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Transgenerational Effects of Toxicants: An Extension of the *Daphnia* 21-day Chronic Assay?

B. B. Castro[1] · A. R. Freches[2] · M. Rodrigues[2] · B. Nunes[3] · S. C. Antunes[2,4]

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Abstract
The assessment of transgenerational effects should be incorporated in standard chronic toxicity protocols for the sake of a realistic extrapolation of contaminant effects to the population level. We propose a simple add-on to the standard 21-day chronic *Daphnia magna* assay, allowing the assessment of the reproductive performance of the offspring (F_1 generation) born from the first clutch of the parental (F_0) generation. The extended generational assay was performed simultaneously with the standard reproduction assay. With this design, we evaluated the lethal, reproductive, and transgenerational effects of four widespread and extensively used substances: a biocide/anti-fouling (copper sulphate), an industrial oxidizing agent (potassium dichromate), a pharmaceutical (paracetamol), and a quaternary ammonium compound (benzalkonium chloride). Benzalkonium chloride was the most toxic in terms of lethality, whereas paracetamol, copper sulphate, and potassium dichromate caused deleterious effects in the reproductive performance of exposed *D. magna*. Adverse effects in the fitness of the daughter (F_1) generation were observed in the case of maternal exposure to paracetamol and copper sulphate, although they were not very pronounced. These findings highlight the usefulness of our approach and reinforce the view—shared by other authors—of the need for a generalised formal assessment of the transgenerational effects of pollutants.

The aquatic environment is a susceptible recipient and reservoir of waterborne contaminants (Cerejeira et al. 2003; Wake 2005; Fick et al. 2009; Monteiro and Boxall 2010), some of which frequently occur in low concentrations, including in drinking water sources (Benotti et al. 2009; Daneshvar et al. 2012). Schwarzenbach et al. (2009) reviewed the scientific challenges of addressing water-quality problems caused by ubiquitous micropollutants, recognising a need to further refine the available toolbox to assess their impact on aquatic life. Many other authors have emphasized the need to understand the subtle effects caused by the long-term exposure to such micropollutants (Daughton and Ternes 1999; Moore et al. 2004; Melvin and Wilson 2013; Nunes 2015). These subtle effects can include neurological and behavioural disorders (Brandão et al. 2013), endocrine disruption (Masteling et al. 2016), and other relevant physiological, histological, cytological, and reproductive alterations (Rocha et al. 2014; Jeong et al. 2015; Rodrigues et al. 2015; Antunes et al. 2016). Other important subtle changes caused by pollutants are transgenerational effects, i.e., the potential negative outcomes on the phenotype, behaviour, and health condition of the progeny resulting from parental exposure to contaminants in critical development stages (Yu et al. 2013).

Transgenerational effects can occur through various mechanisms, such as toxicant-induced developmental changes (in oogenesis and embryogenesis; Baker et al. 2014), contaminant transfer from mothers to offspring (Tsui and Wang 2004), or the integration of multiple sublethal effects (e.g., affecting maternal energy allocation) that result in lower offspring fitness (Rowe et al. 2001). There is strong evidence that transgenerational effects of pollutants can be mediated by epigenetic changes (Vandegehuchte et al. 2010; Baker et al. 2014), which can be transferred to subsequent generations (Youngson and Whitelaw 2008), even if the triggering environmental factor is removed (Vandegehuchte

✉ S. C. Antunes
scantunes@fc.up.pt

[1] Departamento de Biologia, CBMA (Centro de Biologia Molecular e Ambiental), Universidade do Minho, Campus de Gualtar, Braga, Portugal

[2] Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Porto, Portugal

[3] Departamento de Biologia, CESAM (Centro de Estudos do Ambiente e do Mar), Universidade de Aveiro, Aveiro, Portugal

[4] CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental), Universidade do Porto, Porto, Portugal

et al. 2010). Methods and approaches to assess transgenerational effects are still a critical aspect needing improvement, in view of the potentially high ecological significance of "carry-over" effects of pollution onto subsequent generations (Vandegehuchte et al. 2010; Yu et al. 2013; Baker et al. 2014). The fish *Danio rerio* (Baker et al. 2014), the nematode *Caenorhabditis elegans* (Yu et al. 2013), and the crustacean *Daphnia magna* (Vandegehuchte et al. 2010) are well-established model species that have successfully been used in the assessment of transgenerational effects of contaminants.

The standard 21-day chronic *Daphnia* assay was designed to evaluate the reproductive effects of toxicants accompanying multiple reproductive events, but it does not consider transgenerational effects. Early studies with *D. magna* (Sánchez et al. 1999; Hammers-Wirtz and Ratte 2000) already showed the usefulness of incorporating measures of fitness of the daughter generation(s) for the sake of a realistic extrapolation of contaminant effects to the population level. Two recent contributions (Campos et al. 2016; Barata et al. 2017) have proposed and validated a two-generation *Daphnia* reproduction test with toxicants, which allows comparing the sensitivity to toxicants of the parent (F_0) generation to that of their progeny (F_1 generation). This provides a measure of the cumulative effects of a chemical on the offspring born from toxicant-exposed mothers. The few studies that have looked at transgenerational effects in *Daphnia* were focused on substances with a known endocrine disruption profile (Brennan et al. 2006; Chen et al. 2014), including the multigenerational studies from Barata et al. (2017) and Campos et al. (2016). Research going beyond endocrine disruptors is needed.

The existing protocol for the 21-day *Daphnia* reproduction test (ISO 2000; OECD 2012) can be easily adjusted to include an assessment of multigenerational (exposure to contaminants for more than one generation) or transgenerational effects (assessment of progeny fitness after parental exposure to contaminant). However, the increased time and sampling requirements may be problematic in some cases (Guilhermino et al. 1999; Oh and Choi 2012). In part, this problem may be solved with a reduction of the assay duration, by accompanying the initial life stages of *Daphnia* up to the first reproductive events (Guilhermino et al. 1999; Masteling et al. 2016). Indeed, this may be ecologically sounder than following several reproductive events, since it has been estimated that only 10% of individuals in field populations survive to release offspring (first brood) and only 0.1% reach the third brood (Boersma 1997). Also, maternal investment in juvenile fitness seems to be proportionally higher when the mothers are younger (Boersma 1997), and the initial reproductive events contribute the most to population growth (Forbes and Calow 1999; Castro et al. 2007). As such, performing observations up to the first reproductive events in *Daphnia* allows the experimenter to focus on a critical ontogenetic period while saving time that can be used to extend the reproduction assay to perform an assessment of transgenerational effects.

Following this idea, we propose a simple add-on to the standard 21-day chronic *D. magna* assay (ISO 2000; ASTM 2012; OECD 2012). Our goal is to include an assessment of the fitness of the offspring (F_1 generation) born from the first clutch of the parental (F_0) generation, along with the parental exposure to selected contaminants, without exceeding the 21-day period. We feed the alternative vision of assessing toxic effects in the earlier life stages of *Daphnia*, both in the parental and offspring generation. Using this concept, we evaluated the transgenerational effects of four substances widely used in various human activities: an inorganic agrochemical and antifouling (copper sulphate), an industrial oxidizing agent (potassium dichromate), a common pharmaceutical (paracetamol), and a multipurpose biocide (benzalkonium chloride).

Materials and Methods

Toxicants

All toxicants represent substances widely used in various human activities. Copper sulphate is commonly used as herbicide, fungicide, and algaecide (Willis and Bishop 2016), being highly toxic to various aquatic organisms (Kegley et al. 2016). Potassium dichromate is an oxidizing agent used in many chemical and industrial applications (IETEG 2005); this form of chromium (hexavalent) is highly toxic to many forms of aquatic life (Kegley et al. 2016). Paracetamol is a human-use drug classified as a priority pharmaceutical in the aquatic environment (De Voogt et al. 2009), and recent studies have shown it is particularly toxic to crustaceans (Nunes et al. 2014). Benzalkonium chloride is a quaternary ammonium compound widely used as disinfectant and biocide in many industrial and pharmaceutical applications, including disease prevention in aquaculture (Antunes et al. 2016). Although considered overall safe, this synthetic surfactant is toxic to aquatic organisms (Antunes et al. 2016).

Copper sulphate ($CuSO_4 \cdot 5H_2O$), potassium dichromate ($K_2Cr_2O_7$), paracetamol ($C_8H_9NO_2$), and benzalkonium chloride ($[C_6H_5CH_2N(CH_3)_2R]Cl$) were purchased from Sigma Aldrich (Germany) as pure substances. Stock solutions for all toxicants were freshly prepared by dissolving the proper amount of each reagent-grade chemical in synthetic culture medium.

Test Organism

The organism chosen to perform this study was *D. magna*, an aquatic microcrustacean, considered a good indicator for its sensitivity to toxicants. It is widely used as an ecotoxicological model (OECD 2004, 2012) because of its ecological features, which include its key role and intermediate position in lake and pond food webs (Benzie 2005), but also due to logistical advantages, namely its reproductive strategy (asexual parthenogenesis), short generation times, and high fecundity. Environmental influences can be minimized by providing constant culture conditions (Loureiro et al. 2011, 2012), allowing full control to the experimenter. For the purpose of this study, clone A was used (as in Loureiro et al. 2011; Nunes et al. 2014).

Group cultures, constituted by 25–30 asexual females with the same age, were maintained in 1 L glass jars with 800 mL of hard reconstituted water (USEPA 2002; Loureiro et al. 2011), supplemented with a standard organic extract (Loureiro et al. 2011; OECD 2012). Jars were kept in a growth chamber that allowed controlled conditions of light intensity (10–20 µmol m^{-2} s^{-1}), photoperiod ($16h^{L}$:$8h^{D}$), and temperature (20 ± 2 °C). Animals were fed with a microalgae (*Raphidocelis subcapitata*) suspension of 3.0×10^5 cells mL^{-1} every other day. Maintenance of *D. magna* involved daily checks for the presence of newborns (produced asexually), frequent renewal of the culture medium (usually three times per week), and cyclical renewal of the culture (by discarding older mothers and setting up new cultures with newborns). For further details on rearing procedures of *D. magna* and *R. subcapitata* cultures, see Antunes et al. (2004) and Loureiro et al. (2011, 2012). Experiments were performed with individuals less than 24-h old (neonates), born between the third and fifth brood (for homogenization and standardization purposes).

Acute Assays

Acute toxicity assays were conducted according to standard guidelines (OECD 2004), consisting of the exposure of 20 neonates, arranged in four groups of five individuals to each toxicant concentration for 48 h. Toxicant concentrations were chosen according to literature data (Bossuyt et al. 2004; Kreuzinger et al. 2007; Coors et al. 2009; Loureiro et al. 2011; Nunes et al. 2014) and preliminary range-finding tests. The definitive nominal concentrations (geometric series) were: 1.96–6.00 mg L^{-1} (dilution factor 1.15×) for paracetamol; 0.336–2.00 mg L^{-1} (dilution factor 1.25×) for potassium dichromate; 0.061–0.150 mg L^{-1} (dilution factor 1.145×) for copper sulphate; and 0.012–0.242 mg L^{-1} (dilution factor 1.12×) for benzalkonium chloride. A negative control (no toxicant) also was included in the experimental design. Daphnids were not fed and the medium was not renewed during the 48 h; test vessels (100-mL glass beakers) were kept under the controlled conditions described previously. At the end of the assays, immobilized individuals were counted. The purpose of these experiments was to obtain a measure of their relative acute toxicity to help setting up concentration levels for the life history experiments (see "Reproduction Assays" and "Extended Generational Assays").

Reproduction Assays

Reproduction assays with *D. magna* were conducted following general recommendations (OECD 2012) and lasted 21 days. Experiments were initiated with neonates, obtained from a healthy stock, and test vessels (50-mL glass beakers) were kept under the controlled conditions previously described. The experiment consisted of ten individualized organisms exposed to each toxicant concentration. Test nominal concentrations (geometric series) were defined according to the relative acute toxicity of the compounds, simulating subacute exposure levels: 1.3–4.0 mg L^{-1} (dilution factor 1.25×) for paracetamol; 0.05–0.2 mg L^{-1} (dilution factor 1.3×) for potassium dichromate; 0.037–0.197 mg L^{-1} (dilution factor 1.4×) for copper sulphate; and 0.009–0.040 mg L^{-1} (dilution factor 1.3×) for benzalkonium chloride. Daphnids were transferred to newly prepared toxicant dilutions every other day and daily fed with their respective *R. subcapitata* ration (3.0×10^5 cells mL^{-1}). During the assay period, daphnids were monitored daily for mortality and reproductive state; when neonates were present, they were counted and discarded using a plastic pipette. The following parameters determined: age at first reproduction, reproductive output (cumulative number of offspring produced until day 21), and the per capita intrinsic rate of population increase (a measure of population growth potential). Reproductive output considers the contribution of all test organisms, whether they survived until the end of the assay or not. As such, it is a more relevant endpoint than fecundity (number of offspring per surviving female), because it considers the combined effects of stressors on both survivorship and fecundity (OECD 2012; Cuco et al. 2016). For comparative purposes, reproductive output also was calculated at day 12 (see "Extended Generational Assays").

The per capita rate of population increase was iterated from the Euler–Lotka equation, using the survival and fecundity estimates:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where r is the intrinsic rate of increase (in day^{-1}), x is the age class in days ($0 \ldots n$), l_x is the probability of surviving

to age x, and m_x, is the fecundity at age x. Because data from all individuals of each experimental treatment are needed for the calculation, individual pseudo-values for r were generated by jack-knife re-sampling (Meyer et al. 1986).

Extended Generational Assays

To assess the potential transgenerational effects of the tested toxicants, we monitored the life history of one random neonate from the first brood of each test organism from the standard reproduction assay. These organisms (F_1 generation) were individually transferred to test vessels (50-mL glass beakers) with clean reconstituted water (no toxicant) and were monitored daily for mortality and reproductive state. The objective of this assay was to address whether toxicant exposure of parental organisms (F_0 generation) could translate into changes in the fitness of the subsequent generation, via maternal or developmental effects. Apart from the fact that no toxicants were added, the experiment was performed using the same procedures and experimental design as the reproduction assay of the F_0 generation (see "Reproduction assays"). However, this experiment was halted when 95% of all organisms released their first brood (at day 12).

By shortening the duration of the experiment with the F_1 generation (to 12 days), we were able to conduct the transgenerational assay along with the F_0 generation (21-day chronic test). Although a longer assay could allow collection of further data from the F_1 generation, the additional effort would require extending the assay much beyond the 21-day period. Furthermore, we claim that it is much more informative and ecologically relevant to invest time collecting data from earlier phases (juvenile, adolescent, and young adult stages), because (1) although representing only a fraction of *D. magna* lifespan, the first 1–2 weeks of its autonomous life correspond to a very sensitive and demanding ontogenetic period, with strong selective pressures on juvenile growth, ovary development and maturity, and onset of adult life (Masteling et al. 2016); (2) the first reproductive events (which typically start around day 9–10 at 20 °C; Boersma 1997) contribute the most to population growth, and changes in the first reproductive event will affect profoundly the rate of increase (Forbes and Calow 1999); (3) survival of natural *Daphnia* populations is low (due to predation, disease, etc.) and only a small fraction (~ 10%) of individuals will reach adulthood (Boersma 1997).

The following parameters were quantified at day 12 for the F_1 generation: age at first reproduction, reproductive output, and the per capita intrinsic rate of population increase. These endpoints are directly comparable to those quantified in the F_0 generation. In the few cases where F_1 daphnids did not reproduce within the 12-day period, we artificially set age at first reproduction to 14 days (to avoid censored observations).

Statistical Analysis

All analyses were performed with R software, version 3.3.1 (R Core Team 2016). Plots and figures were built in R using the packages *ggplot2* (Wickham 2009) and *cowplot* (Wilke 2016).

Acute EC_{50} values, and respective confidence intervals (using delta method), were determined by fitting a nonlinear concentration–response toxicity model to the *D. magna* survival (immobilisation) data using the *drc* package (Ritz and Streibig 2005) for R software. Immobilisation was modelled as a binomial variable using a two-parameter log-logistic model, where the asymptotes of the curve are fixed to be 0 (no organisms are immobilised) and 1 (all organisms are immobilised), following the rationale of Ritz (2010).

Generalized linear models (GLMs) were used to investigate the effect of the toxicants (using concentration as categorical predictor) on *D. magna* life history parameters using the *glm* function available in R software. Age at first reproduction and reproductive output were analysed as count (Poisson) data with a log link function, whilst the rate of increase was modelled as normally (Gaussian) distributed data (linear model). In the case of count data, we specified a quasi-Poisson error distribution for improved fit (i.e., the dispersion parameter was estimated from the data), as recommended by Szöcs and Schäfer (2015). For inference about the effect of concentration, we used F tests for both linear and quasi-Poisson models, following Szöcs and Schäfer (2015) and references therein. When the F test was significant ($P \leq 0.05$), we used Dunnett contrasts with one-sided Wald t tests (following Szöcs and Schäfer 2015) to identify which concentrations significantly differed from the control group (no toxicant). These post hoc contrasts were carried out using the *multcomp* package (Hothorn et al. 2008) available for R software, specifying Holm's adjustment to correct for multiple testing.

Results

All toxicants were acutely toxic to *D. magna* and their relative toxicity was as follows: benzalkonium chloride (most toxic) > copper sulphate >> potassium dichromate >>> paracetamol (less toxic) (Fig. 1). The acute EC_{50} values (Table 1) allowed setting up the concentrations for the reproduction assays, with the highest concentration tested in the chronic assays approximating the respective acute EC_{50}, with the necessary adjustments.

In the reproduction assays, three of four toxicants caused significant impairment in the life history parameters of *D.*

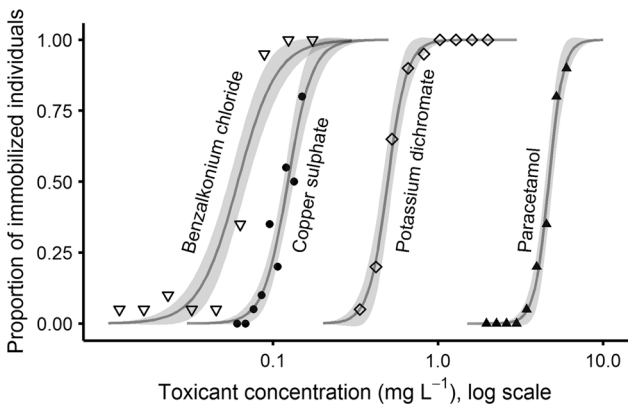

Fig. 1 Acute toxicity data (immobilization of *Daphnia magna*) for the tested toxicants. Different symbols represent observations for each toxicant, and regression lines (log-logistic two-parameter model) are shown along with respective confidence regions

magna. The exception was benzalkonium chloride, which did not cause reproductive impairment at the tested concentrations (Fig. 2), despite its high acute toxicity.

Copper sulphate caused some mortality within test organisms, which lead us to exclude the highest concentration due to substantial mortality (> 80%). This biocide also caused a significant decrease in reproductive output from 0.037 mg L^{-1} upwards (Fig. 2), which was observed irrespectively of the time frame considered: day 12 or day 21. A significant delay in reproduction was also observed in lowest concentrations (Fig. 2).

Paracetamol caused a significant delay and decrease in reproduction from 2 mg L^{-1} upwards, which translated into a significant decrease in population growth (Fig. 2). Curiously, and despite the decreasing trend (Fig. 2), no significant effects could be demonstrated for reproductive output at day 21 (contrary to the same endpoint at day 12).

Potassium dichromate caused a reduction in reproductive output, which was more pronounced when analyzing data for the full duration of the assay, i.e., 21 days (Fig. 2). This reproductive impairment resulted in a significant decrease in population growth from 0.050 mg L^{-1} upwards (Fig. 2).

The extended generational assays (F_1 generation) were successfully conducted while the respective reproduction assays (F_0 generation) were ongoing. In average, females from both the F_0 and F_1 generations released their first young at day 9–10, meaning that the transgenerational assessment of the effects of the toxicants could be completed in 18–20 days. In a worst-case scenario, a maximum of 22–27 days were needed to complete the observation of all experimental units. This maximum only occurred in a few experimental units, where daphnids from both parental

Table 1 Acute toxicity data (mg L^{-1}) of the tested compounds to *Daphnia magna*, from this study (first column to the left) and from available literature (columns to the right)

	EC_{50} (mg L^{-1})	Culture medium and reference
Benzalkonium chloride EC_{50} = 0.0611 (0.0529–0.0692)	0.041	Austrian natural waters (Kreuzinger et al. 2007)
	0.13–0.22	M4–M7 medium (García et al. 2001)
	0.0059	EPA medium (Kaj et al. 2014)
	0.038	Moderately hard water (Lavorgna et al. 2016)
Copper sulphate EC_{50} = 0.123 (0.114–0.132)	0.0826	ASTM medium (Guilhermino et al. 2000)
	0.075	Belgium natural water (Bossuyt et al. 2005)
	0.102	Netherlands natural water (Bossuyt et al. 2005)
	0.134	Netherlands natural water (Bossuyt et al. 2005)
	0.0598	ASTM medium (Loureiro et al. 2011)
	0.0369	ADaM medium (Loureiro et al. 2011)
	0.231	M7 medium (Loureiro et al. 2011)
Paracetamol EC_{50} = 4.68 (4.43–4.93)	9.20	Moderately hard water (Kühn et al. 1989)
	50.0	M4–M7 medium (Henschel et al. 1997)
	30.1	Moderately hard water (Kim et al. 2007)
	4.70	ASTM medium (Nunes et al. 2014)
	2.83	ASTM medium (Oliveira et al. 2015)
Potassium dichromate EC_{50} = 0.494 (0.457–0.532)	1.70	Hard reconstituted freshwater (Berglind 1984)
	0.55–1.16	Belgium natural waters (de Coors et al. 2009)
	0.639	ASTM medium (Loureiro et al. 2011)
	0.792	ADaM medium (Loureiro et al. 2011)
	1.06	M7 medium (Loureiro et al. 2011)
	0.64	M4 medium (Gopi et al. 2012)

Data are shown as 48-h EC_{50}; data from this study are shown with respective 95% confidence interval, extracted from non-linear modelling of immobilisation data

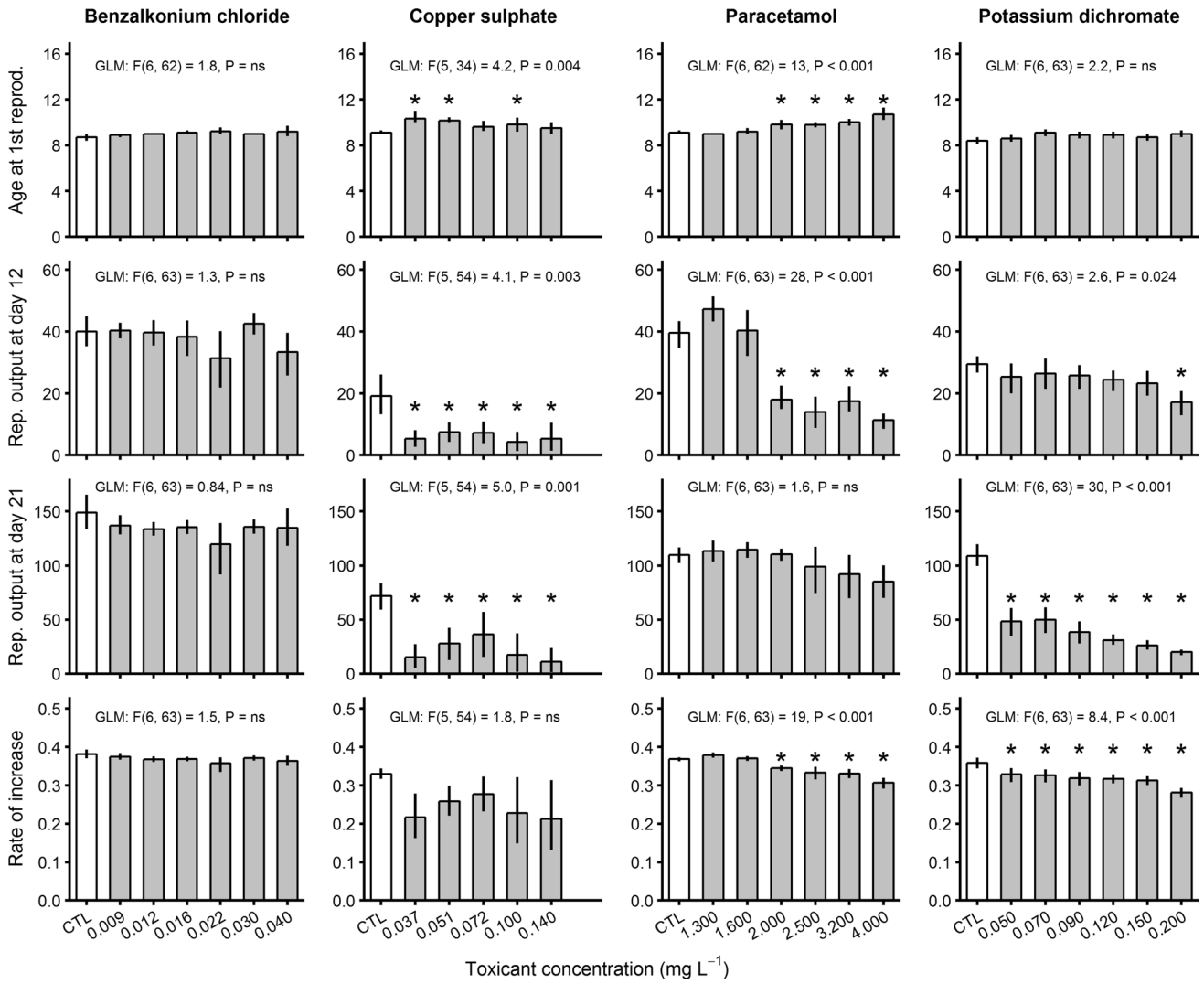

Fig. 2 Chronic toxicity data for the tested toxicants in the parental (F_0) generation: age at first reproduction (day), reproductive output at day 12 (no. offspring), reproductive output at day 21 (no. offspring), and rate of increase (day^{-1}) in *Daphnia magna*. Bars represent mean value for each endpoint and error bars stand for bootstrapped confidence intervals. For each endpoint, the significance of the effect of the toxicant concentration is shown (GLM, generalized linear models), and asterisks are used to highlight statistically significant differences (Dunnett contrasts) relatively to the control (white bar); ns stands for non-significant ($P > 0.05$)

and daughter generations suffered a delay due to the toxicant (e.g., paracetamol—see below). When the F_1 assay was terminated, at day 12, mothers from the F_1 generation had generally experienced 1 or 2 reproductive events.

Transgenerational or carry-over effects were not detected for benzalkonium chloride or potassium dichromate at the tested concentrations (Fig. 3). Slight effects were detected after exposure to copper sulphate, as the daughter generation (F_1) revealed a significant delay in age at first reproduction (Fig. 3) at low concentrations (0.037–0.072 mg L^{-1}). However, paracetamol was the most obvious example of transgenerational effects, given that maternal exposure to this pharmaceutical caused delayed reproduction and lower population growth potential of the daughter generation (unexposed to the toxicant after birth) at the highest concentration (Fig. 3). Despite the decreasing trend also observed in terms of the reproductive output of daphnids previously exposed to paracetamol, no differences were observed relatively to the control treatment (Fig. 3).

Discussion

The evaluation of transgenerational effects brings important added value to our predictive capacity on the effects of chemical substances that ubiquitously occur at low concentrations. We propose an experimental design for the regular evaluation of transgenerational toxicity, using the

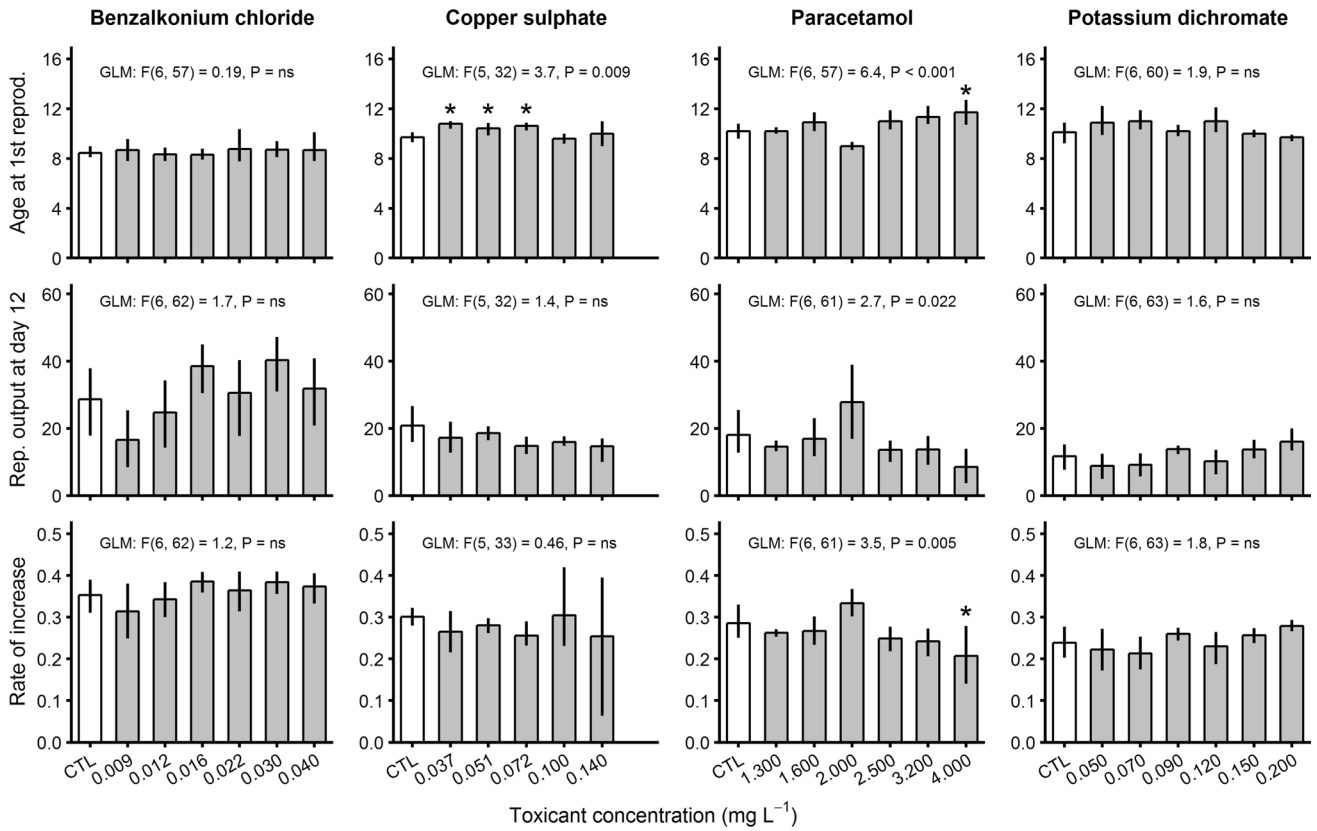

Fig. 3 Transgenerational chronic toxicity data for the tested toxicants in the daughter (F_1) generation: age at first reproduction (day), reproductive output at day 12 (no. offspring), rate of increase (day^{-1}) in *Daphnia magna*. Bars represent mean value for each endpoint and error bars stand for bootstrapped confidence intervals. For each endpoint, the significance of the effect of the toxicant concentration is shown (GLM, generalized linear models), and asterisks are used to highlight statistically significant differences (Dunnett contrasts) relatively to the control (white bar); ns stands for nonsignificant ($P > 0.05$)

crustacean *Daphnia*. This approach allowed assessing the transgenerational effects of common anthropogenic substances, complementing the available literature on this matter (Brennan et al. 2006; Chen et al. 2014), which is biased towards endocrine disruptors. Instead of a cumulative multigenerational approach (Barata et al. 2017; Campos et al. 2016), our focus was on transgenerational effects sensu stricto. Thus, we conducted an assessment of the life history of offspring from toxicant-exposed mothers, providing a direct measure of offspring quality or overall fitness in the absence of toxicant. As such, any changes observed in the life history of the progeny can only be explained by maternal or developmental effects of the in utero exposure to the toxicant. On the contrary, the two-generational *Daphnia* reproduction test (Campos et al. 2016; Barata et al. 2017) looks at the cumulative effect of a toxicant, and assesses the sensitivity of the daughter (F_1) generation to the same compound the parent (F_0) generation was exposed to. Of course, both approaches are complementary and can be expanded to allow further elucidation of the transgenerational mechanisms.

We set up an extended generational assay based on the premise that an early life-stage test (up to the first reproductive events) allows discriminating potential negative effects of parental contaminant exposure in the subsequent generation. By using a shorter duration (12 days), we were able to successfully conduct the transgenerational assay along with the F_0 generation (21-day chronic test), without requiring additional time to perform this assessment (with a few exceptions). An experienced technician or researcher should have no problem in conducting both tests simultaneously, although constant attention is needed when initiating the extended assay (F_1 generation). Most likely, the assay will not start in the same day for all F_1 organisms, and if the toxicant significantly delays reproduction in F_0 daphnids, a few days may mediate between the onset of the assays for the control treatment and the highest concentrations of toxicant (as in the case of paracetamol). The reduced duration of the assay represents an important compromise, because many authors have expressed concern about the duration of the chronic test (Guilhermino et al. 1999; Oh and Choi 2012) because of associated sampling and time × cost

requirements. Data from the F_0 generation show that estimates of reproductive output in *D. magna* at day 21 were not necessarily more informative of the effect of toxicants than at day 12 (with the exception of potassium dichromate). Thus, a shorter duration of the assay does not seem to compromise our ability to predict the toxicity of chemicals (supporting the findings of Guilhermino et al. 1999), whilst allowing substantial savings in time, money and toxic waste. In the perspective of multi- or transgenerational studies, such savings become more important as the number of generations increases; our study with only two generations shows the advantages and feasibility of this option.

A set of "model" pollutants, representative of important human activities, was selected to validate the proposed transgenerational assay; however, our first step was to assess the acute and chronic toxicity of these substances. Benzalkonium chloride and copper sulphate were highly toxic (lethal effects below or close to 100 µg L^{-1}). However, only the latter was causative of reproductive impairment (from 37 µg L^{-1} upwards). Studies on the effects of benzalkonium chloride (BAC) in crustaceans have mostly focused on mortality (Kegley et al. 2016), although Lavorgna et al. (2016) showed its detrimental effects on reproduction (in contradiction to our study) and DNA integrity at very low concentrations. Quaternary ammonium compounds (such as BAC) seem to be extremely toxic to crustaceans (Ivanković and Hrenović 2010), and particularly to *D. magna* (Lavorgna et al. 2016). Unlike copper and BAC, paracetamol was the least toxic to *Daphnia* (lethal and reproductive effects at the mg L^{-1} range). However, this substance elicits a scenario of oxidative stress and reproductive impairment, notably in crustaceans (Nunes et al. 2014; Masteling et al. 2016), thus raising curiosity about its potential transgenerational effects. Potassium dichromate was toxic at the µg L^{-1} range, exerting both lethal and reproductive effects. The standard OECD (2004) protocol defines this substance as a reference toxicant in *Daphnia* assays, stating that the acute EC_{50} at 24 h should be comprised between 0.60 and 2.1 mg L^{-1}; in our study, the 24-h EC_{50} was 0.81 mg L^{-1} (data not shown), which validates the sensitivity of our stock of test organisms. As such, we can confidently compare our toxicity data with reports from the literature, which show an overall concordance for the tested chemicals in terms of acute toxicity (Table 1). Because three of the tested substances caused reproductive impairment, the next step was to assess whether parental (F_0) exposure to the toxicants could affect offspring (F_1) fitness.

Of the four tested substances, two revealed some sort of transgenerational effect, which shows the sensitivity and usefulness of this type of assay. Indeed, parental exposure to copper sulphate and paracetamol caused slight impacts in the F_1 generation. As such, exposure to these toxicants has the potential to cause mild carry-over effects in offspring, even if the offspring are not themselves exposed to the toxicant (except as eggs and embryo). Copper (a metal) and paracetamol (a *p*-aminophenol derivative) have distinct chemical affinities and modes of action, yet they somehow impaired the fitness of the daughter generation. Both substances cause oxidative stress in *D. magna* (albeit through different pathways), even in short-term exposures (Barata et al. 2005; Watanabe et al. 2007; Oliveira et al. 2015; Masteling et al. 2016). Thus, it is possible that oxidative stress may have caused these transgenerational effects by: (1) affecting maternal energy expenditure, which had to be allocated to defence from oxidant insult, resulting in lower investment in reproduction and low offspring quality (Rowe et al. 2001; Smolders et al. 2005); (2) causing effects during egg and embryo development that build up to effects in adulthood (Newman et al. 2015); (3) both mechanisms. However, potassium dichromate also causes oxidative stress (Jemec et al. 2008), but no transgenerational effects were observed; an alternative explanation is needed. The observed transgenerational effects may simply reflect cumulative damage to mothers and offspring, not necessarily caused by an oxidative stress scenario. To bring light to the mechanistic explanations behind transgenerational effects, such experiments can be coupled with ecotoxicogenomic tools (Poynton et al. 2006; Watanabe et al. 2007).

In the specific case of paracetamol, endocrine disruption cannot be excluded as a source of transgenerational effects, although this has not yet been clarified in crustaceans, such as *Daphnia* (Masteling et al. 2016). In mammals, paracetamol can cause overall reproductive senescence (Johansson et al. 2016) and anti-androgenic effects (Kristensen et al. 2011) that may carry-over to offspring from exposed mothers. This has been confirmed in rats (Kristensen et al. 2011), and there also are evidences from human epidemiology (Kristensen et al. 2011; Snijder et al. 2012). These effects are clearly transgenerational and show how maternal influence can condition the reproductive success of the descendants. Given the phylogenetic distance between mammals and crustaceans, it is difficult to extrapolate without further data; however, paracetamol seems to modulate the reproductive responses and outputs in various organisms.

The effects observed here in the fitness of the F_1 generation were either irregular (copper sulphate) or observed at very high concentrations (paracetamol), but the specialised literature shows variable outcomes depending on the studied toxicant. Studies with diazinon (an organophosphate insecticide) were among the first to document transgenerational effects in *Daphnia*, by demonstrating reduced offspring fitness (Sánchez et al. 1999) and increased sensitivity to diazinon in the F_1 generation (Sánchez et al. 2000). Hammers-Wirtz and Ratte (2000) demonstrated that dispersants caused populational effects that could not be predicted from the F_0 reproduction data, because these toxicants caused effects in the population via depressed

offspring (F_1) fitness (smaller size, lower reproduction, and, in some cases, reduced survival of F_2 offspring). This is the type of transgenerational effects that our assay intends to assess, and does so in the course of the 21 days of the standard reproduction assay. Many studies also have looked at the multigenerational exposure to contaminants (similarly to Sánchez et al. 2000), showing an overall build-up effect of contaminants across generations (Brennan et al. 2006; Chen et al. 2014; Jeong et al. 2015; Campos et al. 2016). Some authors (Campos et al. 2016) explain this increase in the sensitivity of daphnids from daughter generations (relatively to the parental generation) as a consequence of decreased offspring quality. This may be an oversimplified explanation, because other studies have identified complex mechanisms that include maternal transfer of contaminants (Tsui and Wang 2004) or transgenerational inheritance (Vandegehuchte et al. 2010), adding to potential effects during egg and embryo development (Baker et al. 2014). This is an exciting field, and one that should advance both discussing the experimental design to evaluate such effects (our study), as well as the ecotoxicogenomic approaches (Poynton et al. 2006; Watanabe et al. 2007; Vandegehuchte et al. 2009) that may clarify putative mechanistic explanations.

Conclusions

Our study proposes a simple add-on to the standard 21-day chronic *D. magna* assay, allowing the assessment of the fitness of the offspring (F_1 generation) born from the first clutch of toxicant-exposed mothers (F_0 generation). This transgenerational assay is focused on early life-stage effects (up to the first reproductive events), allowing the execution of the extended F_1 generation assay along with the standard reproduction test (F_0). With this design, we evaluated the lethal, reproductive and transgenerational effects of four widespread and extensively used substances: a biocide/antifouling (copper sulphate), an industrial oxidizing agent (potassium dichromate), a pharmaceutical (paracetamol), and a quaternary ammonium compound (benzalkonium chloride). Adverse effects in the fitness of the daughter (F_1) generation were observed in the case of maternal exposure to paracetamol and copper sulphate, although they were not very pronounced. These findings highlight the usefulness of our approach and reinforce the view of the need for a generalised formal assessment of the transgenerational effects of pollutants for the sake of a realistic extrapolation of contaminant effects to the population level. Of course, the evaluation of offspring fitness can be expanded by: (1) incorporating other endpoints in the assessment, such as biochemical markers (e.g., oxidative stress parameters), moulting, and other physiological measures; (2) assessing the response of the offspring to a stressor (toxicant, presence of predators, etc.) and comparing their sensitivity to a negative control or to the parental generation.

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