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Effect of paracetamol and diclofenac on population growth of *Plationus patulus* and *Moina macrocopa*

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Abstract

Two non-steroidal anti-inflammatory commonly used in Mexico are paracetamol and diclofenac. These drugs reach natural waterbodies transport through the hydrological areas of agriculture and effluent treatment plants and can affect plankton. In this study, we quantified the chronic effects of paracetamol and sodium diclofenac on the population dynamics of the rotifer *Plationus patulus* and the cladoceran *Moina macrocopa* under laboratory conditions. Both these species of zooplankton are common in freshwaters and are sensitive to chemicals in the medium. Our results showed that densities of both *P. patulus* and *M. macrocopa* decreased with increasing levels of both the analgesic drugs. Both zooplankton species did not survive beyond when paracetamol was applied at 32 mg l^{-1} in the medium. Diclofenac in general had more adverse effect than paracetamol for either zooplankton species. *P. patulus* was more sensitive than *M. macrocopa* to both analgesic drugs. When diclofenac was present in the medium at ≥ 12.5 mg l^{-1}, rotifer reproduction was inhibited, while the tested cladocerans continued to grow but to lower densities compared to control. The rate of population increase (r) per day of *P. patulus* and *M. macrocopa* was significantly and inversely related to the concentration of paracetamol and diclofenac in the medium. However, the relationship between r and drug concentration differed depending on the zooplankton species and drug. In controls, the r of *P. patulus* was 0.18 d^{-1}, for *M. macrocopa* under similar conditions, it was slightly lower (0.16 d^{-1}). The r values of both zooplankton populations became negative (-0.10 to -0.15 d^{-1}) when exposed to paracetamol at 32 mg l^{-1} or diclofenac at 25 mg l^{-1}.

Key words

Cladocera, Pain relievers, Rotifera, Sublethal effects

Introduction

Aquatic ecotoxicology is biased towards the evaluation of toxic effects of substances such as heavy metals, pesticides and fungicides and using aquatic organisms such as fish, invertebrates, plants and algae (Miracle *et al*., 2011). A large group of chemicals including pharmaceuticals enter the aquatic ecosystems but have received much less attention from ecotoxicologists (Fent e*t al*., 2006). Unlike pesticides and heavy metals, which are found in relatively larger quantities in contaminated waterbodies, pharmaceuticals usually occur at extremely low concentrations (e.g., ng l^{-1}) making them more difficult to detect and quantify (Bila and Dezotti, 2003). Nonsteroidal anti-inflammatory drugs (NSAID) are widely available because no medical prescription is needed to purchase or administer them. In Mexico, most widely consumed NSAIDs are acetylsalicylic acid, paracetamol, ibuprofen, diclofenac and naproxen (WHO, 2004). Paracetamol and diclofenac are also the commonly consumed analgesics in medicine and veterinary medicine (Katzung, 1995; Haap *et al*., 2008; Hussein *et al*., 2008).

Many analgesics are xenobiotics and are physiologically active even after the expiry period. This has raised concerns because of possible impact on aquatic organisms when they drain into natural waterbodies (Cleuvers, 2004; Fent *et al*., 2006). Effluents from both domestic and veterinary sources that contain

these substances eventually reach treatment plants and may pass through the final stage of processing without being removed (Araujo and McNair, 2007). In certain European countries, waste water treatment plants have already reported the presence of these drugs in their effluent waters (Ferrari *et al*., 2003). Wastewaters from the Valley of Tula (State of Hidalgo, Mexico) contain high concentrations of diclofenac (2-5 µg l^{-1}) (Gibson *et al*., 2010). Natural waterbodies such as rivers, ponds and lakes and man-made systems such as wastewater treatment plants contain various species of planktonic organisms (Wallace and Snell, 2010). A few studies have shown that planktonic organisms are adversely affected by the presence of analgesic substances, especially after long term exposure. For example, Hussein *et al*. (2008) have shown the adverse effects of paracetamol to plankton.

Freshwater zooplankton is mainly composed of ciliates, rotifers, cladocerans and copepods. Rotifers and cladocerans are widely used as indicators of stress in aquatic environments because they respond rapidly to various dissolved substances in the medium (Sarma and Nandini, 2006). They are also numerically more abundant than copepods in freshwaters and therefore used in risk assessments of drugs and their metabolites (Snell and Joaquim-Justo, 2007). Both rotifers and cladocerans are widely distributed in Mexico and in terms of diversity they represent nearly 15 to 20% of the total species known globally for these two groups (Sarma, 1999; Elías-Gutiérrez *et al*., 2008). In spite of that only few species have been successfully cultured. *Brachionus* among rotifers and *Moina* among cladocerans are widely used in bioassays because they are common in tropical waters and also are the non-target organisms exposed to various industrial, agricultural and pharmaceutical products through effluents (Sarma and Nandini, 2006; Snell and Joaquim-Justo, 2007).

Under stressful conditions, changes in the population growth of zooplankton are considered to be more sensitive than other responses such as feeding or swimming rates (Sarma and Nandini, 2006). Many studies thus focus on the derivation of population growth rates, which are the consequences of changes in the abundances of populations (Lampert and Sommer, 1997; Forbes and Calow, 1999). Population growth rates are thus largely used to interpret the responses of zooplankton exposed to stress (Roex *et al*., 2000). This study quantifies the chronic effects of two analgesics, paracetamol and sodium diclofenac, on the population growth of the rotifer *Plationus patulus* and the cladoceran *Moina macrocopa* under laboratory conditions.

Materials and Methods

Two zooplankton species (*Plationus patulus* and *Moina macrocopa*) were isolated from the main canal of Xochimilco lake (Mexico City). The clonal populations for each species were separately cultured using single celled green alga *Chlorella vulgaris* as food and moderately hardwater as medium (EPA medium). The US EPA medium was prepared by dissolving 96 mg $NaHCO_3$, 60 mg $CaSO_4$, 60 mg $MgSO_4$, and 4 mg KCl in one liter of distilled water (Weber, 1993). *Chlorella vulgaris* was batch cultured using standard culture medium (Bold's basal, Borowitzka and Borowitzka, 1998). Log phase algae were harvested, centrifuged at 3000 rpm for 5 min, rinsed and resuspended in distilled water. Algal density was estimated microscopically using a haemocytometer. For zooplankton cultures, as well as for the experiments, the temperature was set at 23±1°C, pH 7.0-7.4, and fluorescent illumination was continuous but diffuse.

Paracetamol and sodium diclofenac were separately dissolved first in a small volume of (1 ml) of methanol and later diluted using distilled water so as to obtain stock concentrations of 100 mg l^{-1} in 500 ml. Based on the data of median lethal concentration available (Hussein *et al.,* 2008) we chose to test sublethal concentrations for each drug. Thus, for paracetamol, the tested concentrations were 2, 4, 8, 16 and 32 mg l^{-1} and for diclofenac the range was slightly lower (1.56, 3.125, 6.25, 12.5 and 25 mg l^{-1}). Controls contained no added drug. Because the concentration of methanol ending up in the treatments was extremely low (µl per jar) and at this quantity is not toxic to zooplankton (Snell and Janssen, 1995), no negative controls were set up. The drug concentrations were prepared daily just before the commencement of zooplankton quantification.

The experimental design consisted of a total of 66 transparent glass jars of 75 ml capacity (2 zooplankton species X 2 drugs X 5 concentrations X 3 replicates plus 6 controls). Because the body size of the two zooplankton species was different (*P. patulus*: 120µm; *M. macrocopa*: 800µm), we used 25 ml medium for rotifers and 50 ml for cladocerans. The initial zooplankton density in each jar was 1 and 0.2 individual ml^{-1} for *P. patulus* and *M. macrocopa*, respectively. The jars also contained *Chlorella vulgaris* at a density of $1X10^6$ cells ml^{-1}. Following initiation of the growth experiments, we quantified the population density of two zooplankton species and then transferred the living individuals to new test jars containing appropriate drug concentration at the specified algal density. The experiments were terminated after the population density of tested zooplankton species began to decline in most replicates: ca. 25 days for rotifers and 10 days for cladocerans. These data permitted us to calculate the rate of population increase per day (*r*) for each replicate according to the following equation:

$$r = (\ln N_t - \ln N_o) / t$$

where r = rate of population growth, N_o = initial density, N_t = final density after duration t, t = time in days (Krebs, 1985).

Difference in the population growth rates of *P. patulus* and *M. macrocopa* exposed to different concentrations of paracetamol and diclofenac were evaluated by one way analysis

of variance (ANOVA) and regression tests following standard statistical package (SigmaPlot ver. 11, Systat Software Inc., California, USA).

Results and Discussion

Population growth curves of *P. patulus* and *M. macrocopa* exposed to different concentrations of paracetamol and diclofenac showed, in general, decreased zooplankton density with increasing levels of both the analgesic drugs. In controls, *P. patulus* had longer (about a week) lag phase while *M. macrocopa* started to increase its population density immediately after the commencement of the experiments. In addition, rotifer populations in controls reached peak abundances in about 3 weeks, while cladocerans under similar conditions needed much less time. Paracetamol at a concentration of 16 mg l^{-1} elongated the lag phase of *P. patulus* while for *M. macrocopa* at this concentration, the initial lag phase was much pronounced. However, both the zooplankton species did not survive beyond 2 weeks when paracetamol was applied at 32 mg l^{-1} (Fig. 1 and 2). In general, diclofenac had more adverse effect than paracetamol for both the zooplankton species. For this analgesic drug, *P. patulus* was also more sensitive than *M. macrocopa*. When diclofenac was present in the medium at concentrations above 12.5 mg l^{-1}, rotifer reproduction was inhibited, while the tested cladocerans continued to grow but not as rapidly as control. For example, in controls by day 4, *M. macrocopa* reached peak abundances (about 1 ind. ml^{-1}) while at 25 mg l^{-1} of diclofenac, the cladocerans could barely maintain a population.

The rate of population increase (*r*) per day of *P. patulus* and *M. macrocopa* was negatively and significantly affected due to paracetamol and diclofenac in the medium (Fig. 3). However, the relationship between r and the drug concentration differed depending on the zooplankton species and the drug. In controls, the *r* of *P. patulus* was 0.18 d^{-1}, for *M. macrocopa* under similar conditions, it was slightly lower (0.16 d^{-1}). The *r* values of both the zooplankton populations became negative when exposed to paracetamol at 32 mg l^{-1} or diclofenac at 25 mg l^{-1}. ANOVA results showed that the drugs had a significant effect on the zooplankton growth rates. Post hoc (Tukey) tests further revealed that the *r* of *P. patulus* in controls differed significantly ($p<0.001$) from the paracetamol or diclofenac treatments only at higher concentrations (> 16 mg l^{-1} or 12.5 mg l^{-1}, respectively) However, this was not statistically significant at lower concentrations ($p>0.05$, F-test). However, for *M. macrocopa* the *r* in control was significantly lower from treatments containing paracetamol or diclofenac only at the highest tested concentrations.

Both paracetamol and diclofenac are common pain relievers in Mexico. These are the xenobiotics with specific effect on metabolic pathways of vertebrates including birds. For example, veterinary diclofenac is toxic to birds and hence it has been officially banned in some countries such as India (Prakash *et al*., 2007). Touliabah *et al*. (2008) have shown that paracetamol at 8 mg l^{-1} caused 50% reduction in the population abundances of various rotifer species including *Brachionus angularis* under field conditions. In addition, under controlled laboratory conditions it has been documented that rotifers of the family Brachionidae are sensitive to these substances rotifers (Preston *et al*., 2000; Araujo and McNair, 2007). In this work, we also observed that both the rotifers and cladocerans were affected by the presence of these drugs, especially under higher concentrations.

The rotifer *Brachionus calyciflorus* is one the most widely bioassay species under freshwater conditions (Snell and Janssen, 1995). However, *Plationus patulus* is also gaining importance in bioassays especially for pesticides and heavy metals (Sarma *et al*., 2001). *Moina* is considered to be less sensitive than *Daphnia* and *Ceriodaphnia* (Sarma and Nandini, 2006). However, species of *Moina* are capable of reproducing and maintaining populations in harsh conditions such as high temperatures (>30°C) and on particulate organic matter such as excreta from domestic animals (Nandini, 2000; Engert *et al*., 2013). In many freshwater bodies where usually domestic, industrial or hospital effluents are discharged, cladocerans such as *Daphnia* and *Ceriodaphnia* do not survive due to their sensitivity to stress (Könnecker *et al*., 2011). Our data showed that indeed *Moina* is nearly as sensitive as rotifer, *P. patulus* for the tested drugs. For some heavy metals also, *Moina* is as much sensitive as the rotifers (Gama-Flores *et al*., 2009).

Compared to paracetamol, diclofenac is more toxic zooplankton possibly due to its tendency of bioaccumulation through food web (Jones *et al*., 2002; Carlsson *et al*., 2006). The range of concentrations of both the analgesics used in this work is much lower than the recommended doses. In spite of this, both

Table 1 : One-way analysis of variance for the rate of population increase per day of *P. patulus* and *M. macrocopa* exposed to different concentrations of paracetamol and diclofenac.

Source of variation	DF	SS	MS	F	*P*
P. patulus					
Paracetamol					
Among treatments	5	0.101	0.0201	97.03	<0.001
Error	12	0.0025	0.0002		
Diclofenac					
Among treatments	5	0.253	0.0506	85.57	<0.001
Error	12	0.0071	0.0006		
M. macrocopa					
Paracetamol					
Among treaments	5	0.167	0.0333	5.02	<0.05
Error	12	0.0796	0.0066		
Diclofenac					
Among treatments	5	0.146	0.0291	4.97	<0.05
Error	12	0.0703	0.0059		

DF = degrees of freedom; SS = sum of square; MS = mean square; F- F-ratio

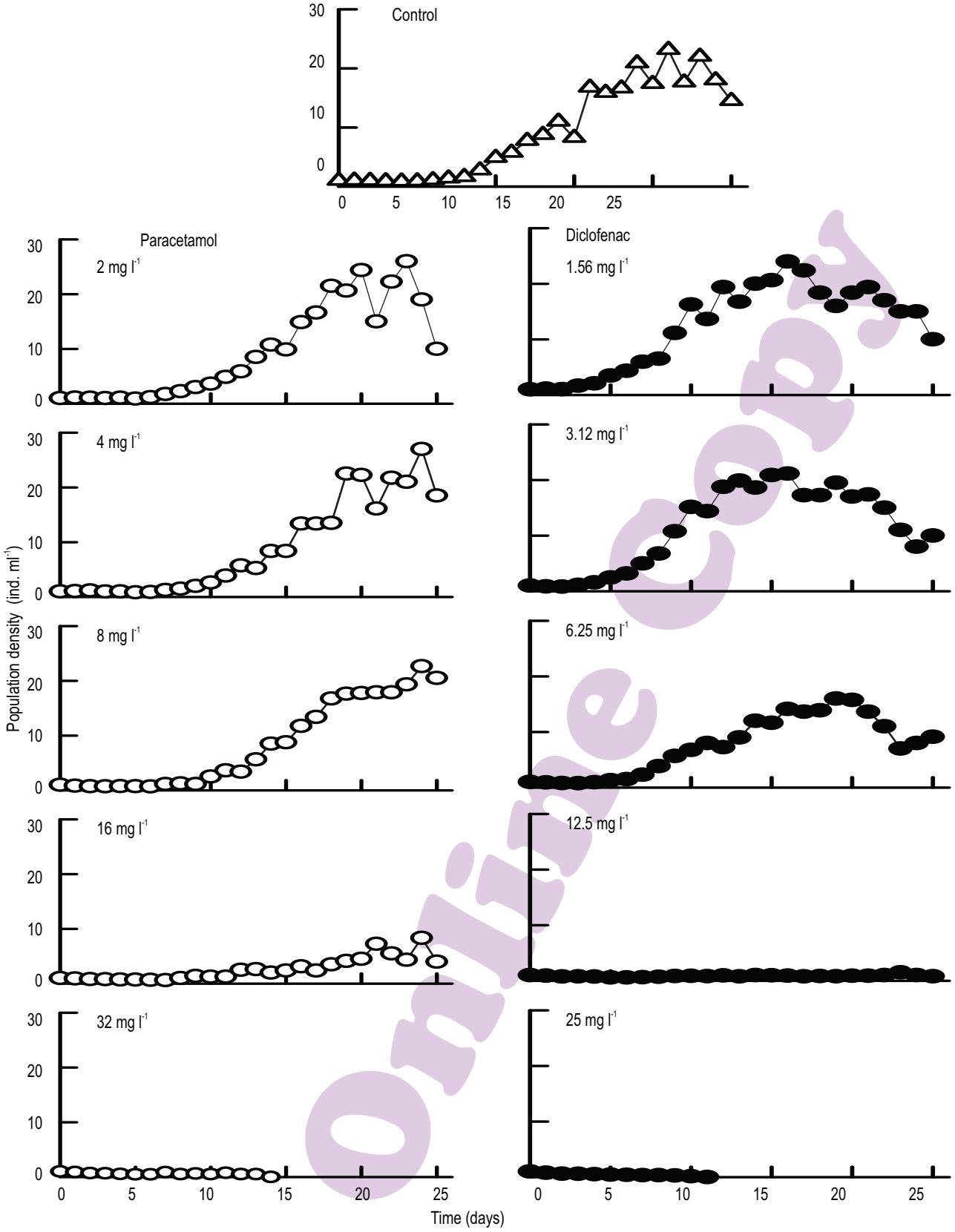

Fig. 1 : Population growth curves of *Platinous patulus* subjected to different nominal concentrations of paracetamol and sodium diclofenac. Values represent means± SE based on four replicates

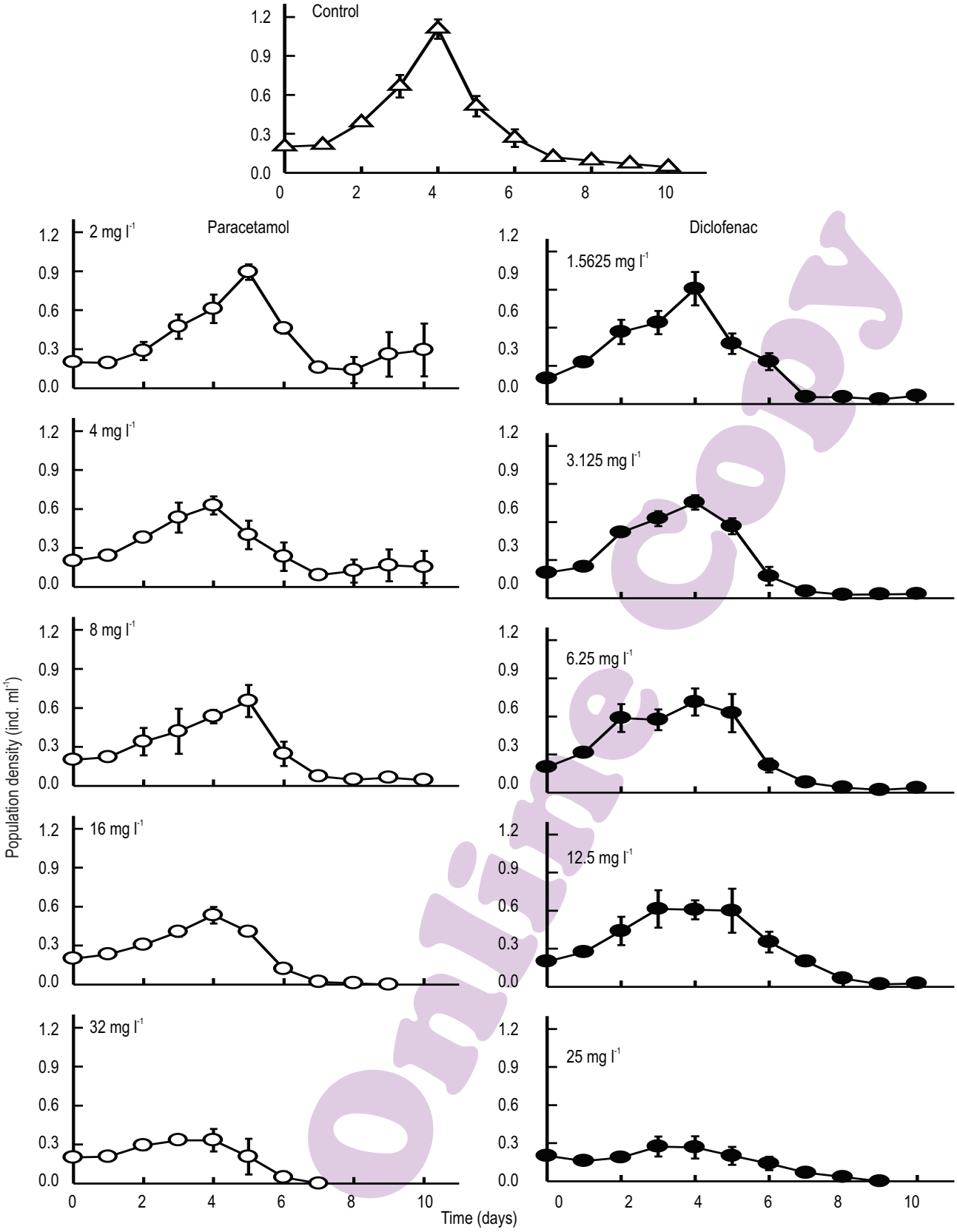

Fig. 2 : Population growth curves of *Moina macrocopa* subjected to different nominal concentrations of paracetamol and sodium diclofenac. Values represent means± SE based on four replicates

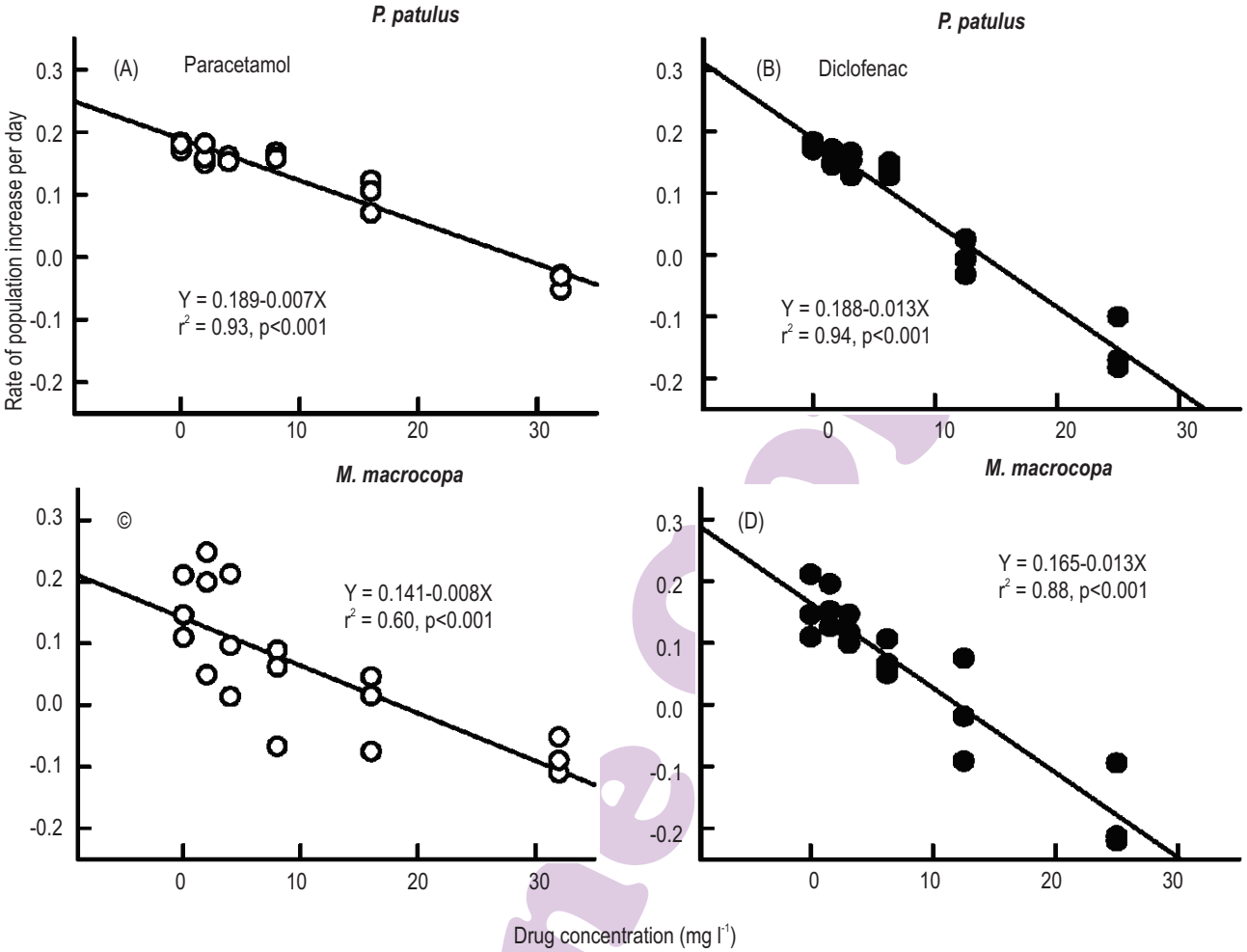

Fig. 3 : Relation between drug concentration and rate of population increase of (A, B) *P. patulus* and (C, D) *M. macrocopa*

these substances caused significantly adverse effect on the population growth of the tested zooplankton species. It has been observed that diclofenac has an adverse effect on both invertebrates and vertebrates (Oaks et al. 2004; Pascoe et al., 2003; Haap *et al*., 2008). The differential effects of diclofenac and paracetamol to the tested zooplankton can be possibly attributed to their chemical structure, environmental fate, farmacodynamics and biological properties (Fent *et al*., 2006). These aspects have not been tested here mainly because our study was based on laboratory conditions which do not permit to evaluate factors like environmental persistence and fate.

Median lethal concentration tests are useful to empirically derive safe levels for a given chemical through application factors such as LC_{50} X 0.1 (Ostrander, 2005). The LC_{50} of paracetamol for different rotifer species is about 8 mg l^{-1} (Hussein *et al*., 2008). For daphniids the LC_{50} for pararacetamol and diclofenac ranged from 20-50 and 65-80 mg l^{-1}, respectively (Henschel *et al*. 1997; Cleuvers 2004). It is known that though two or more zooplankton species may have identical LC_{50} value for a given chemical, at sublethal level, they differ. Our study showed that at sublethal concentrations, the response of rotifers and cladocerans differed. Thus sublethal tests are necessary to complement acute toxicity tests, as recommended in various works on zooplankton (Snell and Janssen, 1995).

Studies on two common pain relievers have shown that these substances have adverse effects on zooplankton. Both *P. patulus* and *M. macrocopa* responded by decreasing population abundances to increasing concentration of paracetamol and diclofenac in the medium. Our study thus indicates that both *Plationus patulus* and *Moina macrocopa* could be useful for evaluating the adverse effects of commonly used pain relievers, as well as other over-the-counter medicines that may pass through wastewater treatment systems. Our results also showed that within a test period of two weeks, the population growth rates of both the tested zooplankton species were adversely affected by the drugs.

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