Hydrobiologia
https://doi.org/10.1007/s10750-023-05348-7

ROTIFERA XVI

Effects of short-chain chlorinated paraffins on the sexual reproduction *of Brachionus calyciflorus*: a populational and biomolecular study

Jeremy Jaegers · Caroline Michaux · Éric Gismondi · Célia Joaquim-Justo

Received: 28 February 2023 / Revised: 8 August 2023 / Accepted: 15 August 2023

Abstract Short-chain chlorinated paraffins (SCCPs) are ubiquitous, persistent chemicals whose toxicity and potential impacts on aquatic ecosystems have been overlooked for many years. This study aims to determine the impact of these chemicals on rotifers. As sexual reproduction is often more sensitive to toxicants than amictic reproduction, this study focuses on endpoints describing sexual reproduction and on the impact on the gene expression of three receptors associated with reproduction: retinoid-x-receptor (*rxr*), retinoid activated receptor (*rar*) and membrane-associated progesterone receptor (*mapr*). *Brachionus calyciflorus* was exposed to environmental concentrations of SCCPs (10,100 and 1000 ng/L) and the impact on sexual reproduction (mixis rate, fertilization rate, resting egg production and viability, inter-generational effects) was assessed. Variations in the gene expressions of *rxr*, *rar* and *mapr* were also studied through RT-qPCR analysis. Our results show that SCCPs increase the mixis rate but decrease the fertilization rate, leading to unaltered resting egg production. Exposure to SCCPs also affected the mRNA levels of the studied receptors in ways which could be linked to observed changes in mixis and fertilization rates.

Keywords SCCP · Rotifer · Real-time PCR · Chronic toxicity · Gene expression

Guest editors: Maria Špoljar, Diego Fontaneto, Elizabeth J. Walsh & Natalia Kuczyńska-Kippen / Diverse Rotifers in Diverse Ecosystems

J. Jaegers (✉) · C. Michaux · É. Gismondi · C. Joaquim-Justo
Laboratory of Animal Ecology and Ecotoxicology (LEAE), Freshwater and Oceanic Sciences Unit of Research (FOCUS), Chemistry Institute, University of Liège, Bât. B6C, 11 allée du 6 Août, 4000 Sart-Tilman, Belgium
e-mail: j.jaegers@uliege.be

Introduction

The phylum Rotifera comprises over 2000 mainly freshwater species, with the most species-rich class being Monogononta (Segers, 2007). They are found all over the world, with high-population densities (1000 individuals/l is a common value) leading to them being classified as one of the three main zooplankton groups in freshwater studies, along with cladocerans and copepods (Segers, 2008). As such, they are a vital component of aquatic ecosystems, as some species may feed on microalgae which are not efficiently grazed by larger organisms and their high reproduction and turnover rates allow for rapid nutrient cycling which supports the higher levels of the food web (Ricci & Balsamo, 2000). The life cycle of most members of Monogononta is complex, alternating between phases of rapid parthenogenetic reproduction and slower sexual reproduction resulting in "resting eggs" which can stay dormant for years waiting for appropriate conditions to develop (Wallace

Published online: 31 August 2023

et al., 2015). Parthenogenetic, or amictic, reproduction is more prevalent than sexual reproduction in low population density situations (Serra & Snell, 2009).

Among Monogononta, members of the genus *Brachionus* are particularly attractive for ecotoxicological assays because of their rapid reproduction, ease of cultivation, and sensitivity to various toxicants and endocrine disruptors (Radix et al., 2002). Numerous endpoints have been used in the laboratory for decades to evaluate the effects of diverse contaminants on rotifers including direct mortality, swimming speed, population growth rate, lifespan and resting egg production and hatching rates (Dahms et al., 2011; Cruciani et al., 2016; Won et al., 2017). More recently, molecular biology techniques have allowed for the sequencing of rotifer genomes (Jeong et al., 2017; Kim et al., 2018), which in turn enabled the study of the expression patterns of genes in response to various conditions (Park et al., 2018; Joaquim-Justo & Gismondi, 2021). Thus, combining well-known endpoints regarding demographic parameters and population growth with molecular techniques may provide a sensitive and integrative tool to study the effects of sublethal doses of contaminants on this important component of planktonic communities.

Emerging pollutants are a concerning topic in this regard, as they are not commonly monitored in the environment and their effects on the wildlife may not be well understood (Geissen et al., 2015), as for example SCCPs. These industrial additives (plasticizers, flame retardants, high pressure fluid components) are aliphatic alkanes that were subjected to chlorination. Their chain length is between 10 and 13 carbons, and their molecular weight may be comprised of up to 70% of chlorine (Li et al., 2017). Environmental concentrations up to 1.2 µg/l were measured in some European rivers (Nicholls et al., 2001; Castells et al., 2004). Their low biodegradability and high octanol/water partition coefficients allow SCCPs to accumulate in abiotic matrices (Chen et al., 2011) as well as wildlife such as fishes (Basconcillo et al., 2015) and amphipods (Li et al., 2017). Furthermore, Ma et al. (2014) observed that biomagnification occurred in the trophic interactions between zooplankton, crustacean and fish. Houde et al. (2008) also detected trophic magnification in lake Michigan, while Sun et al. (2017) reported a phenomenon of trophic dilution in a freshwater pond ecosystem instead, but zooplankton were not incorporated in the latter analyses. Thus, in at least some ecosystems zooplankton have been shown to accumulate SCCPs which are then passed on higher up the food web, yet no studies have been conducted to our knowledge regarding the impact of these exposures on their physiology and reproduction.

While the toxicity, carcinogenicity, and endocrine disrupting properties of SCCPs are increasingly well known in vertebrates such as rats, zebrafish, and humans (in-vitro assays) (Warnasuryia et al., 2010; Liu et al., 2016; Geng et al., 2021; Sprengel et al., 2021), data on invertebrates are scarce, especially for sublethal effects. Currently, available data on invertebrates include a predicted no-effect concentration (PNEC) value of 5.28 mg of SCCPs per kg of soil for terrestrial annelids, collembolans, and nematodes (Bezchlebová et al., 2007) and a 28 day no observed effect concentration (NOEC) for SCCP exposure for the crustacean *Mysidopsis bahia* (Molenock) of 7.3 µg/l (Thompson & Madeley, 1983).

This work aims to determine the sublethal effects of SCCP exposure on populations of *Brachionus calyciflorus* (Pallas) at environmentally relevant concentrations, combining reproductive assays (targeting the intrinsic growth rate, mixis rate (Mr), fertilisation rate (Fr) and diapausing cyst production and viability across two generations) and RT-qPCR molecular biology techniques to investigate the expression of hormonal receptors known to be present in this species and related to sexual reproduction, i.e., retinoid-X-receptor (*rxr*), retinoid-acid-receptor (*rar*) and membrane-associated progesterone receptor (*mapr*) (Joaquim-Justo & Gismondi, 2021). This work will not only allow to uncover potential SCCP effects at environmental concentrations, but also highlight possible mechanisms of action for these contaminants.

Materials and methods

Experimental conditions

The test animals were 3 h old neonate rotifers hatched from cysts of the Gainesville (Florida) strain of *B. calyciflorus* (Snell et al., 1991). The cysts were hatched at 25 °C under continuous illumination for 24 h. The medium used was EPA (US Environmental Protection Agency, 1985), prepared with ultrapure milliQ water.

The algae *Nannochloris atomus*, Butcher (strain CCAP 251/6) was cultivated in Bold's Basal Medium (Andersen, 2005) at 25 °C under continuous illumination until the exponential growth phase before being isolated via centrifugation and re-suspended in EPA medium for rotifer feeding. In all assays, algal concentration in the exposure medium was 3,000,000 cells/ml.

In all experiments, the initial algal concentrations were verified by counting the algae in two randomly chosen replicates using a Thoma cell counting chamber. Every 24 h, the algae concentration was checked in the same manner and adjusted by adding an appropriate algal concentrate in an aliquot of SCCP enriched medium to maintain the initial SCCP concentration.

The exposures took place in the dark in an incubator programmed for an average temperature of 25 °C, with a daily fluctuating temperature (DFT) regimen of ± 2 °C associated with a 12/12 h day/night cycle.

The stock SCCP solution used in the assays was chloroparaffin C10-C13 63% chlorine (CAS no 85535-84-8; lot no G986931CY) from Dr. Ehrenstorfer GmbH (Germany). All SCCP solutions were prepared in acetone before being diluted in the EPA medium (final acetone concentration = 0.01% in all treatments). As such, a solvent control of 0.01% acetone was carried out alongside a pure EPA control to detect possible acetone effects on the studied parameters. In all cases, no significant difference was detected between the two controls, so they were combined.

Demographic parameters

The assay was conducted using three SCCP concentrations (10, 100 or 1000 ng/l).

Six rotifer neonates were placed in 16 × 150 mm glass test tubes containing 12 ml of EPA medium and the number of non-ovigerous females, amictic, mictic unfertilized and fertilized females were counted daily to determine the intrinsic growth rate, the mixis rate (Mr) and the fertilization rates (Fr) (see Cruciani et al., 2016 for calculation details). Eight replicates were used per treatment. The medium was replaced at 48 h and observations stopped at 96 h; at the end of the experiment the number of cysts (both attached to females and laid) was also counted and used to calculate cyst production per female (total of females at 96 h).

Resting egg hatching rate

After the full 96 h duration of the demographic assays described in the previous section, the rotifers were placed in fresh medium for 48 additional hours to produce resting eggs which were then harvested and placed at 4 °C during 21 days before evaluation of the hatching rate. After the 21 day vernalisation period at 4 °C, 10 resting eggs per well were placed in a 24-well plastic microplate, filled with 2 mL of fresh control EPA medium (no contaminant in each well) and four replicates for each of the five exposure conditions described previously including controls were done. The plate was then placed under full illumination at 25 °C and checked twice a day for 5 days for any hatched rotifers, which were tallied then immediately removed from the well.

Transgenerational effects assay

A minimum of 150 resting eggs for each treatment of the demographic assay were made to hatch to start the transgenerational experiment. Of the neonate individuals that hatched for each treatment, 48 were used (i.e. 6 neonates in each of the 8 replicates per treatment). These replicates were prepared and cultivated in the same conditions and number of replicates as previously described (demographic parametres). However, instead of being re-exposed to SCCPs, all rotifers were exposed to a control EPA medium, to evaluate potential effects on their demographic characteristics induced from pre-hatching maternal exposure.

SCCP exposure for mRNA expression analysis

Using the same SCCP concentrations and incubator settings as described above (demographic parametres), the specimens were exposed for either 6, 24, 48, 72 or 96 h in test tubes containing 12 ml of medium with four replicates per exposure time and SCCP concentration. These were placed on a rotator running at a speed of 0.5 rotations/min. Each tube contained 100 neonate rotifers, and algal concentrations in the medium were readjusted to 3,000,000 cells/ml every 24 h as described in the section titled "Experimental Conditions".

At each time of exposure, rotifers were collected on a 20 µm mesh plankton net (one square of netting per test tube) and placed in cryo-tubes to be immediately frozen at − 80 °C. Extraction of the total RNA of rotifers in each test tube took place using the Nucleospin RNA XS kit (Macherey–Nagel, Germany). Concentration and absorbance ratios of the RNA samples were measured using a Nanovue Plus spectrophotometer (GE Healthcare) to check sample quality. The RevertAid RT Reverse Transcription Kit (Thermo Fisher) was then used to transcribe 110 ng of RNA from each sample into cDNA using random hexamers.

Primers for the studied genes were obtained based on sequences from Joaquim-Justo & Gismondi (2021) and based on their data, primer PCR efficiency was tested (Table 1) at a 150 nM concentration on 1 µg of a pooled mixture of cDNA from all samples, diluted 1:2 to 1:128. No-template controls were also performed for each primer pair to confirm the absence of contamination in the process.

RT-qPCR analyses were then performed on a final volume of 10 µl, composed of 10×diluted cDNA solution, iTaq™ Universal Sybr© Green supermix (BioRad), 150 nM primer mix and ultrapure water.

The qRT-PCR program was: 95 °C denaturation for 15 min, followed by 44 cycles of 95 °C denaturation lasting 15 s and 59 °C annealing and amplification for 45 s. A melting curve was then generated for the PCR products (65–95 °C ramping at 0.5 °C increments lasting 5 s each) to verify the absence of non-specific amplification.

The relative transcription level of the genes (fold-change compared to control value) was then calculated according to Livak & Schmittgen (2001) while taking into account primer efficiency. The results were then normalised using the reference genes actin (*act*) and elongation factor 1-alpha (*ef1*).

Statistical treatment

All statistical tests were conducted using XLStat 2021.2.2 (Addinsoft). Normality and homoscedasticity of datasets were verified using the Shapiro–Wilk and Bartlett tests, respectively. When these two criteria were met, significant differences between groups were determined using Student's T-test (demographic parameters, hatching rates and intrinsic growth rate) or ANOVA test followed by Fisher's post-hoc test ($P < 0.05$) for mRNA values.

For all studied parameters, results obtained from negative and solvent controls were first compared for significant differences ($P < 0.05$), in order to test the solvent effect. If no differences were found, the data of the two control conditions were pooled and the contaminated conditions were compared to this control pool. If significant differences were measured between both controls, then the contaminated conditions were compared to the solvent control only.

Table 1 Function, full-name, abbreviation, full nucleotide sequence with number of nucleotids and PCR efficiency of all the primers used in the present study

Function	Gene	Primer	Sequence (5′-3′)	Efficiency (%)
Endocrine signalling	Retinoid-acid receptor	*rar* F, *rar* R	CAA ACA ACA AGC TCT TCA CTG CC (23), GCT TCT TTT GAC ATT CCA ACA GCG (24)	95.4
	Membrane-associated progesterone receptor	*mapr* F, *mapr* R	GAT GGT GTT AAG TCA GAT GGA CG (23), GAG ACG GTT TCA AAT CGC TGA G (22)	96.6
	Retinoid-X receptor	*rxr* F, *rxr* R	CTG CAG CTG CCG CTA TAA ATG (21), ACC CTC ACA ACT ATG CAC ACC (21)	102.5
Reference genes	Actin	*act* F, *act* R	CGA AGC CCC AAT GAA TCC AAA AG (23), GTT CGA CCG GAG GCA TAT AAA G (22)	103.9
	Elongation factor 1-a	*ef1* F, *ef1* R	AGC CGA AAG AGA ACG TGG TAT (21), CGG CTT GTG ATG TAC CAG TG (20)	99.8

Results

Demographic parameters

The growth of rotifer populations exposed to environmentally relevant SCCP concentrations was assessed for 96 h with relevant demographic data being collected at 24 h intervals (Table 2).

The intrinsic growth rate (r) was approximately 1.2 d^{-1} in all treatments and decreased over time until at 96 h it reached approximately 0.9 d^{-1}. There were no significant differences between any treatments for this parameter. Mixis started in the population at 48 h, except for the SCCP 1000 ng/l treatment where no mictic females were observed. At 72 h, the process was underway in all treatments and the mixis rates (Mr) in SCCP 10 ng/l and 100 ng/l populations were significantly higher ($P<0.01$ and $P<0.001$, respectively) to controls. Finally, 96 h after the start of the assay, all Mr converged on values between 56.2 and 63.0% that were not statistically different from the control value.

Fertilised females began to appear after 72 h, with significantly lower ($P<0.05$) fertilization rates (Fr) observed for the SCCP 100 ng/l and 1000 ng/l exposures than in controls (6.3% and 4.0%, respectively, compared to 18.2% in control). Similarly, to Mr, all Fr converged to values without statistical differences with controls by 96 h (between 37.8% and 46.6%). Finally, diapausing cyst production was found to be similar in all conditions, with values between 0.2 and 0.3 cysts per female.

Diapausing cyst hatching rate

As described in the above section, rotifer cysts obtained from the different SCCP exposures were collected in order to test the hatching success in control conditions (Fig. 1). All hatching rates were observed to be between 27.5 and 33.8%, with no significant differences between the initial SCCP treatments and control conditions. In cysts from controls, 97.1% of hatchings occurred within 24 h, while this value was 83.3% in those collected from the SCCP 10 ng/L treatment. The remaining cysts had hatched by the 48 h mark. For the remaining SCCP 100 ng/L and 1000 ng/L treatment cysts, 100% of those that hatched did so within 24 h. No additional cysts hatched between 48 and 72 h in any condition.

Table 2 Values for the intrinsic growth rate r (d^{-1}), the mixis rate (Mr) and fertilisation rate (Fr) (both in percent), cyst production rate (number of cysts produced per female per replicate) and net cyst production (average number of cysts produced per replicate) including standard deviations for all SCCP treatments of the F0 demographic assay

F0	R			Mr			Fr		Cyst production rate	Net cyst production
	48H	72H	96H	48H	72H	96H	72H	96H	96H	96H
Control	1.17 ± 0.13	1.02 ± 0.09	0.92 ± 0.07	2.30 ± 3.12	48.81 ± 9.98	56.23 ± 17.62	18.18 ± 20.14	46.56 ± 14.87	0.32 ± 0.16	75.37 ± 42.13
SCCP 10 ng/L	1.23 ± 0.05	1.10 ± 0.16	0.94 ± 0.05	3.09 ± 1.47	64.62 ± 8.03*	63.01 ± 7.80	5.29 ± 3.43	37.75 ± 9.48	0.24 ± 0.12	56.75 ± 17.22
SCCP 100 ng/L	1.26 ± 0.07	1.09 ± 0.08	0.93 ± 0.03	0.88 ± 1.71	67.78 ± 10.27*	58.24 ± 10.24	6.29 ± 2.93*	41.08 ± 7.18	0.29 ± 0.07	73.00 ± 18.72
SCCP 1000 ng/L	1.26 ± 0.11	1.06 ± 0.13	0.90 ± 0.04	0.00 ± 0.00*	60.58 ± 8.74	60.84 ± 14.32	4.02 ± 2.57*	40.24 ± 13.42	0.27 ± 0.10	79.13 ± 26.49

Values significantly different from controls ($p<0.05$) are marked with an asterisk (*)

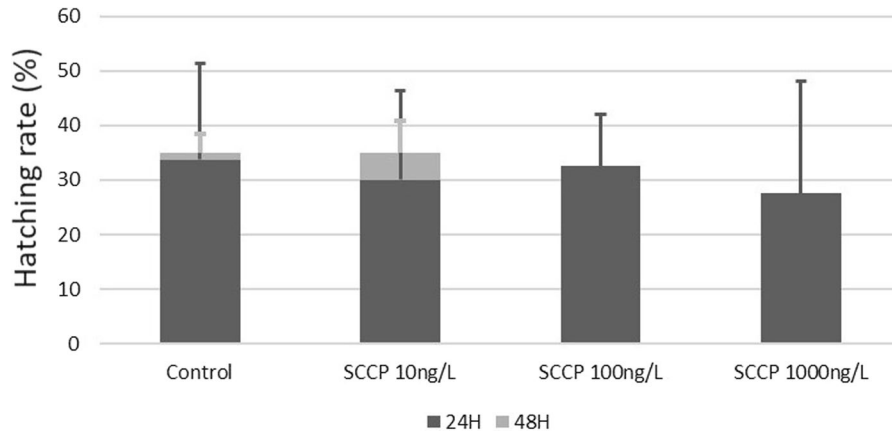

Fig. 1 Graphs of the cumulative hatching rates of *B. calyciflorus* diapausing cysts after 24 and 48 h (in percents) for each parental SCCP treatment. No statistically significant differences were observed between treatments ($p > 0.05$)

Transgenerational demographic assay

Cysts from the treatments of the assay described in the "demographic parametres" section were collected and used to hatch a new generation of individuals (F1) whose parents (F0) had been exposed to control or SCCP treatments. These were placed in similar conditions to the demographic assay and their parameters were recorded during 96 h (Table 3) at 24 h intervals. No significant statistical differences were observed at any point between F1 generation populations descended from SCCP treatments or their controls. Mixis, fertilization and cyst-production values were lower than in the F0 populations, but this was generalized in all treatments and thus not caused by parental SCCP exposure. Notably, cyst production was observed to be between 0 and 6 cysts per replicate, with average values lower than 1 in all conditions but SCCP 1000 ng/L.

Endocrine receptor mRNA assay

Populations of rotifers were exposed to SCCP treatments for durations between 6 and 96 h before RNA extraction and analysis of expression levels for three genes associated with the endocrine system: *rxr*, *rar* and *mapr.* These levels are expressed as a fold-change value compared to control (Fig. 2).

The effects of SCCP treatment on mRNA levels of these endocrine receptors changed depending on exposure time, with most significant alterations compared to control in the very short term (6 h) and chronic (72 h) exposures. The short 24 h treatment resulted in one significant divergence from controls, with *rxr* increasing in the SCCP 100 ng/L treatment while the medium, 48 h, and longest, 96 h, exposure times induced no significant changes from controls. *Retinoid-x-receptor* is the most affected gene by SCCP treatments over time, with 5 of the SCCP treatments out of 15 (3 treatments at 5 exposure times) having significantly different mRNA levels from controls, followed by *rar* (2/15) and *mapr* (1/15).

All genes were affected at least once in the SCCP 10 ng/L treatments, with *rar* also increasing once in an SCCP 100 ng/L exposure and *rxr* being significantly higher than controls in 2 of the SCCP 10 ng/L treatments, 2 SCCP 100 ng/L treatments and 1 SCCP 1000 ng/L exposure.

The two retinoid receptors *rxr* and *rar* were both significantly affected by SCCP treatments at the same time at 6 and 72 h (but, unlike *rxr*, *rar* decreased at 6 h), while *rxr* increased once alone at 24 h and *mapr* was significantly increased compared to controls once, along with *rxr* and *rar*, at 72 h.

Discussion

The present study aimed to evaluate whether SCCPs affected *B. calyciflorus* at the individual or population levels. These more sensitive chronic exposure tests allow to determine contaminant effects at low, environmentally relevant, concentrations.

Demographic parameters

The assessment of demographic parameters in experimental rotifer populations is a common method of

Table 3 Values for the intrinsic growth rate r (d^{-1}), the mixis rate (Mr) and fertilisation rate (Fr) (both in percent) cyst production rate (number of cysts produced per female per replicate) and net cyst production (average number of cysts produced per replicate) including standard deviations for all SCCP treatments of the F1 demographic assay

F1	R			Mr			Fr		Cyst production rate	Net cyst production
	48H	72H	96H	48H	72H	96H	72H	96H	96H	
Control	1.27 ± 0.10	1.24 ± 0.08	1.00 ± 0.05	2.30 ± 3.12	35.11 ± 10.78	13.97 ± 11.33	2.14 ± 2.61	15.20 ± 17.39	0.002 ± 0.001	0.75 ± 0.58
SCCP 10 ng/L	1.22 ± 0.12	1.25 ± 0.08	1.00 ± 0.07	1.98 ± 1.87	40.46 ± 8.36	12.46 ± 7.53	1.31 ± 1.68	12.01 ± 7.55	0.001 ± 0.001	0.38 ± 0.00
SCCP 100 ng/L	1.20 ± 0.10	1.25 ± 0.07	1.03 ± 0.04	1.40 ± 1.98	38.70 ± 6.97	14.42 ± 9.89	1.78 ± 3.24	10.90 ± 6.62	0.002 ± 0.002	0.75 ± 0.44
SCCP 1000 ng/L	1.18 ± 0.12	1.18 ± 0.10	1.03 ± 0.06	2.80 ± 2.66	38.15 ± 6.72	12.95 ± 11.14	1.16 ± 1.31	14.86 ± 8.80	0.002 ± 0.002	1.25 ± 0.53

Values significantly different from controls ($p < 0.05$) are marked with an asterisk (*)

evaluating whether certain contaminants or environmental conditions have the potential to negatively impact a species, via direct toxicity (mortality) or by altering population dynamics. Furthermore, assays involving the sexual reproduction stages of rotifers are some of the most sensitive available for this group of animals (Snell & Carmona, 1994).

Our observations show that asexual reproduction in *B. calyciflorus* is not impacted by SCCPs up to a concentration of 1 µg/l (asexual reproductive NOEC > 1 µg/l). However, this study shows that sexual reproduction in this species is modified during part of their life cycle by exposure to SCCPs. Indeed, the Mr of rotifers exposed to low concentrations of SCCPs (10 and 100 ng/l) were significantly higher than those of the control condition after 72 h. Moreover, Fr were significantly lower in SCCP-treated individuals (100 and 1000 ng/l). Increased mixis has been observed in rotifers exposed to organophosphorous pesticides (Ke et al., 2009) as well to fenitrothion (Lv et al., 2010), perfluorooctane sulfonate (PFOS) and to perfluorooctanoic acid (PFOA) (Zhang et al., 2013, 2014). These latter two molecules have similarities with SCCPs, as they are linear aliphatic alkanes with a high degree of halogenation. However, an increased Mr results in more sexual individuals and, normally, in a higher production of cysts, but we did not observe such an increase in cyst production, likely because the Fr in SCCP-treated individuals (100 and 1000 ng/l) were reduced. It is possible that an increased number of sexual individuals combined with a lower overall rate of fertilisation in the population cancelled each other out. Known molecules that decrease specifically the Fr of brachionid rotifers include nonylphenol, testosterone and flutamide, which are molecules known to have endocrine disrupting effects on vertebrates (Preston et al., 2000).

Notably, these significant disruptions of sexual reproduction parameters disappear after 96 h. Thus, the dynamic appears to be that mixis reaches its peak value earlier in SCCP-treated populations, then stagnates or slightly decreases. Meanwhile, the mixis rate in the control population continues to steadily increase over time, until no more statistical differences are observed with the SCCP treatments. But concurrently to this, Fr is lower in the contaminated treatments and only catches up to controls at 96 h. In any case, this leads to there being no significant differences in diapausing cyst production after

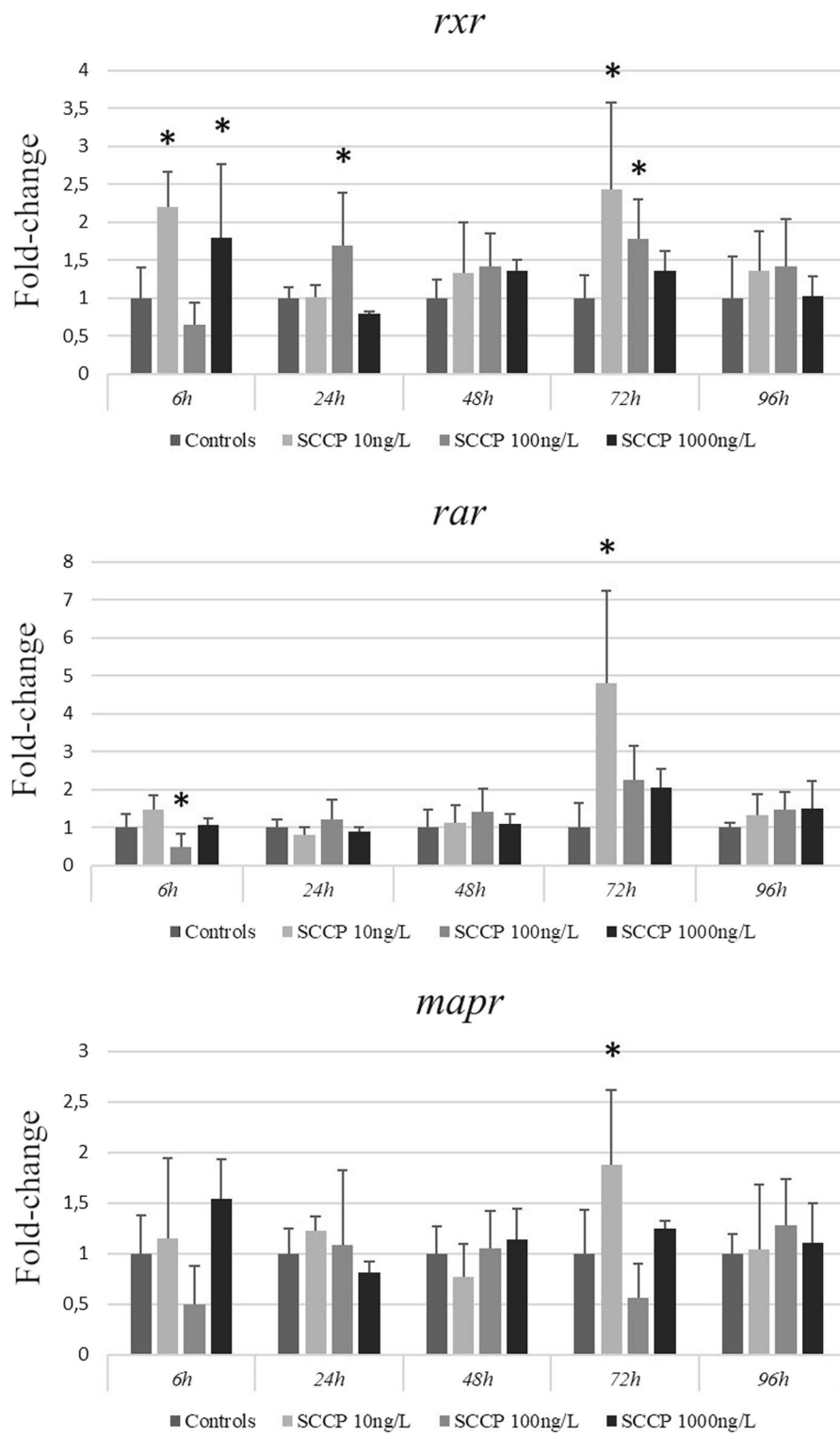

Fig. 2 Graphs of relative mRNA fold-change values compared to controls for each SCCP treatment and studied gene. Significant differences with controls ($p < 0.05$) are marked with an asterisk (*)

96 h, even if 100 and 1000 ng/L SCCP treatments all presented lower average Fr. Furthermore, there is no statistically significant effect of F0 generation exposed to SCCP on cyst viability or the life history traits of the F1 generation according to the present data. It may be notable that no cysts from the SCCP 100 and 1000 ng/L treatments hatched past the 24 h mark in the hatching assay, but the small number of cysts that did so in the control compared to the total means the overall impact is non-significant. Based on our results, showing the lack of significant effects of SCCPs past 72 h and the absence of impacts on cyst production and viability, the data indicate that current environmental SCCP levels are not a threat to the survival of *B. calyciflorus* populations in the wild.

Endocrine receptor transcriptional effect

It has been shown that mRNA levels of the endocrine receptors *rxr*, *rar* and *mapr* change significantly when *B. calyciflorus* is exposed to contaminants that affect reproduction (Joaquim-Justo & Gismondi, 2021). Consequently, as the previous assays showed that SCCPs significantly affect demographic parametres of this species, the expression levels of the genes were measured under SCCP exposure conditions, for a duration up to 96 h that matches the duration of the demographic assay. In this way, the changes in expression can be matched to changes in the demographic assays.

Results indicated that there were two major stages where the gene's mRNA reacted significantly to SCCP treatment: 6 and 72 h. The gene in rotifers whose mRNA levels were most often affected by SCCP treatment was *rxr*, a receptor which is able to form dimers with a broad range of other cellular receptors in order to activate or suppress various transcription cascades. Besides being able to homodimerize, it can also form heterodimers with one of the other receptors studied here, the *rar* (Kubickova et al., 2021). This latter union takes place in a hormone-receptor context and is the main form under which *rar* is active (Germain et al., 2006). Exposure to SCCPs caused rxr fold-change to significantly increase at 6, 24 and 72 h.

Regarding the pattern of *rar*'s mRNA levels significantly diminishing at six hours, before increasing later in the treatment at 72 h, this was shown to occur in another study with pesticides known to affect sexual reproduction in rotifers (Gismondi et al. 2019). The third studied gene, *mapr*, was not highly sensitive to SCCP exposure as it only increased significantly once, with a less than twofold change from controls. This may, however, still have played a role in the reproduction dynamics of *B. calyciflorus*, as exposure to progesterone, this receptor's putative ligand, leads to decreased fertilisation and diapausing cyst production in this species (Yang & Snell, 2010).

The early (6 h) significant fluctuations in receptor gene mRNA are unlikely to have had an impact on sexual reproduction dynamics given that, at this stage in the assay, there are few individuals and mixis has not yet been initiated. The greatest amount of significant variations occurs at 72 h (increase in all three studied genes), which is also the time where the most demographic parametres were observed to be different between control and SCCP treatments (increased Mr and lowered Fr).

As it has been shown that the mRNA levels of these receptors vary significantly when exposed to contaminants that also affect sexual reproduction (Joaquim-Justo & Gismondi, 2021), and that *rxr* in particular is known to be activated by small organic ligands which are lipophilic, making it susceptible to disruption by organic contaminants (Dawson & Xia, 2012), it is possible that this receptor (alone or in combination with *rar*) is reacting with SCCP congeners present in the solution. Reproduction in brachionid rotifers is known to be sensitive to endocrine disruptors such as vertebrate and arthropod-derived hormones, as well as pesticides. Exposure to these molecules resulted in significant effects on mixis and fertilisation (Snell, 2011; Joaquim-Justo & Gismondi, 2021). However, only the transcriptional effects on these receptors are currently known, as well as how they correlate with reproductive parametres, and not the exact mechanisms through which they interact with rotifer physiology and whether they affect reproduction directly. Current genomics research is helping to elucidate the question by identifying the endocrine receptors of brachionid rotifer species (Kim et al., 2017, 2022) but is still ongoing, as the function and putative receptors of these ligands have not yet been conclusively demonstrated. Still, the fact that the present genes react significantly to contaminants which are known to affect rotifer reproduction, could point to a link between these genes and sexual reproduction

in this species. Alternatively, SCCP exposure may exert sub-lethal toxicity on *B. calyciflorus*, leading to altered fitness and behaviour in such a way that reproduction is altered, for example by targeting the germarium of females (Gismondi et al., 2019) or male sperm production.

Conclusion

This work evaluated whether the widespread organic contaminant SCCPs had toxic effects on the brachionid rotifer *B. calyciflorus*. To this end, we conducted demographic parameter assays and compared the results to the mRNA expression levels of key genes putatively associated with endocrine receptors and sexual reproduction in the species. Our results show that environmentally relevant concentrations of SCCPs (in the ng/l range) are able to significantly affect the life history parameters of *B. calyciflorus* which concern sexual reproduction (mixis and fertilisation) during a limited period of time. These effects seem to disappear at 96 h and do not carry over to diapausing cyst production or the development of the following generation. Significant effects were measured regarding the expression of the mRNA of endocrine receptor genes *rxr*, *rar* and *mapr*, suggesting disruption of these receptors could play a role in the alterations of life history parameters that were observed in the demographic assays.

Overall, while our results show that very low SCCP concentrations affect certain stages of *B. calyciflorus* sexual reproduction, no effects were found that would indicate that current environmental levels present a risk to the long-term development and continuation of *B. calyciflorus* populations in the wild. This work opens up the possibility that SCCPs may interact with endocrine receptors not only of vertebrates but also of invertebrates. Considering the large diversity of phyla present in this category, more focused investigations should be conducted on ecologically important groups to elucidate possible impacts and mechanisms of action from exposure in the environment.

Funding This work was supported by the Belgian national funds for scientific research (Fonds National de la Recherche Scientifique—FNRS) under Grant no FC 36575.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

References

Andersen, R. A., 2005. Algal culturing techniques, Elsevier Academic Press, London:, 558.

Basconcillo, L., S. Backus, D. McGoldrick, D. Zaruk, E. Sverko & D. Muir, 2015. Current status of short- and medium chain polychlorinated n-alkanes in top predatory fish across Canada. Chemosphere. 127: 93–100. https://doi.org/10.1016/j.chemosphere.2015.01.016.

Bezchlebová, J., J. Černohlávková, K. Kobetičová, J. Lána, I. Sochová & J. Hofman, 2007. Effects of short-chain chlorinated paraffins on soil organisms. Ecotoxicology and Environmental Safety 67(2): 206–211. https://doi.org/10.1016/j.ecoenv.2006.12.015.

Castells, P., F. J. Santos & M. T. Galceran, 2004. Solid-phase extraction versus solid-phase microextraction for the determination of chlorinated paraffins in water using gas chromatography negative chemical ionisation mass spectrometry. Journal of Chromatography A 1025(2): 157–162. https://doi.org/10.1016/j.chroma.2003.10.069.

Chen, M. Y., X. J. Luo, X. L. Zhang, M. J. He, S. J. Chen & B. X. Mai, 2011. Chlorinated paraffins in sediments from the Pearl River Delta, South China: Spatial and temporal distributions and implication for processes. Environmental Science and Technology 45(23): 9936–9943. https://doi.org/10.1021/es202891a.

Cruciani, V., C. Iovine, J. P. Thomé & C. Joaquim-Justo, 2016. Impact of three phthalate esters on the sexual reproduction of the monogonont rotifer, *Brachionus calyciflorus*. Ecotoxicology 25: 192–200. https://doi.org/10.1007/s10646-015-1579-5.

Dahms, H. U., A. Hagiwara & J. S. Lee, 2011. Ecotoxicology, ecophysiology, and mechanistic studies with rotifers. Aquatic Toxicology 101(1): 1–12. https://doi.org/10.1016/J.AQUATOX.2010.09.006.

Dawson, M. I. & Z. Xia, 2012. The retinoid X receptors and their ligands. Biochimica Et Biophysica (BBA) Acta Molecular and Cell Biology of Lipids 1821(1): 21–56. https://doi.org/10.1016/J.BBALIP.2011.09.014.

EPA, 1985. In Weber, C. I. & W. H. Peltier (eds), Methods for measuring acute Ttxicity of effluents to freshwater and marine organisms. US Environmental Protection Agency, Washington.

Geissen, V., H. Mol, E. Klumpp, G. Umlauf, M. Nadal, M. van der Ploeg, S. E. A. T. M. van de Zee & C. J. Ritsema, 2015. Emerging pollutants in the environment: A challenge for water resource management. International Soil and Water Conservation Research 3(1): 57–65. https://doi.org/10.1016/j.iswcr.2015.03.002.

Geng, N., Y. Luo, R. Cao, X. Song, F. Li, F. Wang, Y. Gong, L. Xing, H. Zhang & J. Chen, 2021. Effect of short-chain chlorinated paraffins on metabolic profiling of male SD rats. Science of the Total Environment 750: 141404. https://doi.org/10.1016/j.scitotenv.2020.141404.

Germain, P., P. Chambon, G. Eichele, R. M. Evans, M. A. Lazar, M. Leid, A. R. De Lera, R. Lotan, D. J. Mangelsdorf & H. Gronemeyer, 2006. International union of pharmacology. LX. Retinoic Acid Receptors. Pharmacological Reviews 58(4): 712–725. https://doi.org/10.1124/PR.58.4.4.

Gismondi, E., H. M. Cauchie, V. Cruciani & C. Joaquim-Justo, 2019. Targeted impact of cyproterone acetate on the sexual reproduction of female rotifers. Ecotoxicology 28(6): 643–649. https://doi.org/10.1007/S10646-019-02063-9/FIGURES/3.

Houde, M., D. C. G. Muir, G. T. Tomy, D. M. Whittle, C. Teixeira & S. Moore, 2008. Bioaccumulation and trophic magnification of short- and medium-chain chlorinated paraffins in food webs from Lake Ontario and Lake Michigan. Environmental Science and Technology 42: 3893–3899. https://doi.org/10.1021/es703184s.

Jeong, C. B., H. S. Kim, H. M. Kang, Y. H. Lee, B. Zhou, J. Choe & J. S. Lee, 2017. Genome-wide identification of ATP-binding cassette (ABC) transporters and conservation of their xenobiotic transporter function in the monogonont rotifer (*Brachionus koreanus*). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 21: 17–26. https://doi.org/10.1016/j.cbd.2016.10.003.

Joaquim-Justo, C. & E. Gismondi, 2021. Expression variations of two retinoid signaling pathway receptors in the rotifer *Brachionus calyciflorus* exposed to three endocrine disruptors. Ecotoxicology 30(2): 343–350. https://doi.org/10.1007/s10646-020-02339-5.

Ke, L. X., Y. L. Xi, C. W. Zha & L. L. Dong, 2009. Effects of three organophosphorus pesticides on population growth and sexual reproduction of rotifer *Brachionus calyciflorus* Pallas. Acta Ecologica Sinica 29(3): 182–185. https://doi.org/10.1016/J.CHNAES.2009.07.008.

Kim, D. H., H. S. Kim, D. S. Hwang, H. J. Kim, A. Hagiwara, J. S. Lee & C. B. Jeong, 2017. Genome-wide identification of nuclear receptor (NR) genes and the evolutionary significance of the NR1O subfamily in the monogonont rotifer *Brachionus* spp. General and Comparative Endocrinology 252: 219–225. https://doi.org/10.1016/j.ygcen.2017.06.030.

Kim, H. S., B. Y. Lee, J. Han, C. B. Jeong, D. S. Hwang, M. C. Lee, H. M. Kang, D. H. Kim, H. J. Kim, S. Papakostas, S. A. J. Declerck, I. Y. Choi, A. Hagiwara, H. G. Park & J. S. Lee, 2018. The genome of the freshwater monogonont rotifer *Brachionus calyciflorus*. Molecular Ecology Resources 18(3): 646–655. https://doi.org/10.1111/1755-0998.12768.

Kim, D. H., E. Byeon, M. S. Kim, et al., 2022. The genome of the marine rotifer *Brachionus manjavacas*: Genome-wide identification of 310 G protein-coupled receptor (GPCR) genes. Marine Biotechnology. 24: 226–242. https://doi.org/10.1007/s10126-022-10102-6.

Kubickova, B., C. Ramwell, K. Hilscherova & M. N. Jacobs, 2021. Highlighting the gaps in hazard and risk assessment of unregulated endocrine active substances in surface waters: retinoids as a European case study. Environmental Sciences Europe 33(1): 1–38. https://doi.org/10.1186/S12302-020-00428-0.

Li, H., J. Fu, W. Pan, P. Wang, Y. Li, Q. Zhang, Y. Wang, A. Zhang, Y. Liang & G. Jiang, 2017. Environmental behaviour of short-chain chlorinated paraffins in aquatic and terrestrial ecosystems of Ny-Ålesund and London Island, Svalbard, in the Arctic. Science of the Total Environment 590–591: 163–170. https://doi.org/10.1016/j.scitotenv.2017.02.192.

Liu, L., Y. Li, M. Coelhan, H. M. Chan, W. Ma & L. Liu, 2016. Relative developmental toxicity of short-chain chlorinated paraffins in zebrafish (Danio rerio) embryos. Environmental Pollution 219: 1122–1130. https://doi.org/10.1016/j.envpol.2016.09.016.

Livak, K. J. & T. D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25(4): 402–408. https://doi.org/10.1006/METH.2001.1262.

Lv, L., T. Snell, J. Yang, S. Li & W. Zhu, 2010. Effects of fenitrothion on life history parameters of the rotifer *Brachionus calyciflorus*. Journal of Freshwater Ecology 25(4): 589–598. https://doi.org/10.1080/02705060.2010.9664408.

Ma, X., H. Zhang, Z. Wang, Z. Yao, J. Chen & J. Chen, 2014. Bioaccumulation and trophic transfer of short chain chlorinated paraffins in a marine food web from Liaodong Bay, North China. Environmental Science and Technology 48(10): 5964–5971. https://doi.org/10.1021/es500940p.

Nicholls, C. R., C. R. Allchin & R. J. Law, 2001. Levels of short and medium chain length polychlorinated n-alkanes in environmental samples from selected industrial areas in England and Wales. Environmental Pollution 114(3): 415–430. https://doi.org/10.1016/S0269-7491(00)00230-X.

Park, J. C., D. H. Kim, M. C. Lee, J. Han, H. J. Kim, A. Hagiwara, U. K. Hwang, H. G. Park & J. S. Lee, 2018. Genome-wide identification of the entire 90 glutathione S-transferase (GST) subfamily genes in four rotifer *Brachionus* species and transcriptional modulation in response to endocrine disrupting chemicals. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 28: 183–195. https://doi.org/10.1016/j.cbd.2018.09.003.

Preston, B. L., T. W. Snell, T. L. Robertson & B. J. Dingmann, 2000. Use of freshwater rotifer *Brachionus calyciflorus* in screening assay for potential endocrine disruptors. Environmental Toxicology and Chemistry 19(12): 2923–2928. https://doi.org/10.1002/ETC.5620191212.

Radix, P., G. Severin, K. W. Schramm & A. Kettrup, 2002. Reproduction disturbances of *Brachionus calyciflorus* (rotifer) for the screening of environmental endocrine disrupters. Chemosphere 47: 1097–1101. https://doi.org/10.1016/S0045-6535(01)00335-6.

Ricci, C. & M. Balsamo, 2000. The biology and ecology of lotic rotifers and gastrotrichs. Freshwater Biology. 44: 15–28. https://doi.org/10.1046/j.1365-2427.2000.00584.x.

Segers, H., 2007. Annotated checklist of the rotifers (Phylum Rotifera), with notes on nomenclature, taxonomy and

distribution. Zootaxa 1564: 1–104. https://doi.org/10.11646/zootaxa.1564.1.1.

Segers, H., 2008. Global diversity of rotifers (Rotifera) in freshwater. Hydrobiologia 595(1): 49–59. https://doi.org/10.1007/s10750-007-9003-7.

Serra, M. & T. W. Snell, 2009. Sex loss in monogonont rotifers. In Schön, I., K. Martens & P. Dijk (eds), Lost Sex. Springer, Dordrecht. https://doi.org/10.1007/978-90-481-2770-2_14.

Snell, T., 2011. A review of the molecular mechanisms of monogonont rotifer reproduction. Hydrobiologia 662: 89–97. https://doi.org/10.1007/s10750-010-0483-5.

Snell, T. W. & M. J. Carmona, 1994. Surface glycoproteins in copepods: potential signals for mate recognition. Hydrobiologia 292–293(1): 255–264. https://doi.org/10.1007/BF00229949/METRICS.

Snell, T. W., B. D. Moffat, C. Janssen & G. Persoone, 1991. Acute toxicity tests using rotifers: IV. Effects of cyst age, temperature and salinity on the sensitivity of *Brachionus calyciflorus*. Ecotoxicology and Environmental Safety 21(3): 308–317. https://doi.org/10.1016/0147-6513(91)90070-6.

Sprengel, J., P. A. Behnisch, H. Besselink, A. Brouwer & W. Vetter, 2021. In vitro human cell-based TTR-TRβ CALUX assay indicates thyroid hormone transport disruption of short-chain, medium-chain and long-chain chlorinated paraffins. Archives of Toxicology 95(4): 1391–1396. https://doi.org/10.1007/s00204-021-02994-5.

Sun, R., X. Luo, B. Tang, L. Chen, Y. Liu & B. Mai, 2017. Bioaccumulation of short chain chlorinated paraffins in a typical freshwater food web contaminated by e-waste in South China: Bioaccumulation factors, tissue distribution, and trophic transfer. Environmental Pollution 222: 165–174. https://doi.org/10.1016/j.envpol.2016.12.060.

Thompson, R. & J. Madeley, 1983. The acute and chronic toxicity of a chlorinated paraffin to the mysid shrimp (*Mysidopsis bahia*), Imperial Chemical Industries PLC, Devon:

Wallace, R. L., T. W. Snell & H. A. Smith, 2015. Phylum rotifera, Thorp and Covich's Freshwater Invertebrates: Ecology and General Biology 4th ed. Elsevier, Amsterdam: 225–271. https://doi.org/10.1016/B978-0-12-385026-3.00013-9.

Warnasuriya, G. D., B. M. Elcombe, J. R. Foster & C. R. Elcombe, 2010. A Mechanism for the induction of renal tumours in male Fischer 344 rats by short-chain chlorinated paraffins. Archives of Toxicology 84(3): 233–243. https://doi.org/10.1007/s00204-009-0489-9.

Won, E. J., J. Han, D. H. Kim, H. U. Dahms, J. S. Lee, E. J. Won, J. Han, D. H. Kim, J. S. Lee & H. U. Dahms, 2017. Rotifers in ecotoxicology. Rotifers. https://doi.org/10.1007/978-981-10-5635-2_10.

Yang, J. & T. W. Snell, 2010. Effects of progesterone, testosterone, and estrogen on sexual reproduction of the rotifer *Brachionus calyciflorus*. International Review of Hydrobiology 95(6): 441–449. https://doi.org/10.1002/IROH.201011267.

Zhang, L., J. Niu, Y. Li, Y. Wang & D. Sun, 2013. Evaluating the sub-lethal toxicity of PFOS and PFOA using rotifer *Brachionus calyciflorus*. Environmental Pollution 180: 34–40. https://doi.org/10.1016/j.envpol.2013.04.031.

Zhang, L., J. Niu, Y. Wang, J. Shi & Q. Huang, 2014. Chronic effects of PFOA and PFOS on sexual reproduction of freshwater rotifer *Brachionus calyciflorus*. Chemosphere 114: 114–120. https://doi.org/10.1016/j.chemosphere.2014.03.099.