. Acute toxicity tests

Depending on the conditions of the experiment, survivors of three selected species of cladocerans were counted under stereozoom microscope after 24 hours of exposed to (62.5, 125, 250, 500 mg/L) at different concentrations of 6.5, 7.5 and 8.5 pH. After 24 hours of exposure, the median lethal concentration (LC50) of the tests selected six species of zooplankton were obtained by a plot of percentage of *Echinisca triserialis*, *Ceriodaphnia cornuta*, *Macrothrix rosea*, against the logarithm of the sample concentrations (Moshi *et al.*, 2010). LC50 values were estimated using a probit regression analysis (Finney, 1952). These different concentrations of Azithromycin (mg/L) calculated as log 10 concentrations of 1.4, 1.7, 2.0, 2.3, 2.6 and 2.9. Mortality rates were recorded after 24 hours. *Echinisca triserialis* was able to survive without dying in control and this species in treatments of Azithromycin declined individuals of survival at 6.5 pH level by probit rates 0, 3.12, 4.16, 3.72 and 3.72 with y value ($y = 1.5179X - 0.2149$). At 7.7 pH level, this *E. triserialis* species was survived all individuals in control than treatments 62.5 to 500 mg of Azithromycin. The survivors in treatments is decreased depend on serial of concentrations of Azithromycin by probit rates 0, 4.05, 3.72, 4.75 and 4.39 with y value ($y = 1.76X + 0.2176$). In 8.5 pH

Level, the species of *E. triserialis* was able to survive from control to treatments. Although, the survival individuals were declined from control to serial concentrations of Azithromycin by probit rates 10, 37, 50, 30 and 50% with y value ($y = 0.4428X + 3.7778$) (Fig. 2)

The survival of *Macrothrix rosea* species decreased from control to treatments 62.5-250 at 6.5 pH by probit rates 4.39, 4.39, 4.92, 4.82 and 6.28 with y value ($y = 0.5919X + 4.0158$) and 7.5 pH by 4.16, 4.67, 4.75, 4.56 and 5.61 with y value ($y = 0.3832X + 4.0611$). Although this species was died nearly all individuals in 500 mg/L of Azithromycin at pH 6.5, individuals in 500 mg/L of Azi were survived a few individuals. At pH 8.5, the *M. rosea* survived from control to treatments by probit rates 3.87, 3.72, 3.72, 3.72 and 4.67 with y value ($y = 0.1305X + 3.7054$) (Fig.3). For *Ceriodaphnia cornuta*, this species was able to survive without dying in controls both of pH 6.5 and 8.5. In treatments, the survival of individuals decreased than controls. Results mortality probit rates were 0.00, 3.36, 4.16, 0.00 and 5.25 with y value ($y = 1.3279X + 0.1666$) at pH 6.5 and by probit rates 0.00, 3.72, 5.84, 5.13 and 5.39. The survival of individuals in pH 7.5 were less than pH 6.5 and 8.5 according to results of probit rates 3.72, 3.96, 4.87, 5.25 and 5.67 with y value ($y = 0.6717X + 3.4864$) (Fig.4).

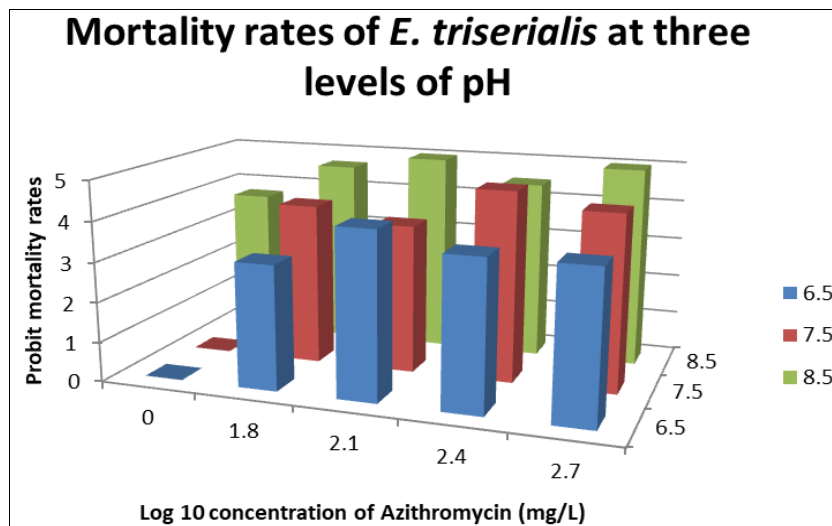


Fig 2: Probit mortality rates of *Echinisca triserialis* at three levels of pH.

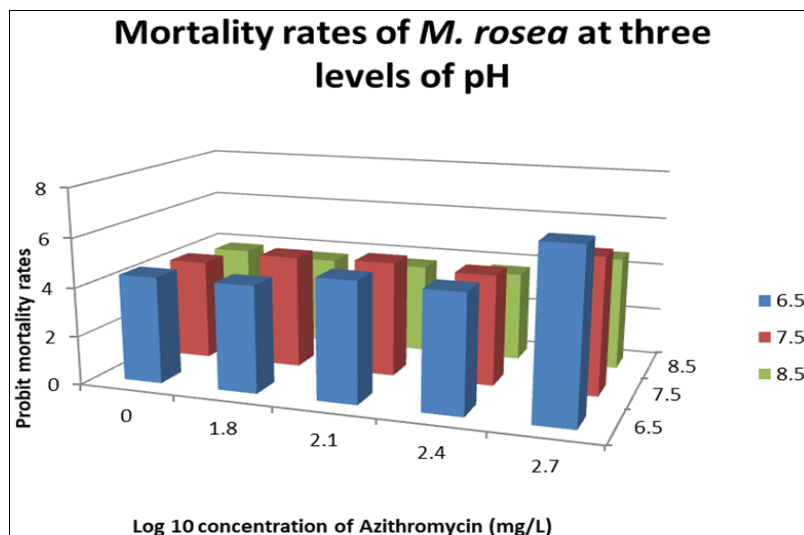


Fig 3: Probit mortality rates of *Macrothrix rosea* at three levels of pH

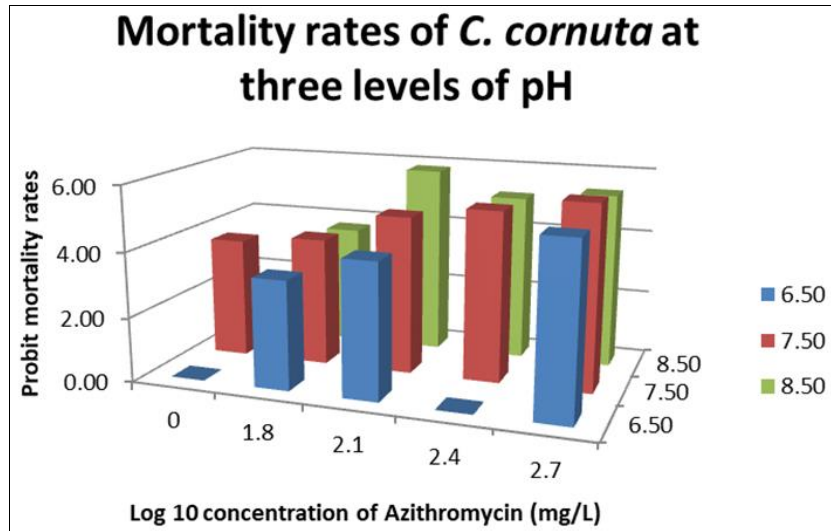


Fig 4: Probit mortality rates of *Ceriodaphnia cornuta* at three levels of pH

Comparison lethal concentration on three species of cladocera at three levels of pH

According to results, in the comparison of three species cladocerans, at pH 6.5, the highest lethal concentration (LC10,50,90) was *Ceriodaphnia cornuta* (Limnetic species) with 26.76, 36.4 and 46.04 followed by *Echinisca triserialis* with 23.1, 31.52 and 39.96 and *Macrothrix rosea* (Benthic species) with (-4.49), 16.63 and 38.25. The higher lethal concentration is the lower toxicity. Thus, *Ceriodaphnia cornuta* (Limnetic species) was tolerance at pH 6.5. At pH 7.5, the highest lethal concentration (LC10, 50,90) was *Echinisca triserialis* (Littoral species) with 19.9, 27.17 and 34.45 followed by *M. rosea* with (-8.9), 24.5 and 57.9 and

The lowest lethal concentration belong to *C. cornuta*. Although, in LC 90, the highest rate was *M. rosea* followed by *C. cornuta* and *E. triserialis*. Even if *E. triserialis* species was less, in LC 90, LC10 and LC50 highest. Therefore, the littoral species, *Echinisca triserialis* was able to be tolerance at pH 7.5. At pH 8.5, the highest lethal concentration (LC10, LC50 and LC90) belong to *Macrothrix rosea* (Benthic species) with 1.12, 99.2 and 197.3 followed by *E. triserialis* with (-1.31), 27.6, 56.51 and the lowest lethal concentration rate was *C. cornuta* with 16.6, 27.55 and 28.5. By the results, the benthic species, *Macrothrix rosea* prefer to survive at pH 8.5 (Table 2 and Fig. 5).

Table 2: Comparison of lethal concentration (LC20, LC50 and LC90) of three species belong to Cladocera at three levels of pH

Taxonomy group	Species	pH 6.5			pH 7.5			pH 8.5		
		LC10	LC50	LC90	LC10	LC50	LC90	LC10	LC50	LC90
Cladocera	<i>Echinisca triserialis</i> (Littoral species)	23.1	31.52	39.96	19.9	27.17	34.45	-1.31	27.6	56.51
	<i>Macrothrix rosea</i> (Benthic species)	-4.49	16.63	38.25	-8.9	24.5	57.90	1.12	99.2	197.3
	<i>Ceriodaphnia cornuta</i> (Limnetic species)	26.76	36.4	46.04	3.48	22.53	41.59	16.60	22.55	28.5

Higher LC = Lower toxicity (more concentration is needed for lethality)

Lower LC = Higher toxicity (less concentration is needed for lethality)

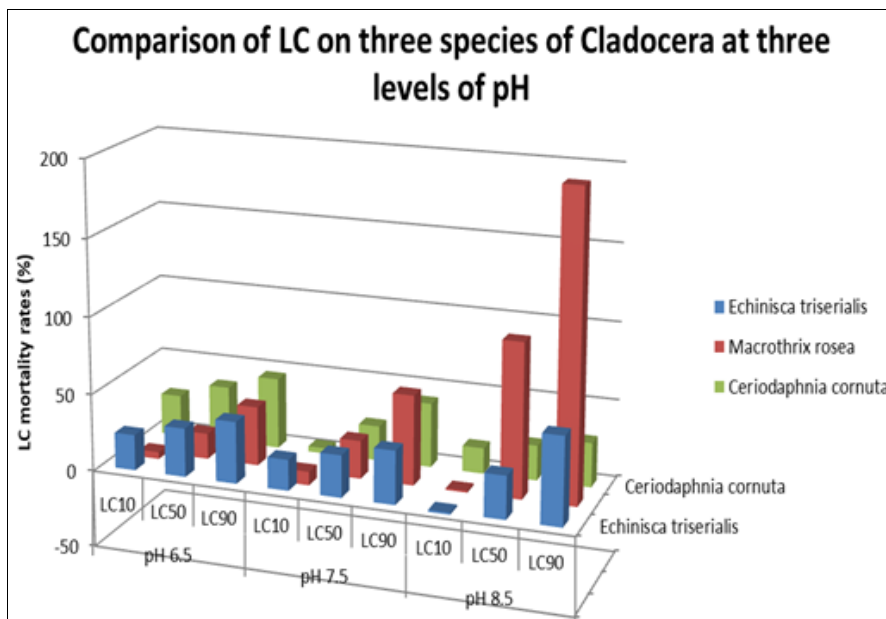


Fig 5: Comparison of lethal concentration (LC 10, 50, 90) on three species at three levels of pH.

Numerical responses of three species of Cladocera

At pH 6.5, *Echinisca triserialis* (Littoral species) started reproducing after 4 day of reproduction reproduced 7 individuals at first reproduction. In 62.5 mg/L of Azi, started reproducing 2 individuals and no reproduce in 125 mg/L of Azi. The maximum population growth rates were 3.30 with 27 individuals in control followed by 2.23 with 9 individuals in and individuals and 1.73 with 6 individuals in 125 mg/L of Azi. At pH 7.5, the highest population growth rates of *E. triserialis* were 3.21 with 25 individuals in control followed by 3.11 with 22 individuals in 62.5 mg/L and 2.51 with 12 individuals in 125 mg/L. At pH 8.5, the population growth rates were 2.12 with 8 individuals in control followed by 2.48 with 12 individuals and 0.51 with 2 individuals. Therefore, for the littoral species of *Echinisca triserialis* was preferred at pH 7.5.

Macrothrix rosea (Benthic species) in control and 62.5

mg/L of Azithromycin treatment except 125 mg/L was not able to reproduce at pH 6.5. This species survived a few individuals in each treatment. The population growth rates were 2.08 with 8 individuals in control followed by 1.10 with 3 individuals in and individuals and 1.47 with 4 individuals in 125 mg/L of Azi. At pH 7.5, the population growth rates of *Macrothrix rosea* were 1.85 with 6 individuals in control followed by 0.00 with 0 individuals in 62.5 mg/L and 0.69 with 2 individuals in 125 mg/L. At pH 8.5, the highest population growth rates were 3.21 with 25 individuals in control followed by 0.00 with one individuals and 0.00 with 0 individuals. Therefore, for the benthic species of *Macrothrix rosea* was preferred at pH 8.5.

The limnetic species, *Ceriodaphnia cornuta* was not ability to reproduce in all treatments including controls at all three levels of pH. Thus, this species was no population growth rates after 10 days (Table 3 and Fig. 6).

Table 3: Comparison population growth rates of three species belong to Cladocera

Taxonomy group	Species	pH 6.5			pH 7.5			pH 8.5		
		Control	62.5 mg/L	125 mg/L	Control	62.5 mg/L	125 mg/L	Control	62.5 mg/L	125 mg/L
Cladocera	<i>Echinisca triserialis</i> (Littoral species)	3.3	2.23	1.73	3.21	3.11	2.51	2.12	2.48	0.51
	<i>Macrothrix rosea</i> (Benthic species)	2.08	1.1	1.47	1.85	0	0.69	3.21	0	0
	<i>Ceriodaphnia cornuta</i> (Limnetic species)	0.92	0	0	0	0	0	0	0	0

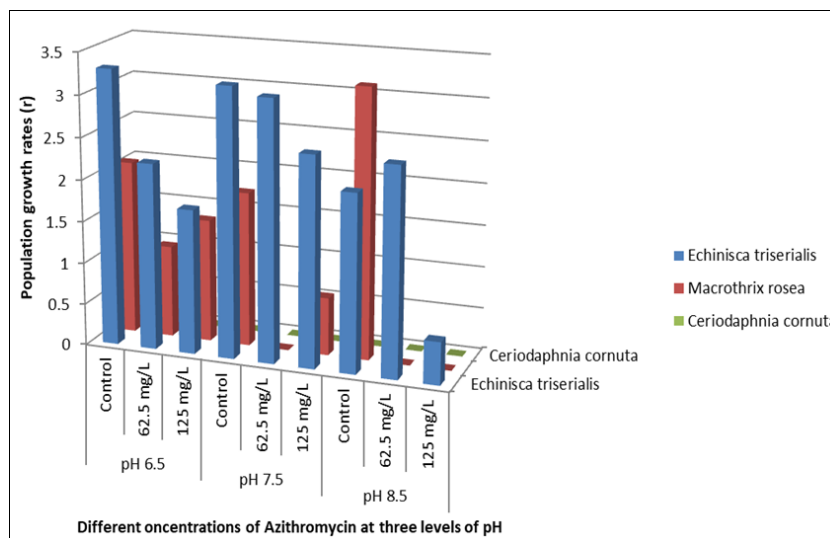


Fig 6: Comparison of numerical responses of three species belong to Cladocera

Discussion

This study investigated the effects of Azithromycin at three levels of Azithromycin on three species of Cladocera. Firstly, three species *Echinisca triserialis* (Littoral species), *Macrothrix rosea* (Benthic species), *Ceriodaphnia cornuta* (Limnetic species) were tested acute responses exposed to 62.5, 125, 250 and 500 mg/L of Azithromycin at three levels of pH. Among three species, *Echinisca triserialis* (Littoral species) was able to survive well at pH 7.5, the benthic species, *Macrothrix rosea* was preferred to survive at pH 8.5 and the limnetic species of *Ceriodaphnia cornuta* survived at pH 6.5 than pH 7.5 and pH 8.5. The survivals of all three species were declined significantly in different concentrations of Azithromycin.

Secondly, these three species were investigated numerical responses exposed to 62.5, 125 mg/L and of Azithromycin. We obtained the highest population growth rate of *E. triserialis* at pH 7.5, the maximum population growth rate of

Macrothrix rosea (Benthic species) recorded at pH 8.5 and for *Ceriodaphnia cornuta* (Limnetic species), just survived only in control at 6.5.

The success of fish hatchery linked to the readily available live feed, zooplankton are important as live feed sources in larval fish culture because of higher nutritional value and economic feasibility for their production in mass culture. In addition to nutrient composition, these organisms are preferred as live feed by early larval fish due to its smaller sizes and higher locomotive behavior. Zooplankton species were high level of proteins, free amino acids, fats and micronutrients. Also pH levels are important in aquacultures and ecosystem. As pH can change due to anthropogenic, the results of changing pH levels can effect on aquatic species. Therefore, we have to inform knowledge of pH level limited. According to present study results, three species (littoral, benthic and limnetic) different live feeds, zooplankton preferred different levels of pH. Moreover,

Azithromycin (antibiotics) at different levels of pH effected negative impact on their survivals and reproducing of three species of Cladocera. For algae and zooplankton form the basis of aquatic food webs, drastic and sudden changes in their population growth would likely cause a rippling effect through the entire ecosystem.

Conclusions

Therefore, it is important to assess impacts of emerging pollutants like antibiotics on nontarget species, which we have done in this study.

This study suggests carefully discarding the biomedical wastes and application of traditional medicines and natural antibiotics are more eco-friendly then these xenobiotic compounds.

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