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Population level responses of rotifers (*Brachionus calyciflorus* and *Plationus patulus*) to the anti-diabetic drug, metformin

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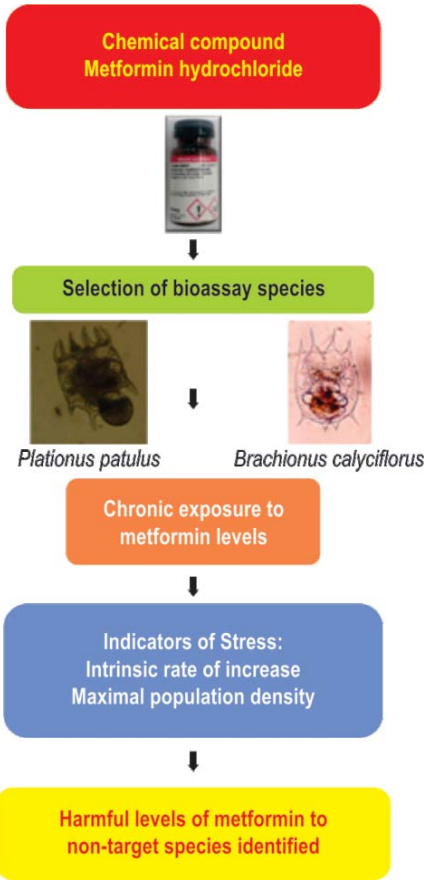
Abstract

Aim: *Brachionus calyciflorus* and *Plationus patulus* are the two brachionid rotifers that are widely recognized as suitable bioassay species for testing the effects of toxicants and xenobiotics. However, their relative sensitivities vary depending on the duration of exposure, nature of the toxicants and the ecological tools used. Most ecotoxicological works on rotifers have considered one species at a time and mainly tested the effects of pesticides and heavy metals. We quantified the population level changes in the rotifers *B. calyciflorus* and *P. patulus* subjected to different concentrations of metformin, a widely used anti-diabetic drug.

Methodology: *B. calyciflorus* and *P. patulus* were separately cultured starting with a single parthenogenetic individual. The single-celled green alga, *Scenedesmus acutus* was used as diet for both the species of rotifers. The experiments were separately conducted using 25 ml of test medium with one of the five concentrations of analytical grade metformin (0 (control), 25, 50, 100 and 200 µg l^{-1}. The initial density of each rotifer was 1 ind. ml^{-1}. Daily we quantified the number of rotifers living in each jar and then transferred the surviving individuals to fresh jars containing appropriate metformin-alga combination. The experiments were terminated after 16 days.

Results: Peak population abundance and the rate of population increase (*r*) of both the rotifers were adversely affected due to metformin. The *r* in controls of *B. calyciflorus* and *P. patulus* were 0.38±0.02 and 0.26±0.01 per day, respectively. For*B. calyciflorus* and *P. patulus* the r decreased to 0.19±0.01 and 0.12±0.02 per day, respectively, when exposed to 200 µg l^{-1} of metformin.

Interpretation: The environmental levels of metformin in effluents are similar to those used in this work. Therefore, metformin should be considered as harmful for rotifers on long term exposure at concentrations of as low as 25 µg l^{-1}.



Introduction

The increased incidence of diabetic problems among the world population is a concern not only to medical profession but also to the pharmaceutical industries (Dunkle *et al.,* 2014). As many as 16% of the Member States of the United National Organization has at least 12% of their population with documented cases of diabetes (Martinez, 2013). This has led to the increased production of antidiabetic medicines, especially those administered orally. For example, the industrial production and consumption of two of the most commonly prescribed oral antidiabetic (against type II diabetes) drugs (metformin (N,N-Dimethylimidodicarbonimidicdiamide) and glibenclamide5-chloro-N-(2-(4-((((Cyclohexylamino)carbonyl)amino)sulfonyl) phenyl) ethyl)-2-methoxybenzamide) has increased considerably in recent years (Schwabe and Paffrath, 2012). With the increase in drug production, environmental problems related to their adverse effects on non-target organisms has become more common (Larsson, 2014). Many drugs eventually reach water bodies through the unregulated release of the expired or unused medicines. In addition, various metabolic products of drugs are excreted via urine or other wastes which also reach freshwaters. In fact, 90% of the consumed metformin remains unchanged in urine even after passing through the human body (Santos, 2012).

Compared to glibenclamide, metformin is more widely prescribed by medical practitioners worldwide and Mexico is no exception to this (Montoya-Eguía *et al.,* 2015). Therefore, metformin is more likely to be found in domestic effluents. For example, in German influent waste waters, metformin has been reported up to 100 µg l^{-1} (Scheurer *et al.,* 2012). Though the half-life of metformin is just a few hours (ca. 6 h), its constant use by the population results in persistent levels in fresh waters and thus affects the aquatic organisms. Among the different invertebrates that inhabit fresh waters, rotifers are generally numerically more abundant than others due to their high taxonomic diversity (ca. 2000 species), smaller body size (average body length ca. 100 µm), short generation time (<one week) and rapid rate of population growth (up to 2 per day) (Wallace *et al.,* 2015). A given water body may have higher than 100 species (Dumont and Segers,1996). However, because of their high sensitivity to stress, only a few species occur in contaminated water bodies (Snell and Jansen, 1995)

The diversity and density of rotifers are also affected by natural factors such as temperature and food level etc., which vary through seasons (Sarma *et al.*, 2001; Vázquez-Sánchez *et al.*, 2014). So, it is difficult to separate the effects due to anthropogenic factors from those natural stresses. Hence, laboratory growth studies on rotifers are advisable since the influence of abiotic factors can be controlled. Population level studies on rotifers are gaining importance in ecotoxicology (Sarma *et al.*, 2014) since the seminal works of Halbach during the 1980s (Halbach,1984). Compared to many other ecological variables (e.g., feeding rates, swimming speed etc.), population growth permits the quantification of certain other sensitive variables such as peak population density and the rate of population increase.

Brachionus calyciflorus and *Plationus patulus* are the two brachionids that are widely recognized as suitable bioassay species for testing the effects of toxicants and xenobiotics (Clesceri, 1999; Rios Arana *et al.*, 2007; Martinez Gomez *et al.*, 2015). However, their relative sensitivities vary depending on the duration of exposure (acute vs chronic), nature of the toxicants used (heavy metals vs pesticides) and the ecological tools used (life table demography vs population dynamics). Most ecotoxicological works on rotifers have considered one species at a time; only in a few cases, two or more species were the subject of evaluation in toxicity testing (Moreira *et al.,* 2016).

The aim of the present work was therefore to quantify the population level changes in the rotifers *Brachionus calyciflorus* and *Plationus patulus* subjected to different concentrations of metformin.

Materials and Methods

Two rotifer species, *B. calyciflorus and P. patulus* were first isolated from local waterbodies from Aguascalientes City and Mexico City, respectively. Clonal cultures for each species were separately established starting with a single parthenogenetic individual. The single-celled green alga, *Scenedesmus acutus* was used as diet for both the species of rotifers. Moderately hard water (here after EPA medium) was used as medium for culturing rotifers. The EPA medium was prepared by dissolving 96 mg of $NaHCO_3$, 60 mg of $CaSO_4$, 60 mg of $MgSO_4$ and 4 mg of KCl to 1 l of distilled water (Weber, 1993). The alga was batch-cultured using Bold's basal medium (Borowitzka and Borowitzka, 1988) in 2 l transparent glass bottles with continuous fluorescent light and aeration. The medium was supplemented every third day with 0.5 g of $NaHCO_3$ as a source of carbon. Log phase alga was harvested, centrifuged and re-suspended in distilled water. The algal density was estimated using a haemocytometer. The algal and the rotifer cultures as well as the experiments were maintained at a temperature of 21±1°C.

Metformin hydrochloride (Analytical grade) was obtained from Sigma-Aldrich and a stock solution of 1000 mg l^{-1} was prepared by first dissolving in distilled water in a standard volumetric flask and stored in a refrigerator until ready to use. Fresh metformin solution was prepared every day. From this stock solution of metformin, the desired concentrations were obtained through serial dilution using the EPA medium. Due to the lack of water treatment plants and when they are present, the inefficiency of the same in developing countries, we based on the metformin concentrations in water reported by Scheurer *et al.*

(2012), we chose the following levels for the population growth experiments of both rotifer species: 0 (control), 25, 50, 100 and 200 µg l^{-1}. Each test jar also received *Scenedesmus acutus* at a density of $1.0X10^6$ cells ml^{-1}. Since the addition of algae to the test jars increase the test volume and also dilutes the concentration of metformin, we doubled the concentration of metformin and algae and halved the volume from each of them so that we would obtain the final levels of drug and algae as well as the test medium in the jars as desired (Rao and Sarma, 1986).

The population growth experiments of the two rotifer species were separately conducted in 50 ml transparent jars containing 25 ml of the test medium. Into each jar, we introduced 25 individuals of *B. calyciflorus* or 10 individuals of *P. patulus* using Pasteur pipettes under a stereoscopic microscope (Nikon SMZ-745T, Japan). Following initiation of the growth experiment, daily we quantified the number of rotifers living in each jar and then transferred the surviving individuals to fresh jars containing appropriate metformin-alga combination. The experiments were terminated when rotifer population in most treatments reached stabilization or a declining phase.

Based on the data collected, we obtained the peak population density and derived rate of population increase (*r*) per day using the following exponential equation (Krebs; 1985):

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

where *r* = rate of population increases; N_0 and N_t = initial and the maximum population densities, respectively; *t* the day on which the maximum density was reached.

Data on the peak population density and the population growth rates (*r*) were statistically analyzed using one-way ANOVA and for multiple comparisons, post hoc (Tukey) analysis was applied.

Results and Discussion

Population growth of both the rotifer species was adversely affected by the presence of metformin in the medium. However, the magnitude of this negative effect differed depending on the rotifer species and the concentration of the drug. In controls of both rotifer species, the population steadily increased with time, reaching a peak after about 10 days but thereafter declined. This trend differed in treatments containing metformin. For example, at the lowest metformin concentration (25 µg l^{-1}), *B. calyciflorus* continued to grow nearly exponentially until almost towards the end of the experiment. However, for *P. patulus* at the same metformin level, the population reached a stabilization phase after a week of the initiation of the experiment. With further increase in the drug level, the population growth curves of *P. patulus* showed much stronger oscillations, especially during the first few days. Peak population density of both the rotifer species was significantly affected by the presence of metformin in the medium ($p<001$, one-way ANOVA, F-test, Table 1, Fig. 2). The rates of population increase (mean ± standard error) in controls of *B. calyciflorus* and *P. patulus* were 0.38±0.02 and 0.26±0.01 per day, respectively. The increase in drug level decreased the *r* of both the rotifer species significantly, but more adversely of *P. patulus* ($p<0.01$, Table 1, Fig. 3).

Data on the effects of metformin to zooplankton are scarce. For the cladoceran *Daphnia magna*, the acute immobilization concentration (EC_{50}) of metformin was 64 mg l^{-1} (Cleuvers, 2003). In the present study, we used metformin concentrations on a wider scale (25-200 µg l^{-1}). Considering both rotifers and cladocerans have comparable sensitivities to toxicants (Sarma and Nandini, 2006; Moreira *et al.*, 2016), it is evident that the brachionids in this work are more sensitive than *Daphnia* (Cleuvers, 2003). In this work both the rotifer species continued to survive and reproduce over a period of time, even under the highest metformin concentration. This is possibly due to the role of algae in the detoxification of xenobiotic substances, such as pharmaceuticals (Sarma *et al.*, 2014). In chronic toxicity tests, as is the case here, a certain concentration of algal diet must be added to the experimental jars so that the test species do not die of starvation. As reported previously (Podemski and Culp, 2001), live algal cells interact with toxicants in the test jars and change the nutritional quality or even detoxify them to some extent. In the present experiment, we added live *Scenedesmus acutus* to the test jars containing different concentrations of metformin which would be expected to reduce the effect of the drug. In spite of this, metformin as low as 25 µg l^{-1} had a significant effect on the growth rate of *P. patulus*. Based on the EC_{50} data, the European Union Directive has classified chemicals as very toxic (<1 mg l^{-1}), toxic (1-10 mg l^{-1}) or harmful (10-100 mg l^{-1}) (see Cleuvers, 2003). However, compared to acute toxicity tests, sublethal evaluations based on demographic approach are much more sensitive as also evident here.

Since the pioneering work of Halbach(1984) on the use of rotifer population growth as a tool in evaluating the sub-lethal levels of toxicants, several publications have appeared in the literature on zooplankton, especially rotifers and cladocerans (reviewed in Sarma and Nandini, 2006; Snell and Joaquim-Justo, 2007). These studies, in general showed some common trends: (a) when exposed to toxicants, most test populations deviated strongly from the typical growth curves consisting of an initial lag phase, a long exponential phase and a short retardation component, (b): both peak population abundance and rate of population growth decreased with increasing level of toxicant in the medium, (c): in populations where the body size data are available, the toxicant-exposed individuals were significantly smaller than those in controls and (d): in only a few cases, test populations exposed to extremely low levels of toxicants, hormetic effects were reported.

In the present study, we were able to observe the first two conditions. We did not measure the body size of either rotifer

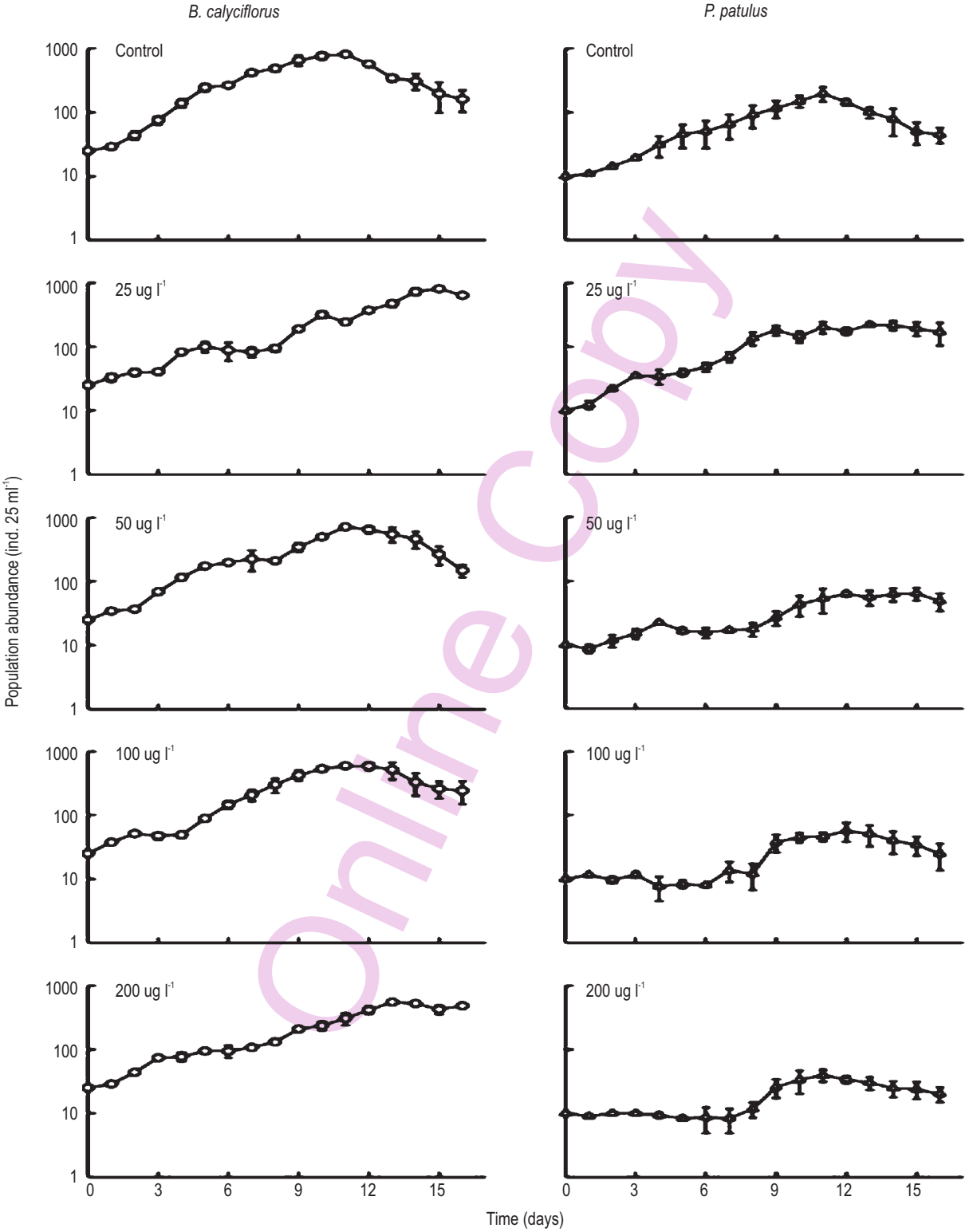


Fig. 1 : Population growth of the rotifers *Brachionus calyciflorus* and *Plationus patulus* in relation to different concentrations of metformin. Shown are the mean±standard error based on three replicates

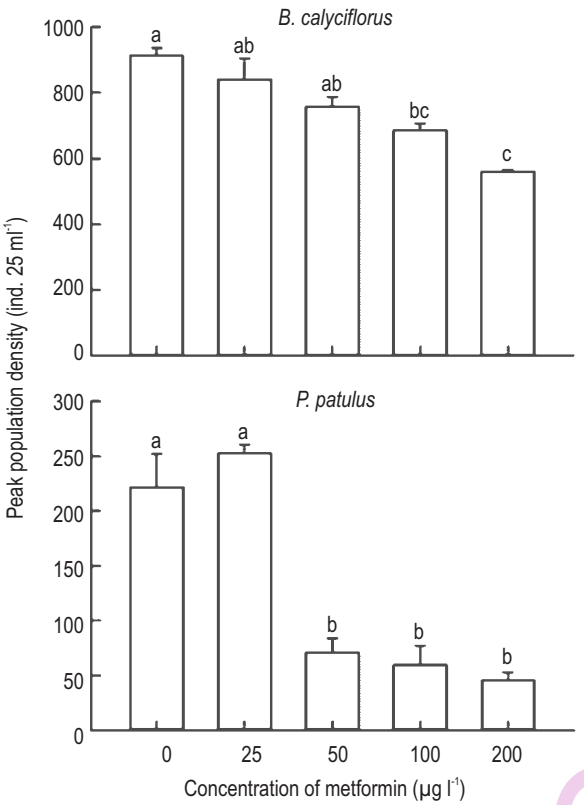


Fig. 2 : Peak population density of *Brachionus calyciflorus* and *Plationus patulus* exposed to different concentrations of metformin. Shown are the mean±standard error based on three replicates. Data bars carrying identical alphabet are not significant (p>0.05, Tukey test)

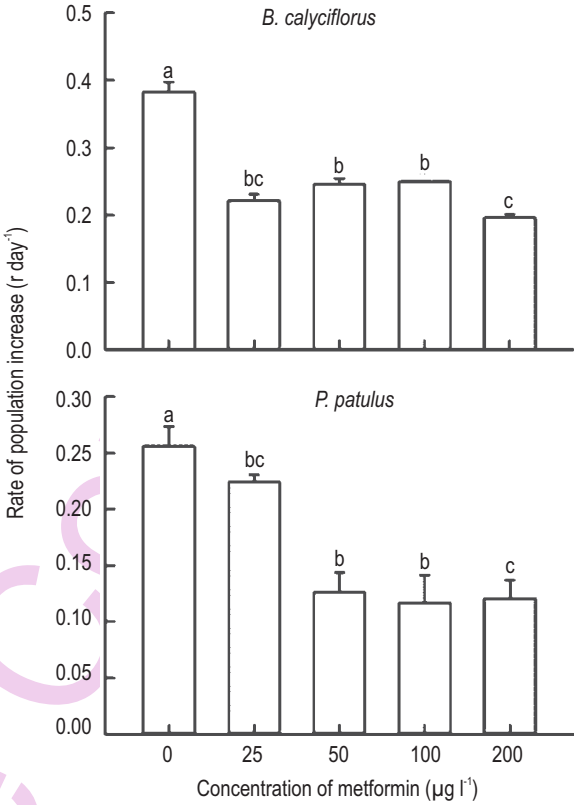


Fig. 3 : Rate of population increase per day of *Brachionus calyciflorus* and *Plationus patulus* exposed to different concentrations of metformin. Shown are the mean±standard error based on three replicates. Data bars carrying identical alphabet are not significant (p>0.05, Tukey test)

species in the test jars. However, during the daily observations, we found that most individuals of both rotifer species were smaller in treatments containing metformin. Sarma *et al.* (2008) also observed that when *P. patulus* was exposed to heavy metals, the body size became smaller compared to controls.

Hormesis occurs when the test populations are exposed to sub-inhibitory levels of toxicants, as a mechanism of over compensation against stress. Thus, compared to controls, certain toxicants at very low levels produce hormetic effects in zooplankton (Calabrese and Baldwin, 1998). In the present work, the peak population density of *P. patulus* exposed to 25 µg l^{-1} of metformin, appeared to be higher than controls, but it was not statistically significant suggesting that hormesis was not evident in this work.

Peak density and the intrinsic rate of increase are two significant population level parameters, sensitive to stresses such as the presence of competitors, toxins or unfavourable abiotic factors such as temperature (Sarma *et al.*, 1999). Under stressful levels, the birth rate decreases and the death rate increases causing a reduction in the population abundance, even though food resources are not limiting. This, in turn, reflects the peak population density. Thus, in the present study, for both *B. calyciflorus* and *P. patulus*, this parameter became statistically significant. The rate of population increase, which sums up the different life history variables such as rate of egg production, hatching as well as their survival and the age at first reproduction etc. (Godfray and Rees 2002) is long considered as one of the most sensitive variables in the demography of zooplankton. In the present study, this variable was sensitive only to certain high levels of metformin. The lack of significant influence of metformin at low levels implies that the impact of the drug was not strong enough to cause changes in the physiology of the test species.

The molecular mechanism of metformin effects on mammals is well documented. For example, metformin is involved in the suppression of hepatic gluconeogenesis as a consequence of mitochondrial inhibition (Rena *et al.*, 2013). It is possible that different mechanisms of action of metformin occur in

Table 1 : One way ANOVA obtained from the data observed in maximum peak of population density and rate of population increase (r) by *Brachionus calyciflorus* and *Plationus patulus*, exposed to different concentrations of metformin. *p<0.001

Source	DF	SS	MS	F
Peak pop density				
B. calyciflorus				
Between Groups	4	226215	56553	15.71*
Residual	10	35997	3599	
P. patulus				
Between Groups	4	117256	29314	32.24*
Residual	10	9093	909	
Rate of population increase				
B. calyciflorus				
Between Groups	4	0.062	0.016	63.12*
Residual	10	0.002	0.0002	
P. patulus				
Between Groups	4	0.053	0.013	14.4*
Residual	10	0.009	0.001	

lower invertebrates. For example, some studies indicate that metformin has no effect on the mortality rates of insects while others show that it is involved in the life extension of nematodes (Lucanic *et al.,* 2013). Since we did not study the age-specific lifespan of rotifers, whether or not metformin affects the duration of life of rotifers remains to be solved. Like most other synthetic pharmaceutical products, metformin also undergoes some kind of transformation into other compounds due to bacterial action in nature. For example, it is known that metformin is mainly degraded to guanylureain treatment plants. In waste waters, the concentration of guanylurea has been recorded up to 20 µg l^{-1} and the ecotoxicological effects of this compound to zooplankton is not known (Scheurer *et al.,* 2012). Thus, it is evident that, regardless of the mode of action, metformin at concentrations as low as 25 µg l^{-1} had some adverse effect on the population growth of the tested rotifer species. And these concentrations are found in nature causing long term impacts (Scheurer *et al.,* 2012).

Considering its impact on the maximal population abundances and the intrinsic rate of increase, metformin is toxic to rotifers. Thus, the environmental levels of metformin in effluents are similar to those used in this work. Therefore, this drug should be considered as harmful for rotifers possibly more than the crustacean zooplankton. It remains to be seen how the populations of other groups of zooplankton, mainly cladocerans respond to the tested concentrations of metformin.

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