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Exposure to single and binary mixtures of fullerenes and triclosan: Reproductive and behavioral effects in the freshwater snail *Radix balthica*

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Abstract

Emerging pollutants occur in complex mixtures in rivers and have the potential to interact with freshwater organisms. The chronic effects of nominal exposure to 3 µg/L of fullerenes (C_{60}) and 1 µg/L of triclosan (TCS) alone and in a binary mixture, were evaluated using the freshwater snail *Radix balthica*. Pollutants accumulation, reproductive output and feeding behavior were selected as sublethal endpoints. After 21 days of exposure, we did not observe interactive effects between TCS and C_{60} on the studied endpoints, except for the accumulation of C_{60} in *R. balthica* in TCS + C_{60} treatment, which was lower than when the fullerenes were alone. Neither TCS nor C_{60} caused significant effects on reproduction, expressed as number of eggs per individual, but an increase in the clutch size was observed in treatments with TCS at the third week of exposure, independently of the presence of C_{60} (16.15 ± 1.67 and 18.9 ± 4.01 eggs/egg mass in TCS and TCS + C_{60} treatments, respectively, vs. 13.17 ± 4.01 in control). The presence of C_{60} significantly enhanced the grazing activity of *R. balthica* during the first seven days (4.95 ± 1.35 and 3.91 ± 0.59 % of the area grazed per individual in C_{60} and TCS + C_{60} treatments, respectively, vs 2.6 ± 0.39 % in control). The accumulation of TCS was quite similar in treatments where this pollutant was present (BAF ≈ 1007 L/kg d.w.); however, the accumulation of C_{60} was higher when the nanoparticles were alone (BAF = 254.88 L/kg d.w.) than when it was in the binary mixture (BAF = 7.79 L/kg d.w). Overall, although TCS has been listed as an endocrine disrupter compound, no significant effects on reproduction were observed in the assayed conditions. Regarding C_{60}, the limited effects on feeding activity and the low BAF obtained in this experiment indicate that fullerenes do not have ecological consequences of relevance at the studied environmental concentrations in freshwater snails.

Keywords: C_{60} fullerenes, chemical mixtures, accumulation, freshwater invertebrates, simple food web

1. Introduction

One major reason for the biodiversity decline affecting freshwater organisms is organic and inorganic pollution (Strayer and Dudgeon 2010; Vörösmarty et al., 2010). Emerging pollutants (Geissen et al. 2015, Richardson and Ternes, 2018) massively occur throughout European rivers (Loos et al., 2009, Gravilescu et al, 2015), with high potential ecotoxicological effects (Kuzmanovic et al., 2015). These pollutants are usually present in complex mixtures and interact with physical, chemical or biological stressors, potentially modifying the toxicological effect expected for a single substance (Altenburger et al., 2015). Hence, a realistic risk assessment of pollutants requires an understanding of the combined effects of different pollutants on organisms.

Among the organic pollutants discharged in the environment as a result of human activities, a varied range of substances are thought to cause endocrine disruption in aquatic organisms that are sensitive to these substances. That is the case for triclosan [TCS, 5-chloro-2-(2,4-dichlorophenoxy)-phenol], a broad-spectrum antimicrobial agent present in medical, household and personal care products such as deodorants, toothpastes, shampoos, medical skin creams and detergents (Bhargava & Leonard, 1996; Daughton & Ternes, 1999; Schweizer, 2001). After their use, TCS reaches wastewater treatment plants (Singer et al., 2002), where it is not completely removed (Adolfsson-Erici, Pettersson, Parkkonen & Sturve, 2002) and may achieve concentrations up to 2 µg/L in freshwater ecosystems (Dann & Hontela, 2011). TCS is a suspected endocrine disruptor compound with possible toxic effects on reproduction and other hormone-mediated systems (Dann and Hontela, 2011; Wang and Tian, 2015). Mediating biological activity, TCS can degrade to byproducts such as methyl triclosan [MeTCS, 5-chloro-2-(2,4-dichlorophenoxy)anisole)], which is more persistent, lipophilic, bioaccumulative and stable than TCS (Lindström, 2002). Beyond its effects on animals, TCS is highly toxic to bacteria (Franz et al., 2008; Ricart et al., 2010) and can bioaccumulate in biofilms (Huerta et al., 2016). Bioaccumulation also occurs in aquatic molluscs (Serra-Compte et al., 2018, Coogan & La Point, 2008), and although environmental concentrations may not be lethal for this group of organisms (Guasch et al., 2016), effects on physiology, growth and behavior may occur (Canesi et al., 2007; Binelli et al., 2009; Brown et al., 2012).

Fullerenes (C_{60}) are carbon-based nanoparticles with 60 atoms of carbon formed by bounded pentagons and hexagons defining a spherical cage, which provides them with relatively large surface-area relationships, thermal stability, conductivity, adsorption and catalytic capacity. These nanoparticles have been detected in different water bodies (Freixa et

al., 2018a). Usually, C_{60} concentrations in freshwaters lie within the ng/L range (Sanchis et al., 2015a), well below the described ecotoxicological effects on freshwater organisms (Freixa et al., 2018a), which are observed at much higher concentrations (Lovern et al., 2007; Tervonen et al., 2010; Leinonen et al., 2012). Beyond the low actual toxicity of C_{60}, these materials can easily aggregate and participate in sorption processes with organic matter, aquatic organisms and organic pollutants (Bundschuh et al., 2016). TCS may interact with carbon nanoparticles, which are also discharged through sewage effluents (Farré et al., 2011). The binding of TCS to fullerenes may either reduce or increase their potential toxicity. This may be due to the reduced bioavailability and intake of the adsorbed organic pollutant (lower toxicity), or alternatively, that they act as carriers and enhance bioavailability (higher toxicity). This remains an important research issue since the role of C_{60} as vectors for other contaminants is still largely unknown. Sanchís et al. (2015b) demonstrated synergistic effects in *D. magna* mobility for malathion but not for TCS. Other studies have reported either synergistic or antagonistic effects for organic pollutants in bacteria or fish (Yang et al., 2010; Ferreira et al., 2014; Hu et al., 2015). These results suggest that the magnitude and direction of the interactive effects of fullerenes and organic compounds depend on a number of factors.

Freshwater snails are grazers ubiquitous in aquatic ecosystems, playing an important role as consumers of primary producers, controlling their populations, and serving as a bridge in the transfer of matter and energy in river systems (Lagadic et al., 2007). These organisms are sensitive pollution bioindicators (Oehlmann et al. 2007; Alonso and Camargo 2011; Tallarico 2015) and have been previously used in ecotoxicological studies also because of their ease of collection and maintenance (Alonso et al., 2016). Their response to toxicants can provide information on the effects of pollutants on reproduction (Duft et al., 2007; Oehlmann et al. 2007), behavior (Bernot et al., 2005; Musee et al., 2010) and animal physiology (Guerlet et al., 2006; Tufi et al., 2015). Their role as grazers also allows for studying the effects of chemical stressors on simple food webs (López-Doval et al., 2010; Guasch et al., 2016).

In this study, we aimed to understand the sublethal effects of environmentally relevant concentrations of C_{60} and TCS (alone and in binary mixture) on grazing activity and reproduction in a field population of *R. balthica*, a common freshwater snail in European rivers. Bioaccumulation of the contaminants was also evaluated. Lethal effects of these substances are not expected under realistic environmental conditions (Freixa et al., 2018a; Guasch et al., 2016), however, sublethal effects can occur. For this reason, behavioral (e.g., feeding activity) and reproductive responses (e.g., number of eggs and/or clutches) are end

points that can integrate information on the sublethal effects of toxicants in organisms at several levels (from molecular to organ systems level), since in both responses, a considerable number of biological processes and organ systems are involved (Hellou, 2011). Furthermore, alterations in behavior or reproduction have consequences on an individual's performance, energetic balance (e.g., less food intake in the case of lower grazing activity), and the viability of populations (e.g., reduced fertility). The hypotheses we wanted to test were that a) the two pollutants would bioaccumulate in freshwater snails, affecting their feeding activity and reproduction and b) the mixture of C_{60} and TCS would produce assembled effects, differing from those produced as a single compound.

2. Materials and Methods

2.1. Chemicals and chemical preparation

Test substances were added to the respective treatments from intermediate solutions. An aqueous C_{60} stock solution of 100 mg/L was prepared by adding C_{60} fullerene powder (sublimed, 99.9% purity; reference 572500, Sigma-Aldrich) to 100 mL of filtered rainwater (see below for water characteristics) and stirring for at least 1 month at a controlled temperature (20 ºC). A specific volume of this solution was added to experimental units to reach a nominal concentration of 3 µg/L. This method was chosen because of its ecotoxicological suitability (Oberdöster et al., 2006; Sanchis et al., 2015b) since no organic solvent is used. Particle size of C_{60} suspension in stock solution was previously characterized by transmission electron microscopy (TEM) (Zeiss EM 910). TEM images (recorded using digital CCD Gatan Orius 200 camera) indicated that the C_{60} contained round-shaped aggregates with particle size between 100 and 200 nm, and the particles were clearly dispersed homogeneously after long-time stirring (Freixa et al., 2018b). A stock solution of TCS (≥97%; Sigma-Aldrich) of 1000 mg/L was prepared in methanol (LC-MS grade; Merck). Intermediate dilutions were prepared with filtered rainwater to achieve a final nominal concentration of 1 µg/L in the respective experimental units. The final concentration of methanol in the water of the experimental units was negligible (1µL/L) and therefore did not affect the ecotoxicological responses in the organisms (OECD 2016a, 2016b).

2.2. Experimental design

The experiment was performed using 12 experimental units at the mesocosm facility of the Catalan Institute for Water Research (ICRA) (Freixa et al., 2018b). Each experimental unit consisted of a cylindrical glass aquaria (25 cm Ø x 15 cm high) with a glass column in the

middle to generate a toroid-like structure. This geometrical disposition allowed for the circulation of water in a single direction, since water was propelled by means of a glass blade connected to an electric engine (12 V, 2.2 W, 60 rpm, Philips). The water velocity was maintained at 3.5 cm/s, and each experimental unit was filled with 4 L of rainwater filtered by active carbon filters. All the experimental units were covered with tiles colonized by biofilms, and 9 freshwater snails were introduced (see below and figure S1). The experiment lasted for three weeks and was performed under a controlled temperature (20 ºC) and night-day cycle (12:12). The illumination was provided by LED lamps (Lumina LED 62, 48 W). The mean conductivity of the rainwater was 354 ± 26 µS/cm, the pH was 8.25, and the oxygen concentration was 10.25 mg/L. The mean concentration of dissolved organic carbon (DOC) in the rainwater was 2.14 mg/L, the total hardness was 115.8 mg $CaCO_3$/L, the N-NO_3 concentration was 1.17 mg N/L, and the P-PO_4 concentration was 0.01 mg/L. Ammonium and nitrite were below the limit of detection (0.001 mg/L and 0.004 mg/L respectively).

The experiment consisted of 4 different treatments with 3 replicates (experimental units) for each (C_{60}, TCS, C_{60}+TCS and control). The C_{60} treatment received a concentration of 3 µg/L of C_{60} nanoparticles. The TCS treatment received 1 µg/L of TCS. The interactive effects of TCS and C_{60} (1 µg/L and 3 µg/L, respectively) were analyzed in the C_{60} + TCS treatment. Finally, a control treatment without pollutants was considered as a reference condition. Environmental concentrations of the pollutants were selected for the experiment following previous experiments, as well as by considering the potential processes of adsorption and aggregation (Freixa et al., 2018b, Santos et al., 2019). The water was completely renewed every 3-4 days to maintain the concentration of the pollutants and nutrients during the 21 days of the experiment. Homogenization of the TCS and C_{60} nanoparticles added each time were ensured by the turbulence and flow produced by the glass blade.

2.3. Test organisms

The freshwater snail *R. balthica* (Linnaeus, 1758) was selected as the test organism. *R. balthica* is a Pulmonate freshwater snail (F. Lymnaeidae) that is common in European rivers. This organism has been used in previous experiments with TCS (Guasch et al., 2016), and because of its size and biomass, it provides enough material for bioaccumulation analyses. The organisms were identified following Bech (1990) (see also Bargues et al., (2001) and Pfenninger et al., (2006) for a discussion on taxonomy and identification). The organisms were collected from a known wild population in the Llémena River (Girona, NE Spain 34T 467911E, 4657062N) in a sampling site with low anthropogenic alteration, which have been previously

used to obtain organisms and river biofilm inoculums for exposure experiments. No TCS and fullerenes were detected (data not shown) in water samples from this sampling point. We used wild individuals instead of individuals reared in lab conditions to i) have a genetically diverse set of individuals and ii) to have individuals that during their life cycles have been exposed to real fluctuations of environmental conditions and stressors; therefore, the use of animals from a wild population makes our observations more realistic. These organisms were transported to the laboratory in less than 1 h and acclimated in starvation for 48 h before starting the experiment. This allowed us to discard organisms with poor health status while emptying their digestive systems. In first instance, organisms were classified by their shell width (4 - 8 mm) by means of sieves. A total of 130 organisms were selected and shell length was measured in each individual (mean length 10.46 ± 0.26 mm). A portion (n= 108) was used in the experiment, and the remaining 22 (20% of the total selected population) were monitored for their initial conditions (size and weight). These 22 organisms were used as proxy of the initial biomass (17.93 ± 10.31 mg dry weight). From the 108 organisms reserved for the experiment, 9 snails were randomly added to each experimental unit and the organisms were exposed for three weeks to the designed treatment. After the exposure period, the organisms were left for a further 24 h period in the absence of food (but exposed to their respective conditions) to allow them to clear their guts.

2.4. Feeding substrate

The freshwater snails fed on lab-grown biofilms. Five weeks before the beginning of the experiment and during the experiment, we cultured river biofilms on frosted glass tiles (1.5 x 1.5 cm and 14 x 2 cm) that were used as a colonizing substrate in artificial stream channels (Acuña et al., 2015), using the same water, temperature and light conditions as the experimental units. Biofilms from the Llémena River were used as the inoculum for the colonization of the frosted glass tiles. The colonized glass tiles were later transferred to the experimental units. Up to 9 (1.5 x 1.5 cm) and 4 (14 x 2 cm) biofilm-colonized frosted glass tiles were added to the bottom of each experimental unit every seven days to replace the grazed tiles and provide fresh food to the snails. The amount of biofilm added to each experimental unit was based on the nutritional needs to support 9 snails for 7 days (Muñoz et al., 2000) to guarantee that no food limitation occurred. The grazed tiles that were removed were used to measure the grazing activity (see below).

Colonized biofilms were previously exposed to the contaminants for 72 h at the same nominal concentrations of TCS and C_{60} as those used in the experimental units (for TCS, C_{60} or TCS + C_{60}

treatments). Then they were introduced into their respective experimental units. The objective was to accumulate sufficient pollutants in the biofilm to ensure their potential transfer to the grazers. Previous studies have shown that 72 h is time enough for the accumulation of the contaminants (Sanchís et al., *submitted*; Santos et al., 2019).

In vivo chlorophyll-a (Chl-*a*) fluorescence (Timoner et al., 2012) was used to check for the quality of the biofilm before and after every renewal. Measurements were performed at random on 5 different glass tiles in each mesocosm using a portable pulse amplitude modulate fluorimeter (Diving PAM, Walz, Germany) to measure the basal fluorescence (F_0), as approximation to Chl-*a* content, and the photosynthetic efficiency (Y_{eff}) of the Chl-*a*, as approximation to physiological status.

2.5. Measured endpoints of R. balthica.

Mortality. The number of dead and live snails was counted and recorded every day. A snail was considered dead when no movement was observed and when the snail did not respond to an external stimulus (e.g., contraction of the soft body into the shell when touched by forceps). Dead animals were withdrawn from the experimental units.

Biomass. Individual biomass was measured as dry weight (d.w.) after freeze-drying the soft body. For the analysis of the biomass, individuals were collected, flash-frozen in liquid N_2 and stored at -80 ºC. At the moment of the analysis, the organisms were defrosted, and the soft body was separated from the shell and freeze-dried. The weight of the soft body after freeze-drying was considered the dry weight. The biomass was calculated for the initial population pool (n = 22) as well as for the surviving individuals from each treatment at the end of the experiment.

Reproduction. *R. balthica* lays eggs grouped in gelatinous masses (egg masses or clutch) that are attached to aquatic surfaces. Once a week, in order to minimize manipulation in the experimental units, during water renewal, each experimental unit was inspected for the presence of egg masses attached to the surfaces (glass walls and tiles). The egg masses were carefully detached from the surfaces with a spatula and saved for further counting. Clutches observed during the first week were discarded because the organisms could be previously fertilized (e.g., in the river or during the acclimation period). The total number of egg masses per experimental unit was counted as well as the number of eggs inside each mass using a

stereomicroscope. The cumulative number of egg masses (and their number of eggs) laid during the week and the number of eggs/egg mass were counted at the end of weeks 2 (period from day 8 to day 14) and 3 (period from day 15 to 21). Data was normalized by the number of individuals alive in the respective experimental unit on day 14 or 21 respectively and the data was expressed as the number of egg masses/individual, as the number of eggs/individual and as the number of eggs/egg mass.

Grazing activity. The grazing activity was weekly measured. The freshwater snails fed on lab-grown biofilms. The replaced grazed tiles were collected for analysis of the total area grazed after a period of 7 days. Three different periods were considered: week 1 (from day 1 to day 7), week 2 (day 8 to 14) and week 3 (day 15 to 21). Photographs of 4 large and 3 small tiles chosen randomly from each experimental unit were taken at equal conditions of light and distance (Canon PowerShot A 3500 IS). The pictures were subsequently transformed to grayscale, and the remaining area was calculated using ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/). The grazed area was calculated as the percentage grazed from the total area of the tiles (total area = 118.75 cm^2) and normalized by the number of snails in the respective experimental unit at the end of each week (days 7, 14 and 21, respectively).

2.6. Chemical analysis of water, biofilm and freshwater snails

The conductivity, pH, temperature, oxygen concentration, nutrient concentration, and DOC were measured for the initial conditions and after 3-4 days in each experimental unit. The physical variables (pH, oxygen, conductivity, and temperature) were measured using portable handheld probes (WTW, Weilheim in Oberbayern, Germany). The NO_2^-, NO_3^-, and NH_4^+ concentrations were analyzed by ionic chromatography (Dionex, ICS 5000), and the PO_4^{3-} concentration was analyzed spectrophotometrically by the ascorbate-reduced molybdenum blue method. DOC was quantified using a total organic carbon analyzer (Shimadzu TOC-V CSH).

The concentrations of TCS, MeTCS and C_{60} in the water were analyzed during the experiment in the two hours following the addition of the pollutants and before each water renewal in the respective experimental units. Triclosan and MeTCS were determined by online HS-SPME-GC-MS/MS according to Santos et al. (2019, see SM for further details). The concentration of C_{60} was determined by liquid chromatography coupled to high-resolution

mass spectrometry (HPLC-HRMS) using the instrumentation and methodology described in Sanchís et al. (2015).

The concentrations of TCS and MeTCS were analyzed in biofilms and snails at the end of the experiment. The extraction of TCS and MeTCS from the biota samples was made with a method adapted from Santos et al. (2019). Briefly 20 mg of freeze-dried snail soft tissue (obtained from a pool of 3 organisms *per* replicate) or 100 mg of freeze-dried biofilm (integrated sample from all the replicates in the same treatment) were extracted with 1 mL of citric buffer pH 4:acetonitrile (1:1, *v/v*) using a mini bead beater (Vortex-Genie 2, MoBio Laboratories, Inc, USA) with glass beads followed by a clean-up step using solid phase extraction (SPE) and further analyzed by online HS-SPME-GC-MS/MS. More detailed information on the biota samples preparation and validation parameters of the analytical methodology can be found in the Supplementary Material.

Evidence for, and the values of, the accumulation of C_{60} in the biofilms were obtained in a previous experiment with fullerenes and biofilm in similar conditions as the present experiment (Sanchís et al., 2018, submitted). Therefore, accumulation of C_{60} in the biofilm was not analyzed in this experiment, and only the snails (composite of 2 individuals from each replicate) were analyzed. Because C_{60} could adsorb to the surface and tegument of the freshwater snails, the concentration of fullerenes was first analyzed on the surface of the freeze-dried organisms before homogenization. Superficial aggregates were determined with a solid-liquid extraction (SLE) with toluene and vortex agitation (30 seconds 500 rpm) of the organisms without homogenization to "clean" the surfaces and drag the nanoparticles. After cleaning the snails, the organisms were homogenized and analyzed to determine the C_{60} ingested and accumulated in the freshwater snails. The total fullerene body burden was estimated after homogenizing the snail by ultrasound-assisted SLE in an ultrasonic bath for 60 min with toluene as solvent. The extracts were centrifuged for 3 min at 4,000 rpm, and the supernatants were analyzed as indicated above for water samples (Sanchís et al., 2015).

Bioaccumulation factors (BAFs) were calculated for C_{60} and TCS by dividing the concentration of the pollutants in the snail homogenates (ng/g d.w.) by the mean concentration of each pollutant in the water during the experiment (µg/L). The concentrations in the water samples collected after 3 or 4 days were taken considering that the system reached equilibrium. Although concentrations of pollutants in individuals are calculated and given in ng/g d.w., BAF are given in L/Kg wet weight (w.w.) to allow comparison with

regulatory values. For this purpose, knowing the percentage of water lost in our samples in the freeze drying process a correction factor of 0.095 was applied to the d.w. to estimate the w.w.

2.7. Statistical analysis

For the exposed organisms, data on the grazing activity was recorded in all the replicates (n = 3) three times during the experiment after 7 days of grazing activity by the snails. The grazed surface (in percentage of the total colonized area) was corrected by the number of individuals alive at the end of the grazing period. The number of egg sacs and the number of eggs in each egg sac were recorded in all the replicates (n = 3) at the end of weeks 2 and 3. These values were corrected by the number of individuals alive at the end of the analyzed period. The mortality was checked daily in all the treatments and replicates (n = 3), and the biomass was analyzed at the end of the experiment; however, for these parameters, we only studied significant differences for the values obtained after 21 days of exposure (end of the experiment). The data were checked for normality and homogeneity of variance by Smirnov-Kolmogorov and Levene's tests. One-way ANOVA with a *post hoc* Dunnett's test was applied to detect significant differences in the responses in each of the treatments against the control. In addition, when significant differences were detected, a two-way ANOVA with TCS and C_{60} as factors (two levels: presence or absence of each contaminant) was applied for the mixture to identify the effect of each factor on the significance of the responses and whether an interaction between the factors existed. These analyses were performed using the IBM SPSS v21 software (IBM Corp. released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

3. Results

3.1. Water parameters

The water quality was suitable for *R. balthica* and constant over the experiment and between treatments (Table 1). The concentration of DOC increased by double after 3-4 days. After water renewal, the ammonium concentration was below the limit of detection but increased with regard to the initial values after 3-4 days; however, the final concentrations were nontoxic following ASTM (2015) guidelines (≤0.035 mg N/L) for *R. balthica* (Berenzen et al., 2001)

The initial concentrations of TCS in the water (Table 2) were slightly below the predicted nominal levels in the TCS and TCS + C_{60} treatments (0.86 ± 0.15 µg/L and 0.95 ± 0.07

µg/L, respectively) which could be attributed to both abiotic degradation and sorption processes (Santos et al., 2019). The TCS concentration decreased in both the TCS and TCS+C_{60} treatments down to nearly 50% after 3-4 days of exposure. No TCS was detected in the control and C_{60} treatments. No significant differences in the concentration of TCS were detected between treatments for the samples taken after or before the water renewal. In contrast, the concentration of MeTCS was lower in the TCS + C_{60} treatment after 3-4 days (Table 2)

The initial C_{60} concentrations ranged from 2.5 to 7.5 µg/L in the C_{60} treatment and from 2.9 to 5.7 µg/L in the TCS + C_{60} treatment (Table 2). After 3-4 days, the concentrations of C_{60} in water considerably decreased (approximately until 1 µg/L), indicating high aggregation, adsorption to surfaces, or incorporation into snails. C_{60} concentrations in the control and TCS treatments were below the method detection limit (Table 2). No significant differences in the concentration of C_{60} were detected between the treatments for the samples taken after or before water renewal.

3.2. Basal resources

The quality of the biofilm, measured as basal fluorescence (F_0), was similar between the treatments at the beginning of each feeding period. A two-way ANOVA (week of introduction in the experimental units x treatment as factors) demonstrated no significant differences in F_0 between the treatments, but significant differences were related to week ($F = 8.372$, $p = 0.002$), where the basal fluorescence was slightly higher in the fresh biofilm at week 2, indicating more chlorophyll. No significant differences were observed for Y_{eff} due to week or treatment.

The biofilms exposed to C_{60} and TCS accumulated these compounds during the 72h acclimation period. TCS and MeTCS were detected in the biofilms (≈ 146 ng/g and ≈ 27 ng/g for TCS and MeTCS, respectively, Table SM2). The accumulation of fullerenes in the biofilms was not investigated in this experiment but investigated in a previous experiment under similar experimental conditions (Sanchís et al., submitted).

3.3. Mortality and biomass

The snail mortality did not differ between the treatments at the end of the experiment. The average mortality in the control accounted for 22% (table SM3), which is slightly higher than OECD recommendations (OECD 2016a, 2016b). The mechanical action of the blades on the snails also accounted for mortality (3 individuals in a C_{60} replicate and 1 in one control

replicate). The biomass was highly variable between the organisms selected for the exposure experiment (17.93 ± 10.31 mg). However, there were no significant differences in the biomass between treatments and between the initial and final conditions. Mean biomass in the experimental units at the end of the experiment was 13.3 ± 2.65; 13.99 ± 0.76; 13.78 ± 0.9; 14.7 ± 1.87 mg for control, C_{60}, TCS and TCS + C_{60} treatments, respectively.

3.4. Bioaccumulation

Because our samples were freeze-dried for the chemical analysis, concentrations are expressed as function of the d.w. of the sample. However, we applied a conversion factor of 0.095 for BAF values to obtain w.w. and to compare our values with regulative thresholds. The organisms exposed to C_{60} accumulated these nanoparticles after 3 weeks showing different patterns of accumulation depending on the treatment. Organisms from the treatment with only C_{60} showed higher concentrations of fullerenes in their bodies (average 1,527 ng/g d.w.) and tegument surfaces (average 10.2 ng/g d.w.), while those in the TCS + C_{60} treatment had lower body burden average values of 77.2 ng/g d.w. and an average 1.51 ng/g d.w. in the tegument surfaces. Fullerenes were not detected in organisms from the control or TCS treatments (see Table 3). The mean BAFs were 253.93 L/Kg w.w. and 7.78 L/Kg w.w. for the C_{60} and TCS + C_{60} treatments, respectively.

The organisms exposed to TCS accumulated this compound and the MeTCS in their tissues after 3 weeks of exposure in treatments exposed to TCS (see table 3). The body burden of MeTCS was significantly higher in the organisms in the TCS treatment than in those from the TCS + C_{60} treatment (145.7 ± 6.3 ng/g d.w. and 8.4 ± 2.0 ng/g d.w. respectively, $F = 1{,}408.77$; $p < 0.001$) whereas no significant differences were observed in the TCS accumulation among treatments. For the BAFs, the TCS values were highly similar in both treatments with mean values of 1034.42 L/Kg w.w. and 998.01 L/Kg w.w. for the TCS and TCS + C_{60} treatments, respectively.

3.5. Reproduction

No significant differences between treatments were observed for the number of eggs/individual in the second or third week (Table SM 4). The number of egg masses laid per individual did not show significant differences between the treatments when considering a one-week period; however, significant differences between weeks were detected ($F = 6.448$; $p = 0.022$, two-way ANOVA with treatment and week as factors), being higher in the third week (from day 15 to 21). Weak significant differences between the treatments for the number of

eggs/egg mass (one-way ANOVA, F = 4.108; p = 0.049) occurred only in the third week (from day 15 to day 21). At that time, the number of eggs/egg mass was higher in the treatments with TCS (Table SM 4 and figure 1). The effect of TCS was significant (F = 9.854; p = 0.014), contributing to 55.2% of the variability (two-way ANOVA with TCS and C_{60} as factors). The binary mixture did not produce a different pattern of response than TCS alone.

3.6. Grazing activity

Significant differences with respect to the control only occurred in the C_{60} and TCS + C_{60} treatments and only during the first week (table SM 3 and figure 2, one-way ANOVA with Dunnett's test, F = 4.459; p = 0.040). The area grazed was higher in the treatments with fullerenes (two-way ANOVA with TCS and C_{60} as factors, F = 11.30; p = 0.01). The presence of fullerenes alone explained 58.6% of the variance (Eta square). In the TCS + $C_{60,}$ treatments, the responses were not different from those observed in the treatment with fullerenes alone.

4. Discussion

Our experimental design, based on a long-term exposure, focused on sublethal effects and using natural biofilm as feeding source, is aligned with the recent discussion on the needs of greater realism in micro- and mesocosms tests at conditions close to the real-world environment in comparison to conventional chemical testing (Holden et al., 2016). This is the first time that the chronic effects of fullerenes in freshwater snails have been assessed and described. We observed an accumulation of these pollutants in *R. balthica*. Overall, the nanoparticles did not cause any significant mortality with regard the control, nevertheless, mortality was slightly above of the OECD recommendations for experiments with freshwater snails (20% in control conditions, OECD 2016a, 2016b). In some cases, this mortality was caused by the effects of blades, but maybe an acclimation time of 48h could not be enough to eliminate organisms with poor health status. Our results showed that grazing activity was affected by the presence of C_{60} the snails exposed to fullerenes had enhanced grazing activity after the first 7 days of exposure. However no significant effects on reproduction were observed that could affect the viability of the population. These observations partially support the hypothesis that sublethal concentrations of these emerging pollutants may cause significant effects on individual performance. Our study also showed that when we co-exposed TCS and C_{60}, the responses in the studied endpoints were not different than the responses observed when the contaminant was alone, discarding the potential interaction between these two pollutants at the studied environmental concentrations.

4.1. R. balthica accumulated TCS, MeTCS and fullerenes

In this experiment, the freshwater snails had two potential ways of contamination: through the water and/or the assimilation of biofilm via feeding activity. TCS and C_{60} were always present in water, despite the reduced concentration after 3-4 days. In the case of biofilm, TCS was detected (table SM2), and the accumulation of C_{60} at values of 2.76 ± 2.38 ng/g was described in a previous experiment (Sanchís et al., *submitted*) using similar experimental conditions. Since the experimental conditions in both experiments were very similar, we used this information as evidence of C_{60} accumulation in the biofilms. These results ensured that these contaminants were available via feeding activity. With this information we can consider that biofilm can be a source of TCS and C_{60} contamination to freshwater snails. Finally, in *R. balthica*, we observed an overall accumulation of C_{60} in the homogenates as well as on the external surfaces (i.e., teguments). The bioaccumulation factors (BAFs) were 253.93 L/Kg w.w. and 7.78 L/Kg w.w. for the C_{60} and TCS+C_{60} treatments, respectively. These BAF factors can be considered very low following European Union regulation, that considers bioaccumulation when values are ≥ 2000 (Gobas et al., 2009). Previous studies have indicated that C_{60} entering via feeding activity accumulates in the gut lumen rather than in the tissues or cells (Tervonen et al., 2010; Pakarinen et al., 2011; Mendoça et al., 2011; Waissi-Leinonen et al., 2012), although Hu et al., (2015) detected fullerenes in different organs of fishes exposed to concentrations of mg/L of carbon nanoparticles, a concentration much higher than the studied here. The BAFs values of C_{60} observed in the snails in our experiment, indicate that possibly fullerenes do not penetrate massively into the organism rather they accumulate in the digestive system, thus they do not arrive at other tissues and do not truly bioaccumulate in *R. balthica*. These values could be the result of residual contents in the gut of fullerenes introduced with the grazed biofilm. Although the organisms were starved at the end of the experiment for 24 h before being sacrificed with N_2 to allow for the excretion of feces and the emptying of their guts, fullerenes could remain adsorbed to their digestive surfaces. It is also interesting to note that significant lower concentrations of C_{60} were observed in the homogenates and tegument surfaces in organisms in the TCS+C_{60} treatment than in those with C_{60} alone. This suggests that the presence of TCS could diminish the accumulation of C_{60} in exposed organisms, potentially due to the adsorption of TCS to the fullerenes, which reduces its bioavailability. Since the concentrations of C_{60} in the water and biofilm were not altered by the presence of TCS, it can be hypothesized that TCS has a possible effect on the physiology of *Radix* sp. that could enhances C_{60} elimination or avoids accumulation. It is known that gastropods produce pedal mucus as a primary defense against environmental stressors, and its

secretion can be increased (Lopez-Doval et al., 2013) or decreased (Davies 1992) in the presence of some pollutants. The secretion of mucus reduces the bioaccumulation and toxic effects of metals (Jugdaohsingh et al., 1998, Davies et al., 1999) in different gastropods. Mucus also plays an important role in feeding activity, since it is secreted in the mouth and agglutinates the food through the digestive system (Dillon, 2004). A potential mechanism could be that TCS (but not fullerenes) stimulated mucus secretion, which chelated the fullerenes and increased their elimination. Unfortunately, mucus secretion was not measured, and further research is needed to confirm this hypothesis.

TCS and MeTCS were also detected in the snails' homogenates. Bioaccumulation of TCS has been previously described in freshwater snails (Coogan and La Point, 2008), but the results are hardly comparable because the data were standardized by lipid content. Riva et al. (2012), studied the bioaccumulation of TCS in the freshwater mussel *Dreissena polymorpha*. After seven days of exposure to 580 ng/L the concentration in organism's tissues was 248 ng/g d.w., a concentration in tissues considerably lower than our concentration, maybe because the lower time of exposure and concentration in water. No significant differences in TCS accumulation between the TCS or TCS + C_{60} treatments were observed, and in this case, the BAFs were 1034.42 and 998.01 L/kg w.w. for the TCS and TCS + C_{60} treatments, respectively, higher than in the case of the C_{60} nanoparticles but following European regulations, bioaccumulation cannot be considered (Gobas et al., 2009). However, MeTCS showed significant differences depending on the treatment, with the concentration of MeTCS approximately 9.5 times lower in the TCS + C_{60} treatment than in the treatment with only TCS. Despite the lack of statistical evidence of interactions between TCS and C_{60} observed in the studied endpoints, the homogenate concentrations indicate some type of interaction between these pollutants affecting their accumulation pattern, but whether this is due to chemical interactions between the two compounds or because the effects are mediated by alterations in the detoxifying and excretory mechanisms of the snails is not yet known.

4.2. No effects of TCS and fullerene exposure on fertility

Effects of C_{60} in reproduction have been described in different organisms and at concentrations in the range of mg/L in other organisms (Oberdörster et al., 2006), but not in freshwater snails. TCS is a well-known endocrine disruptor compound (EDC) and has effects on hormone expression and metabolism (in the ng/L range) and on the reproduction of different freshwater organisms at concentrations ranging from 40 to 220 µg/L (Dann & Hontela, 2011 and references therein). The effects of TCS in the fertility of freshwater snails have been

already described. Geiß et al. (2016) demonstrated the increased production of embryos from concentrations of 0.66 µg/L after 28 days of exposure to TCS in *Potamopyrgus antipodarum*. However, in our experiment, no significant effects between treatments were observed in the total reproductive output, expressed as number of eggs/individual or number of egg masses laid/individual in each seven-day period. Only a slightly significant difference between treatments was observed, related to an increase in the number of eggs/egg mass during the third week in those treatments with TCS (treatments TCS and TCS + C_{60}). A similar response has been observed in other Pulmonates exposed to different chemical pollutants. In *in situ* experiments in Mediterranean rivers, De Castro-Català et al. (2013) correlated environmental concentrations of TCS and other EDCs in rivers with a higher number of eggs/egg mass in *Physa acuta* and Lopez-Doval et al. (2010) also observed an increase in the number of eggs/egg mass for the same organism exposed to the herbicide diuron under experimental conditions. Clutch size is a parameter sensitive to the exposure to pollutants (Coutellec and Lagadic, 2006), but also sensitive to organisms' density and environmental factors such as experimental conditions (Brown, 1985; Coutellec et al., 2008). Nevertheless, considering the lack of effects on the number of eggs and egg mass per individual and the slight effects on the number of eggs per egg mass, effects of C_{60} or TCS on reproduction are not conclusive.

4.3. Feeding behavior as a sensitive sublethal endpoint of fullerene exposure

Changes in feeding activity can lead to alterations in the energetic balance in individuals and eventually to modifications in other vital traits and individual performance (Barata et al., 2004). In our study, the grazing activity was significant higher in the treatments with fullerenes, but only during the first week, suggesting that some adaptation could also exist after a short time. Freixa et al., (2018b) studied the effects of TCS and C_{60} on biofilms at concentrations very similar to those used in this experiment and observed similar effects of both substances in biofilms. Since TCS and C_{60} caused similar toxic effects on the quality of the biofilms, the differences in the grazing activities observed in our experiment should be considered direct effects of C_{60} on snails rather than indirect effects due to changes in the nutritional value of the biofilm caused by the pollutants. Lovern et al. (2007) described an increase in the feeding activity of *D. magna* after exposure to 260 µg/L of C_{60} for 2.5 h, suggesting that this was a response to compensate for the intake of nanoparticles. Organisms with lowered capacities of assimilation should increase their feeding effort to compensate for this reduction and maintain their energetic demands. Nanoparticles have indeed shown the capability to reduce food assimilation by means of mechanical obstruction in the guts of

experimental organisms. Mendoça et al. (2011) observed the deposition of nanocarbon particles in the gut lumen of *D. magna* and considered it to be a possible cause of the inhibition of food absorption. Tervonen et al. (2010) studied the accumulation of fullerenes in *D. magna* and found nanoparticles only in the gut lumen. Finally, Waissi-Leinonen et al. (2012) observed the aggregation of nanoparticles and damage in the gut microvilli of *Chironomus riparius* exposed to C_{60}, causing impairment in food assimilation and diminution in growth and biomass. Similar results were obtained by Pakarinen et al. (2011) in long-term experiments with *L. variegatus*. Overall, it is very likely that fullerenes can impair, to some extent, the ability of individuals to assimilate nutrients, inducing higher grazing activity as compensatory behavior. In our study, we cannot provide a feasible explanation for the mechanism of C_{60} on the grazing activity of *R. balthica*, but mechanical obstruction in the gut lumen and the reduction of food assimilation capability, as described in cited literature, could be the causes of the increase in grazing activity. In our study, we consider that the assimilation of C_{60} *via* biofilm intake is a feasible way of contamination. Nevertheless, this did not lead to significant changes in biomass, perhaps because this enhanced feeding activity was only observed during the first 7 days of exposure.

5. Final remarks

Although interactions between both pollutants have not been observed in the studied endpoints, our study provides evidence of the possible effects of TCS on C_{60} accumulation in homogenates and surface of *R. balthica*. Our results showed significant effects on clutch size however no significant effects on the overall reproductive performance were observed after the TCS exposition. Finally, the determined effects of C_{60} on feeding activity and the low BAF of this pollutant indicate that it does not have ecological consequences of relevance at the environmental studied concentrations for freshwater snails

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Figure captions

Figure 1. Fertility on week 2 (from day 8 to 14) and 3 (day 15 to 21), expressed as number of eggs/egg mass. Only in the third week significant differences were observed in treatments with TCS (n = 3, F = 4.108; p = 0.049). Asterisk indicates treatments with significant factors after two-way ANOVA with TCS or C_{60} as factors.

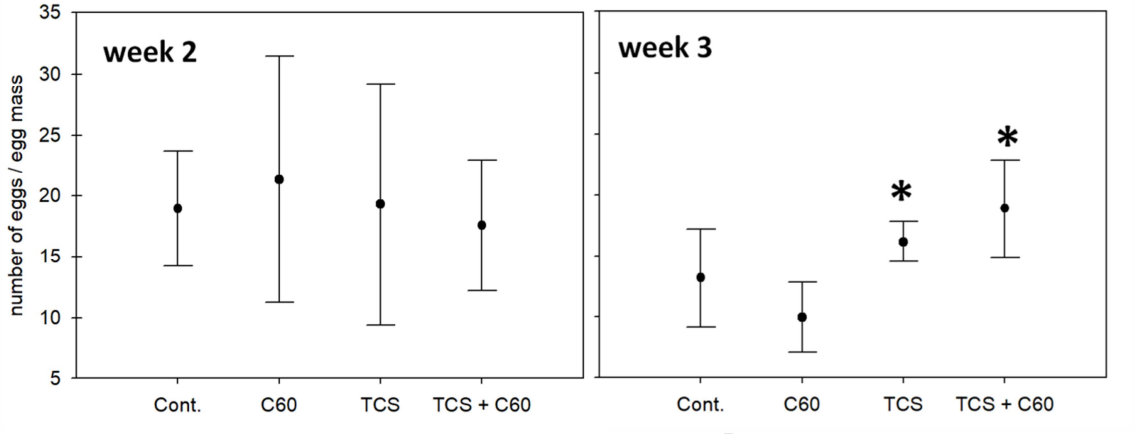
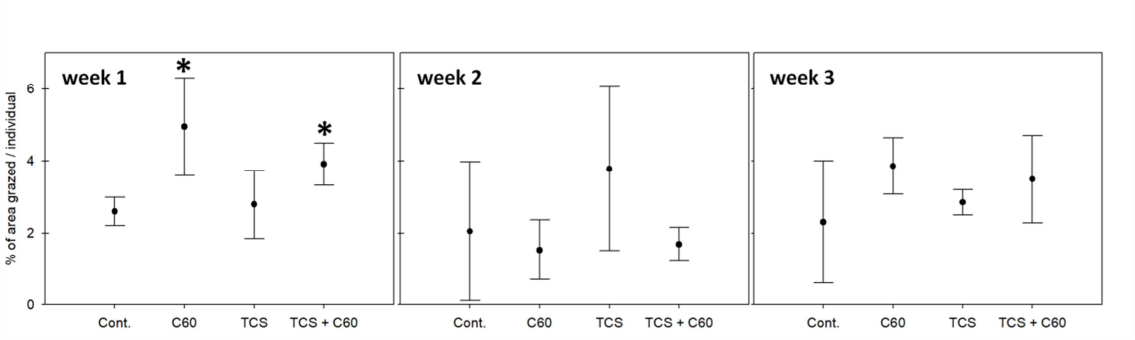


Figure 2. Grazing activity measured as area grazed by individual after 7 days during the experiment. Values are expressed as percentage of grazed area. Only in the 1st week significant differences were observed in treatments with C_{60} (n = 3, F = 4.459; p = 0.040). Asterisk indicates treatments significantly different after two-way ANOVA with TCS or C_{60} as factors.



1 Tables

2 Table 1. Physical and chemical quality of water in all treatments after 3-4 days. Mean ±
3 standard deviation.

	Cont	C_{60}	TCS	TCS + C_{60}
Conductivity	354.52 ± 29.30	354.36 ± 26.63	354.27 ± 26.40	355.64 ± 26.34
Temperature º C	17.47 ± 0.22	17.53 ± 0.21	17.63 ± 0.34	17.55 ± 0.38
pH	8.37 ± 0.22	8.27 ± 0.10	8.21 ± 0.10	8.24 ± 0.09
DOC (mg C/L)	5.103 ± 1.031	4.775 ± 1.055	4.596 ± 0.721	4.858 ± 0.857
N-NH_4 (mg N/L)	0.031 ± 0.025	0.029 ± 0.016	0.036 ± 0.021	0.039 ± 0.021

4

5

Table 2. Concentration of TCS, MeTCS and C_{60} in water in the different treatments before and after the water renewal. Data expressed in µg/L (mean ± s.d., n = 3). MLOD = below method detection limit.

treatment	day	TCS	(max - min)	MeTCS	(max - min)	C_{60}	(max - min)
Control	d_0	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
	d_{3-4}	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD	< MLOD
C_{60}	d_0	< MLOD	< MLOD	< MLOD	< MLOD	2.74 ± 0.46	3.50 - 2.03
	d_{3-4}	< MLOD	< MLOD	< MLOD	< MLOD	0.54 ± 0.54	0.69 - 0.11
TCS	d_0	0.864 ± 0.146	1.054 - 0.731	0.001 ± 0.069	0.002 - < MLOD	< MLOD	< MLOD
	d_{3-4}	0.480 ± 0.062	0.531 - 0.402	0.064 ± 0.021	0.085 - 0.044	< MLOD	< MLOD
TCS + C_{60}	d_0	0.953 ± 0.070	1.031 - 0.877	0.001± 0	0.001 - <MLOD	4.31 ± 0.95	5.70 - 2.95
	d_{3-4}	0.444 ± 0.125	0.602 - 0.308	0.006 ± 0.002	0.010 - 0.004	0.91 ± 0.26	1.27 - 0.55

Table 3. Concentration of TCS, MeTCS and C_{60} in homogenates and surface of *R. balthica*. Data expressed in ng/g d.w. (mean ± s.d., n = 3). MLOD = below method detection limit.

	TCS	**MeTCS**	**C_{60} homog.**	**C_{60} surface**
Cont.	< MLOD	< MLOD	<MLOD	<MLOD
C_{60}	< MLOD	< MLOD	1,527.03 ± 1,664.86	10.17 ± 10.32
TCS	5,122.8 ± 545.2	145.7 ± 6.31	<MLOD	<MLOD
TCS + C_{60}	4,468.1 ± 895.0	8.39 ± 2.05	77.18 ± 96.54	1.52 ± 2.78

Highlights

- Effects of TCS and C_{60} on a freshwater snail were investigated alone and binary mixtures.

- The accumulation of TCS in snails was higher in comparison to C_{60}.

- Clutch size was sensitive to TCS at the end of the experiment.

- Feeding behavior was sensitive to C_{60}.

- No synergistic or antagonistic effects were observed for the binary mixture.