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Tardigrades as potential bioindicators in biological wastewater treatment plants

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ABSTRACT

The aim of this study was the evaluation of the relationship between the presence of tardigrades and various levels of sewage pollution in different tanks of a wastewater treatment plant. The study was carried out in the wastewater treatment plant located near Poznań (Poland) during one research season. The study was conducted in a system consisting of three bioreactor tanks and a secondary clarifier tank, sampled at regular time periods. The presence of one tardigrade species, *Thulinius ruffoi*, was recorded in the samples. The tardigrades occurred in highest abundance in the tanks containing wastewater with a higher nutrient load. *Thulinius ruffoi* was mainly present in well-oxygenated activated sludge and its abundance was subject to seasonal fluctuations; however, its preference for more polluted tanks seems to be consistent across the year. Although more detailed experimental study is needed to support the observations, our data indicate that *T. ruffoi* has a high potential to be used as a bioindicator of nutrient load changes.

KEYWORDS

Bioindication; wastewater treatment; sludge; water bears

INTRODUCTION

The activated sludge system is one of the most widely used sewage treatments. It relies on developing microorganisms in a form of foamy suspension called activated sludge. Thus, the activated sludge is actually a heterotrophic ecosystem, in which organic matter decomposition predominates the processes of synthesis (e.g., Fiałkowska et al., 2010). First, these processes are regulated by heterotrophic bacteria, mainly *Zooglea ramigera* Butterfield, 1935, which form a flocculated structure in the sludge (Williams & de los Reyes, 2006; Németh-Katona, 2008; Kocwa-Haluch & Woźniakiewicz, 2011). The entire process of sewage treatment with activated sludge depends, to a large extent, on the control of the activated sludge conditions, including the structure and size of flocs as well as the composition of species and the population structure of microorganisms (Eikelboom & van Buijsen, 1983; Fiałkowska et al., 2010).

Tracking the changes of biodiversity within the sludge is a crucial part of evaluating the wastewater plant's functioning parameters. Physicochemical analysis provides information on the situation and the condition of the sludge at the time of sampling. In contrast, observations of the ecosystem concentrating on bioindication reveal the condition of the sludge in a specified time interval (Németh-Katona, 2008). Thus, to obtain information on sewage quality and/or the condition of the individual wastewater treatment plant tanks, appropriate biotic indexes and coefficients are used and their correlations with the physicochemical parameters are assessed (Fiałkowska et al., 2010).

The main prokaryotic composition of the activated sludge is morphologically very homogeneous, which is why bioindication often relies on larger eukaryotic organisms such as protozoans (flagellates, amoebas, ciliates) and small invertebrates (rotifers, nematodes, gastrotrichs and oligochaetes) (e.g., Stout, 1978; Utsugi, 2005; Chen et al., 2004). Ciliates are the most commonly used bioindicators and have been extensively studied. Due to their great variability and susceptibility

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to changes in environmental conditions, their evaluation reflects activated sludge pollution very well (Nicolau et al., 2001). Similar studies conducted on other eukaryotic organisms are rather scarce, and only a few papers have been published on such non-ciliate bioindicators (e.g., Ratsak et al., 1993; Chen et al., 2004).

Tardigrada are small invertebrates with an elongated and cylindrical body, divided indistinctly into four body segments and a head (e.g., Nelson et al., 2015). Tardigrades are phyto-, bacterio- and detritiphagous animals, but many of them are predators and some are also parasites (e.g., Shill et al., 2011). They inhabit almost all biotypes throughout the world, both terrestrial and aquatic, from the highest mountain peaks to the deepest parts of the ocean. The majority of tardigrades (especially those inhabiting terrestrial environments) have the ability to enter into cryptobiosis. This unique metabolic state can be characterized by complete (or almost complete) suspension of metabolism (e.g., Clegg, 1973). 'Cryptobiotic' tardigrades can survive extreme environmental conditions (i.e., desiccation, freezing, high doses of various toxic substances, UV and ionizing radiation, etc.) and return almost immediately to active life when conditions became suitable (Rebecchi et al., 2007). Wide ecological tolerance allows tardigrades to inhabit activated sludge, whose structure resembles their natural ecosystems – especially the particles present in aquatic ecosystems called 'lake snow' or 'marine snow' (Grossard & Simon, 1993; Fiałkowska et al., 2010).

In the published literature, a few papers have reported the successful use of tardigrades as potential pollution bioindicators. These papers considered the influence of DDT, heavy metals and air pollution on terrestrial tardigrade species diversity (e.g., Barret & Kimmel, 1972; Steiner, 1994a, b; Hohl et al., 2001; Vargha et al., 2002). However, the presence of Tardigrada in activated sludge of wastewater treatment plant bioreactors was discussed in only two studies. Utsugi (2001), in Japan, reported the presence of one species from the family Hypsibiidae – *Isohypsibius myrops* (Du Bois-Reymond, 1944) in all the analysed samples. Additionally, Sobczyk et al. (2015) extracted the specimens of *Thulinius ruffoi* (Bertolani, 1981) from the activated sludge of a wastewater treatment plant in Poland and tested their resistance to various concentrations of ammonia.

In this paper, we present the results of a pilot study on using tardigrades to evaluate the quality of wastewater in a mechanical-biological wastewater treatment plant. We start with a hypothesis that tardigrade communities differ significantly in terms of quality or quantity between the sludge in particular stages of treatment, and that these differences are visible in subsequent seasons of a year.

1. MATERIALS AND METHODS

The study was conducted at the Central Wastewater Treatment Plant (CWTP) located in the village of Koziegłowy (Czerwonak Commune) in the N-E part of Poznań County, Poland (52.4534° N; 16.9804° E). The CWTP is a mechanical-biological wastewater treatment plant with elevated removal of nutrients and complete processing of activated sludge. The main technological elements are grates, sand grit tanks and primary clarifiers (Fig. 1), all designed to mechanically purify sewage from the suspended solids (Podgórniakowa, 2001). Subsequent stages of sewage processing involve the use of bioreactors (volume 25,000 m^3 each) and secondary clarifiers, in which biological purification by microorganisms takes place (Podgórniakowa, 2001). The sewage is delivered from Poznań and neighbouring communities (areas populated by ca. 670,000 people in total). Following treatment, the waste water is discharged into the Warta River.

The study was conducted in one of the six complete systems of bioreactor tanks and clarifier tanks. Samples were taken from the bioreactor tanks marked 1 to 3 and a secondary clarifier marked as tank 4 in Fig. 1. In the tanks, the average sludge age ranged from 24 to 29 days. The tanks differed in regard to the oxygen conditions and concentrations of nutrients, and this was a main criterion in the choice of sampling sites (Table 1). Activated sludge was sampled every three weeks between 22 March and 24 November, in 2011, excluding 8 August to 6 October, during which an environmental audit was conducted.

The sludge was collected using a plastic bucket (500 ml) fixed on a two-meter long arm. Five 20 ml subsamples were

Figure 1. Sampling sites within the Central Wastewater Treatment Plant in Koziegłowy: 1 – bioreactor tank 1; 2 – bioreactor tank 2; 3 – bioreactor tank 3; 4 – secondary clarifier.

Table 1. Characteristics of the studied tanks in the Central Wastewater Treatment Plant in Koziegłowy

Tank	Number (Fig. 1)	Process	Relative nutrient load value
1st bioreactor tank	1	Dephosphatation	The highest nutrient load
2nd bioreactor tank	2	Nitrification	High nutrient load
3rd bioreactor tank	3	Hypoxic zone	Moderate nutrient load
Secondary clarifier	4	Pumping and removing of sludge	Low nutrient load

collected by pipette from the bucket and combined into a 100 ml sample. In total, 42 samples were collected, one sample was collected from each tank on 13 different dates in three week intervals (the bioreactors and secondary clarifer), and additionally, three samples from tank 1 were collected as a reference at the beginning, in the middle and at the end of the sampling period (22.03.2011; 27.06.2011; 24.11.2011). All the samples were placed in 100-ml plastic containers and fixed with 75% ethanol.

The number of samples taken from the first tank of the bioreactor was lower than from the others, since it was not sampled during all the occasions. Consequently, these samples were not included in the analysis of seasonal changes, and they were not incorporated into our final conclusions. The data from the first tank were used only as a reference for comparisons between the remaining tanks. All the samples were stored in a refrigerator at about 5°C until analysis, for no longer than two weeks (pilot sampling showed that such low temperature and storage time do not influence the number and diversity of tardigrades in the samples). For each 100 ml sample, five random 10 ml subsamples (after stirring) were collected for analysis with an automatic pipette. In each subsample, tardigrades were counted and initially identified under stereomicroscope (Olympus SZ51). Later, some specimens were selected randomly, fixed on microscopic slides and measured. Final identification to the species level was made by Phase Contrast Microscopy (Olympus BX41). Because identification of the species from the genus *Thulinius* is difficult due to morphological similarities, the studied specimens were identified using the key in Kaczmarek et al. (2010) and original descriptions and redescriptions of the species from that genus. The mean number of specimens per one litre of sludge calculated for each sample was used for the analyses throughout the paper.

The results of the chemical analyses were obtained from the accredited laboratory of the CWTP. These results allowed us to assess the levels of raw (tank 1) and treated sewage pollution (tank 4). However, they only comprised the background of the study due to a lack of precise data from the two remaining bioreactor tanks. Chemical oxygen demand (COD) analysis was performed according to standard PB/PFO-24 ed. 3 from the 01.07.2013 and PN-ISO 6060:2006, biochemical oxygen demand (BOD_5) according to standard PN-EN 1899-1:2002 and PN-EN 1899-2:2002, total suspended solids (TSS) according to standard PN-EN 872:2007+Ap1:2007, total Kjeldahl nitrogen (TKN) according to standard PN-EN 25663:2001 and total phosphorus (TP) according to standard PN-EN ISO 6878:2006+Ap1+Ap2/2010.

The data on the number of tardigrades were analysed using descriptive statistics under Statistica 7.1 software. The significance of differences in the density of tardigrades between particular tanks, and in the physicochemical parameters between tanks 1 and 4, were tested using randomised Analysis of Variance (ANOVA) with Bonfferoni correction. The significance of correlations between tardigrades and physicochemical parameters in tanks 1 and 4 were tested with the Reduced Major Axis regression (randomised version). These calculations were made using RundomPro 3.14 software.

2. RESULTS

In all the 42 analysed samples, only *T. ruffoi* was found. The number of specimens varied greatly between the samples and ranged from 0 to 8300 ind. l^{-1} of sludge (SD = 0 to 1946.3).

Differences in tardigrade abundances between the three tanks sampled systematically were statistically significant when the data from all the sampling days were included to the analysis (ANOVA: F = 4.1866; p = 0.012; DF = 2). The post-hoc test showed differences between all the pairs of tanks ($p < 0.05$) except for the tanks 2 and 3 (p = 0.533). Among tanks 2, 3 and 4, the highest average abundance of tardigrades was found in the second tank of the bioreactor, that is, 2291 ind. l^{-1} (SD = 2187.8). The lowest was in the tank four: 423 ind. l^{-1} (SD = 794.2). Comparing all the four tanks only on the three sampling dates that included all of the tanks (Fig. 2), clearly the highest average density was observed in tank 1 (4207 ind. $^{-1}$; SD = 2906.2).

Time changes in the abundance of tardigrades in the tanks 2–4 of the bioreactor are presented on Fig. 3. Tanks 2 and 3 initially had relatively constant abundances, not exceeding 860 ind. l^{-1} (SD = 366.6). However, from June 27th to October 20th, significant fluctuations were observed. In July, the abundance of tardigrades decreased from 2040 ind. l^{-1} (SD = 527.6) of sludge in tank 2, and from 1920 ind. l^{-1} (SD = 449.0) of sludge in tank 3, to 1400 ind. l^{-1} (SD = 352.1) of sludge in both tanks. Other increases of tardigrade abundances were observed in tanks 2 and 3, on October 6th (24% increase) and August 8th (23% increase). On November 3rd, the number of tardigrades in tank 2 decreased rapidly and remained at a low level until the end of the study period. The number of tardigrades in tank 3 had been decreasing regularly and gradually starting in August.

In contrast, the number of tardigrades in the secondary clarifier (tank 4) was very variable, ranging from 0 to 3060 ind. l^{-1} (SD = 1233.8) of sludge. An abundance peak was observed on October 6th, similar to tank 2. Subsequently, the number of tardigrades decreased rapidly, and from November 3rd, no tardigrades were observed in the sludge of tank 4.

Results of the analyses of sludge's physico-chemical parameters are presented in Table 2. No significant correlations between the densities of tardigrades and physicochemical parameters were found in the two tanks where chemical analyses were performed (COD: $p = 0.3652$, $R^2 = 0.0194$; BOD: $p = 0.5883$, $R^2 = 0.0149$; TSS: $p = 0.5661$, $R^2 = 0.0152$; TKN: $p = 0.2137$, $R^2 = 0.0301$; TP: $p = 0.7732$, $R^2 = 0.0044$). However, the differences in the physicochemical parameters between tanks 1 and 4 were statistically significant (in all cases: $F > 67$, $p < 0.0001$).

3. DISCUSSION

Microbial communities form a multi-tiered trophic structure on the flocs of activated sludge (Caron et al., 1982). The diversity of these structures depends on the quality of the treated sewage and growth rates of particular microorganisms (Eikelboom & van Buijsen, 1983). The biological composition of the activated sludge, in addition to its physical and chemical conditions (such as quality and availability of nutrients, water oxygenation and temperature), significantly impacts the process of its formation (Eikelboom & van Buijsen, 1983; Chen et al., 2004).

Thulinius ruffoi has so far been mostly found in freshwater habitat types (rivers, lakes and small ponds) across Europe (McInnes 1994). However, only one paper concerning its presence in the activated sludge of wastewater treatment plants has been published (Sobczyk et al., 2015) and a large number of specimens of *T. ruffoi* were found in such plants in Germany (Kaczmarek Ł., unpublished data). Moreover, unidentified tardigrades have been found during other studies (Fiałkowska et al., 2010), mainly in lightly burdened wastewater treatment plants (< 0.1 BOD_5 kg d.m.$^{-1}\cdot$d^{-1}). Our results confirm these findings because CWTP is a lightly burdened wastewater treatment plant. Yet, the population of *T. ruffoi* occurring there was characterized by significant variation in abundance when particular tanks were compared (bioreactor tanks and the secondary clarifier). Since tardigrade density was highest in the first most polluted tank, it could be inferred that *T. ruffoi* prefers tanks with relatively higher pollution levels and higher amounts of suspended solids. However, more detailed study is needed to verify this observation.

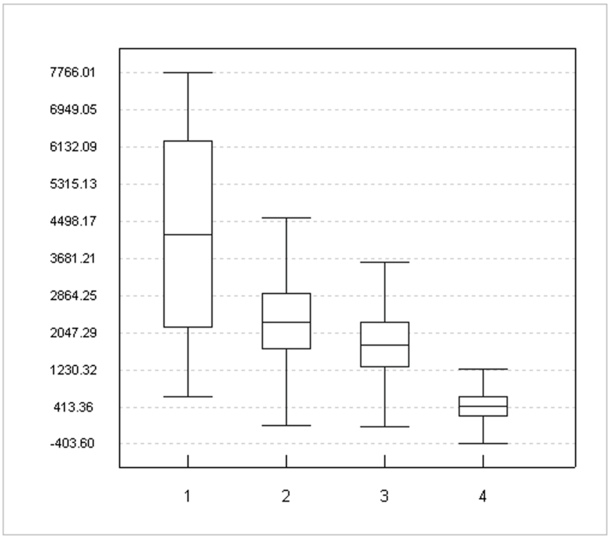

Figure 2. The average abundance of tardigrades (vertical axis, ind. l-1) in tanks 1 to 4 (horizontal axis) in three sampling dates when all the tanks were sampled. Middle line: mean; box range: standard error; whiskers: standard deviation.

The highest average density of tardigrades was observed in the first tank of the bioreactor where dephosphatation takes place. This tank receives a sewage purified only using mechanical methods. In the subsequent bioreactor tanks, the number of tardigrades gradually decreased. At the last stage of sewage treatment, in the secondary clarifier where the sludge is separated from the treated sewage, the number of tardigrades was almost four times lower than in the first tank of the bioreactor.

This disparity in tardigrade densities may be related to the availability of nutrients. The trophic pyramid present in the activated sludge has a significant influence on the stability and development of this microecosystem. The amount of detritus, flocculation of bacteria and the availability of both detritivorous and bacteriophagous invertebrates (including tardigrades) inhabiting activated sludge was studied by Utsugi (2001) and Kocwa-Haluch & Woźniakiewicz (2011). As shown by Fiałkowska et al. (2010), changes in the subsequent links of the trophic chain are analogous to those in natural ecosystems, and we think there is a direct correlation between the availability of food and tardigrade growth.

Although the exact life history of *T. ruffoi* is unknown, based on studies conducted on other tardigrade species (e.g., *Milnesium tardigradum* Doyère, 1840 (Suzuki 2003)) and tak-

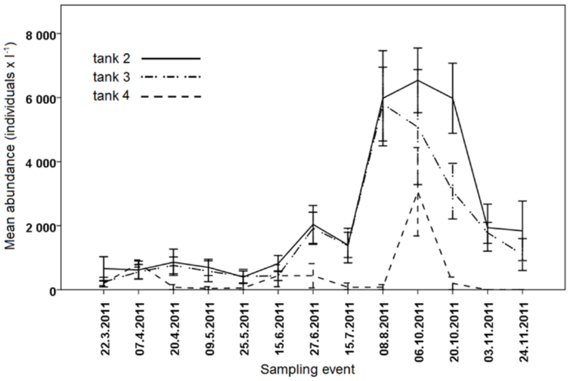

Figure 3. The mean abundance of tardigrades (vertical axes) in tanks 2, 3 and 4 at respective sampling dates (horizontal axis). Whiskers: standard deviation.

ing into consideration a relatively old sludge age (24 to 29 days), it appears that four weeks is a sufficient time to reach maturity after hatching from an egg. Thus, differences in the life stages and the fact that comparing to other organisms occurring in sludge, the maximum size of *T. ruffoi* (ca. 550 µm) and its weight are relatively high, could also potentially influence the results. The stages and size of tardigrades may be different between the consecutive tanks, and particular stages could be transferred to subsequent tanks with different success. Thus, the lower number of tardigrades in the secondary clarifier (tank 4) might be caused by the higher sedimentation rate of the larger specimens (larger specimens of *T. ruffoi* might settle down in bioreactor tank 3 and not flow to the secondary clarifier).

Interestingly, it seems that *T. ruffoi* inhabiting the activated sludge are resistant to many toxic substances (e.g., heavy metals, Podgórniakowa, 2001) that may occur in municipal sewage. Such resistance is in contrast with known vulnerability of some terrestrial tardigrades to toxic substances including heavy metals (see Vargha et al., 2002). It was also shown that anthropogenic impact cause a decrease in the abundance and species diversity of tardigrades (Vargha et al., 2002). Similar results were also reported by Steiner (1994a, b) and Hohl et al. (2001) who showed that air pollution (i.e., SO_2 emissions) negatively influence both diversity and density of tardigrades. High abundance of one tardigrade species in the present study may indicate that *T. ruffoi* common in the entire microecosystem is highly resistant to disturbances connected with anthropogenic pollution. Chen et al. (2004) showed that an increase in tardigrade abundance was observed in nitrification tanks where the concentrations of toxic compounds, such as ammonia, decreased. During the nitrification process, ammonia is oxidized to harmless nitrates, and our results may indicate lack of influence of ammonia on *T. ruffoi* in the contrary to Gastrotricha (Fiałkowska et al., 2010). Also, the experimental studies conducted by Sobczyk et al. (2015) on *T. ruffoi*, extracted from activated sludge, showed susceptibility of this species to high concentrations of ammonia.

In the tanks 2–4 of the bioreactor, notable variability in tardigrade abundance between the sampling periods is visible, which could be due to seasonal fluctuations. If so, the abundance of *T. ruffoi* during summer and early autumn may suggest a preference for higher sewage temperatures. Density of tardigrades in bioreactor tanks 2 and 3 was relatively constant excluding the samplings conducted during the warm summer months (June to October), when a clear peak of abundance was recorded (Fig. 3). From the beginning of November, the number of tardigrades decreased rapidly and remained at a low level until the end of the study. In the secondary clarifier (tank 4) a similar abundance peak was observed in the beginning of October and lasted till November, when no tardigrades were observed in the sludge.

In the same timeframe, the chemical parameters in tanks 1 and 4 changed continually and differed significantly between the tanks (Table 2), although they were not significantly correlated with tardigrade abundance. The concentration of nutrients decreased in between the tanks, for example, the concentration of total phosphorus in the last tank reached twofold lower level than in the first tank, and in the same tank, the total Kjeldahl nitrogen (TKN) level was even 30 times lower in comparison to the first tank (Tab. 2). All the parameters indicating the amount of solids suspended in the sewage (BOD, COD and TSS), changed continually as well. The treated sewage had TSS values from 2 to even 206 times lower than the raw sewage.

Our observations suggest that *T. ruffoi* prefers warmer, well-aerated sewage, with BOD_5 values of ca. 400 mg l^{-1} and

Table 2. Values of physicochemical parameters (in mg l-1) recorded in the tanks 1 and 4 of the Central Wastewater Treatment Plant in Koziegłowy during the period of the study: COD - chemical oxygen demand, BOD - biochemical oxygen demand, TSS - total suspended solids, TKN - total Kjeldahl nitrogen and TP - total phosphorus, in raw and treated sewage in the study season.

Date	Raw sewage (tank 1)					Treated sewage (tank 4)				
	COD	BOD	TSS	TKN	TP	COD	BOD	TSS	TKN	TP
22.03.2011	1080	500	636	81.2	10.2	61	8	33.2	5.97	1.20
07.04.2011	1280	450	668	87.7	13.8	45	4	8.4	3.47	0.71
20.04.2011	1760	850	1050	104.0	15.6	453	110	354	31.3	8.40
09.05.2011	1000	520	576	88.9	12.8	53	4	12.6	2.83	0.72
25.05.2011	1460	800	928	90.6	13.5	46	3	10.8	3.85	0.99
15.06.2011	1710	810	1100	117.0	20.6	42	4	10.8	3.88	1.08
27.06.2011	783	370	322	73.8	12.3	27	2	6.4	3.52	0.56
15.07.2011	1280	610	548	103	13.9	32	2	3.6	3.99	0.52
08.08.2011	842	360	444	74.8	10.0	41	3	5.5	4.40	0.35
06.10.2011	1090	420	500	87.2	10.9	37	4	9.6	3.46	1.22
20.10.2011	1100	400	468	95.7	14.3	44	3	6.0	5.78	0.82
03.11.2011	1250	440	613	99.2	16.2	38	6	7.4	6.21	1.25
24.11.2011	1110	420	412	89.6	16.3	41	2	2.0	5.34	0.30

a TSS load of ca. 500 mg l^{-1}. For comparison, the optimal values of BOD_5 for Oligocheta, Nematoda and Rotifera range from 0 to 20 mg l^{-1} (Fiałkowska et al., 2010).

Our results also indicate that the presence of tardigrades in sludge is probably seasonal. Moreover, their concentration is contingent on sludge age and the availability of suspended solids (which probably constitute a rich food base). Presence of high concentration of nutrients and potentially toxic compounds seems to influence tardigrade abundance to a lesser extent. Although statistical analyses did not confirm correlations between individual chemical elements and tardigrade abundance, such relation is highly possible and needs to be studied with a higher number of samples.

Although Sobczyk et al. (2015) stated that *T. ruffoi* is not a good bioindicator candidate for total ammonia nitrogen because its LC50 in the concentration of total ammonia nitrogen is several times lower than norms in treated waters, we believe it could be useful for other pollutants. Though more detailed, experimental study is needed to support the observations, our data suggest that *T. ruffoi* could have a potential to be used as a bioindicator of nutrient load changes. The use of tardigrades as such bioindicators has some important advantages when compared to nematodes and rotifers currently used for such purposes. Probably only few tardigrade species live under the conditions of wastewater treatment plants (only two known up to now), so the possibility of misidentification is definitely smaller than in the case of invertebrates currently used as bioindicators. Even if the number of tardigrade species occurring under such conditions was higher, they are far less difficult to determine to species level by a non-specialist than rotifers or nematodes, they are also larger so the possibility of missing them is smaller.

4. CONCLUSIONS

Recorded abundances of tardigrades were the highest in tanks with higher pollution levels and higher amounts of suspended solids although the abundance of *T. ruffoi* was subject to seasonal fluctuations. Although the direct influence of toxic compounds on the biology of *T. ruffoi* is unknown, it seems that this species is highly resistant to these compounds, or is able to adapt to unfavourable environmental conditions. Tardigrades, in general, are well-known for their high tolerance of extreme environmental conditions (e.g., Rebecchi et al., 2007). For future research, it would be useful to conduct detailed measurements in all of the bioreactor tanks to ascertain the present conclusions on *T. ruffoi* presence and development. In addition, other wastewater treatment plants should also be studied to evaluate the possibility of using tardigrades as effective bioindicators.

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