Published in final edited form as:

Sci Total Environ. 2017 February 01; 579: 1073–1083. doi:10.1016/j.scitotenv.2016.11.066.

Validation of a two-generational reproduction test in *Daphnia magna*: An interlaboratory exercise

Carlos Barata^{a,*}, Bruno Campos^a, Claudia Rivetti^a, Gerald A. LeBlanc^b, Stephanie Eytcheson^b, Stephanie McKnight^b, Marysia Tobor-Kaplon^c, Selinda de Vries Buitenweg^c, Suhyon Choi^d, Jinhee Choi^d, Elena I Sarapultseva^e, Marie-Agnès Coutellec^f, Maïra Coke^g, Pascal Pandard^h, Arnaud Chaumotⁱ, Hervé Quéauⁱ, Nicolas Delormeⁱ, Olivier Geffardⁱ, Fernando Martínez-Jerónimo^j, Haruna Watanabe^k, Norihisa Tatarazako^k, Isabel Lopes^l, João L.T. Pestana^l, Amadeu M.V.M. Soares^l, Cecilia Manuela Pereira^m, and Karel De Schamphelaere^m

^aDepartment of Environmental chemistry, Institute of Environmental Assessment and Water Research (IDAEA, CSIC), Jordi Girona 18, 08017, Barcelona, Spain ^bToxicology Program, Department of Biological Sciences, North Carolina State University, Box 7633, Raleigh NC 27695-7633, 850 Main Campus Drive, Raleigh, NC 27606, USA ^cCharles River Laboratories Den Bosch B.V., Dept. of Discovery and Environmental Sciences, Hambakenwetering 7, 5231, DD's-Hertogenbosch, Netherlands ^dEnvironmental Systems Toxicology Lab., School of Environmental Engineering, University of Seoul, 163, Seoulsiripdae-ro, Dondaemun-gu, Seoul, 02504, Republic of Korea ^eNational Research Nuclear University "MEPhI", Kashirskoe Highway, 31, Moscow 115409, Russian Federation ^fESE, Ecology and Ecosystem Health, INRA, Agrocampus Ouest, 35042 Rennes, France ^gU3E, Unité d'Ecologie et d'Ecotoxicologie Aquatique, INRA, 35042 Rennes, France ^hINERIS, Direction des Risques Chroniques, Unité EXES, Parc technologique ALATA, BP, 2, 60 550 Verneuil en Halatte, France ⁱIRSTEA, UR MALY, Laboratoire d'écotoxicologie, F-69616 Villeurbanne, France ^jInstituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, Lab. de Hidrobiología Experimental, Prol. Carpio esq. Plan de Ayala S/N, Col. Santo Tomas, México, D. F. 11340, Mexico ^kNational Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan ^lDepartment of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal ^mGhent University (UGent), Laboratory of Environmental Toxicology (GhEnToxLab), Coupure Links 653, B9000 Gent, Belgium

Abstract

Effects observed within one generation disregard potential detrimental effects that may appear across generations. Previously we have developed a two generation *Daphnia magna* reproduction test using the OECD TG 211 protocol with a few amendments, including initiating the second generation with third brood neonates produced from first generation individuals. Here we showed the results of an inter-laboratory calibration exercise among 12 partners that aimed to test the robustness and consistency of a two generation *Daphnia magna* reproduction test. Pyperonyl butoxide (PBO) was used as a test compound. Following experiments, PBO residues were

*Corresponding author at: Institute of Environmental Assessment and Water Research, Spanish Research Council (IDAEA, CSIC), Jordi Girona 18, 08034 Barcelona, Spain. cbmqam@cid.csic.es (C. Barata).

determined by TQD-LC/MS/MS. Chemical analysis denoted minor deviations of measured PBO concentrations in freshly prepared and old test solutions and between real and nominal concentrations in all labs. Other test conditions (water, food, *D. magna* clone, type of test vessel) varied across partners as allowed in the OECD test guidelines. Cumulative fecundity and intrinsic population growth rates (r) were used to estimate “No observed effect concentrations” “NOEC using the solvent control as the control treatment. EC₁₀ and EC₅₀ values were obtained regression analyses. Eleven of the twelve labs succeeded in meeting the OECD criteria of producing >60 offspring per female in control treatments during 21 days in each of the two consecutive generations. Analysis of variance partitioning of cumulative fecundity indicated a relatively good performance of most labs with most of the variance accounted for by PBO (56.4%) and PBO by interlaboratory interactions (20.2%), with multigenerational effects within and across PBO concentrations explaining about 6% of the variance. EC₅₀ values for reproduction and population growth rates were on average 16.6 and 20.8% lower among second generation individuals, respectively. In summary these results suggest that the proposed assay is reproducible but cumulative toxicity in the second generation cannot reliably be detected with this assay.

Graphical Abstract

Keywords

Daphnia reproduction; Multigeneration assay; Interlaboratory; OECD 211; Contaminants; Life-history; Offspring quality

1. Introduction

Most eco-toxicological test guidelines only evaluate the effects observed within one generation, thus disregarding those potential detrimental effects that may appear across generations. The toxicity of chemicals may decrease, increase, or remain unchanged across generations. Toxicity can also “emerge” across generations, with modes of action which do not operate in the first generation of exposure (e.g. epigenetics) (Marczylo et al., 2016). However, in many cases pollutants affect both exposed organisms and their progeny, hence adverse effects may become more severe in subsequent generations (Campos et al., 2016). This is the case for some endocrine disruptors that by altering hormonal levels in the mother

can have detrimental effects in their offspring (Colborn et al., 1996). In this regard OECD Work Related to Endocrine Disrupters <http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm> recommends that in vivo assays be used that provide more comprehensive data on adverse effects on endocrine relevant endpoints over extensive parts of the life cycle of the organisms. Reproductive toxicity assays in mammals typically assess detrimental effects on the parental and offspring generations (Janer et al., 2007). Indeed there are standardized test procedures designed to assess multigenerational effects in mammals and fish (Janer et al., 2007; Nakamura et al., 2015), but there are no equivalent tests for invertebrates, despite their short life-cycles, which allow for cost-effective assessments of toxicity over multiple generations (OECD, 2006; Oliveira-Filho et al., 2009a; Oliveira-Filho et al., 2009b; Verslycke et al., 2007).

Among existing ecotoxicological assays, the *Daphnia magna* reproduction test is probably the most employed standardized life cycle test in aquatic toxicology. Indeed the “Daphnia Multi-generation Assay” is already mentioned in Level 5 of the OECD Conceptual Framework for endocrine disrupters. From the available literature on multi-generational effects of chemical contaminants and other stressors (UV radiation and radioactivity) in *D. magna* or related species, it was not always possible to evaluate whether concentration effect levels increased, decreased or remained unchanged across generations as it is established by OECD; (Baldwin et al., 2001; Brennan et al., 2006; Campos et al., 2016; Dalla Bona et al., 2015; Dietrich et al., 2010; Faassen et al., 2015; Hammers-Wirtz and Ratte, 2000; Huebner et al., 2009; Jacobasch et al., 2014; Jeong et al., 2015; Kim et al., 2012; Kim et al., 2014; LeBlanc et al., 2013; Li et al., 2016; Li et al., 2014; Massarin et al., 2010; Muysen and Janssen, 2004; Papchenkova et al., 2009; Parisot et al., 2015; Plaire et al., 2013; Sarapultseva and Dubrova, 2016; Völker et al., 2013; Ward and Robinson, 2005; Yang et al., 2013). About half (13 out of 25) of the above mentioned studies followed OECD TG211 or related protocols, which allowed to compare effect concentration levels across generations and hence demonstrating the potential of this protocol for evaluating toxicity beyond a single generation.

In summary there is experimental evidence indicating that it is possible to study multigenerational effects by just extending the OECD TG 211 protocol (OECD, 2012) to an additional generation, but there is a need to harmonize testing protocols and to assess interlaboratory variability. In a previous study Campos et al. (2016) showed that selection of the clutch size to initiate the second generation is important and that third brood neonates allowed to obtain more consistent results across generations and contaminants. Campos et al. (2016) also reported that piperonyl butoxide (PBO) was one compound showing significant multigenerational effects on reproduction. Effects of this compound were mostly related with embryonic development arrest. PBO is a well-known and widely used insecticide synergist, known to inhibit the activity of the insect cytochrome P450 detoxification system (Hardstone et al., 2015). This compound inhibits steroid hydroxylases in daphnids (Baldwin and LeBlanc, 1994), which are necessary for embryo development (LeBlanc et al., 2000). Furthermore, as a broad inhibitor of cytochrome P450 enzymes, this compound can alter metabolic pathways in exposed embryos. In zebrafish embryos PBO is embryotoxic (Wang et al., 2012). Therefore, exposure of second-generation organisms to PBO during their embryo development may adversely influence their reproductive competence.

This study aimed on assessment of test validity using interlaboratory variability in assessing multigenerational effects on reproductive responses in *D. magna*. More specifically it was studied the consistency in measuring multigenerational cumulative toxic effects of PBO across 12 different labs using a two-generation *D. magna* reproduction protocol developed previously. The experiment was designed in accordance with TG211 requirements. This provided a way to test the robustness of the guideline without testing all possible combinations of parameters that are allowed to vary in this guideline. For example, all laboratories did not use the same clone and culture conditions, because TG211 does not recommend the use of a particular clone or culture conditions, i.e., the test is considered reliable irrespective of the clone or culture conditions used.”

2. Material and methods

2.1. Chemicals

Piperonyl butoxide (PBO, CAS 51-03-6) was purchased from Sigma-Aldrich. All other chemicals were analytical grade and were obtained from Merck.

2.2. Participating laboratories

Up to 12 different laboratories from 10 countries from Europe (6), Asia (3) and America (2) participated in this inter-laboratory calibration exercise (detailed addresses are in authors affiliations). 1. Department of Environmental chemistry, (IDAEA, CSIC), Spain; 2. Toxicology Program, Department of Biological Sciences, North Carolina State University, (NC-USA); 3. Charles River Laboratories Den Bosch, Netherlands (WilRes); 4. School of Environmental Engineering, University of Seoul, Korea (USeul); 5. National Research Nuclear University, Russian Federation (RusFed); 6. ESE, Ecology and Ecosystem Health, INRA & U3E, Unité d'Ecologie et d'Ecotoxicologie Aquatique, INRA, Rennes, France; 7. INERIS, France; 8. IRSTEA, France; 9. Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, México, 10. National Institute for Environmental Studies, Onogawa, Japan; 11. Departamento de Biologia & CESAM, Aveiro, Portugal; 12. Ghent University (UGent), Belgium.

For confidentiality purposes partner number depicted in results do not correspond to the order of participating labs.

2.3. Experimental animals and culture conditions

Following OECD TG 211 guidelines, different clones of *D. magna* were used and animals were maintained individually in artificial reconstituted water (ASTM, M7, M4, COMBO) or in de-chlorinated tap water at 20 °C. The photoperiod regime was 16:8 h or 12:12 h light: dark cycle. Animals were cultured in 50 or 100 ml of media and fed with different algae species using different food rations and food additives. Culture and test media were changed every other day. A brief summary of cultured conditions across labs is depicted in Table 1.

2.4. Experimental design

Experiments were conducted following the *D. magna* reproduction test OECD guidelines (OECD, 2012) with minor modifications (Campos et al., 2016). Treatments included five

concentrations of PBO (50, 100, 200, 400, 800 µg/L) although some labs included an additional lower concentrations (25 µg/L). Selected chemical concentrations allowed to fully define concentration-response curves for cumulative offspring production and to estimate low concentration effects. Stocks of PBO ($\times 20,000$) were prepared by the study coordinator in pure ethanol and sent to each partner in sealed chromatographic vials. All PBO treatments and the solvent control received 0.05 ml/L of ethanol. A control without solvent was also included.

Once first generation females, hereafter referred as parental generation (F0), released the third clutch of offspring, we initiated the second generation (F1). Individuals from the F1 generation were maintained as those of the parental one for 21 days, following also the OECD guidelines (OECD, 2012). Measured life-history traits were: juvenile and adult survival; age at first reproduction; clutch size and intrinsic population growth rates (r), computed from the age specific survival and reproduction rates according to the Lotka equation (Barata et al., 2002b). Most partners provided detailed information of number of dead and aborted embryos/neonates releases in each brood. These data was used to estimate the percentage of non-viable offspring.

2.5. Chemical analyses

pH, oxygen levels and temperature were measured on freshly and old (24 or 48 h) test solutions using each partner's electrode devices. Upon termination of the test, each partner sent test samples to the coordinator for analysis of PBO levels. Chemical analyses of PBO were limited to the freshly and old test solutions of 50, 100, 200 and 400 µg/L of PBO. Samples of 25 µg/L solution were not analyzed because this concentration was not studied by all laboratories. Actual concentrations of PBO were measured by Ultra Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS) following previous methods (Campos et al., 2016; Mayer-Helm et al., 2008). In short, PBO was measured using an Acquity Ultra Performance LC system (Waters, Mildford, MA, USA) connected to a Triple Quadrupole Detector Acquity, using a Luna C18 (150 mm $\times$ 2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A, then increased to 40% A in 4 min, 60% A in 7 min, reaching 100% A in 11 min and then return to initial conditions within 4 min. The system was operated at room temperature, the flow rate was set at 200 µL/min and 10 µL were injected. Acquisition was performed in SRM mode under positive electrospray ionization (ESI+) using two transitions from $[M + H]^+$ precursor ion to daughter ions. The transitions used as well as the cone voltages and collision energies were in accordance with Mayer-Helm et al. (2008). Quantification was based on external calibration standard 8 point curve ($r^2 > 0.98$, range between 10 and 1000 µg/L). Limits of detection and quantification defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, were determined from the spiked water samples. The data were acquired and processed using the MassLynx v4.1 software package.

2.6. Data analyses

Within (E) and between lab (L) variation in cumulative offspring production and population growth rates across PBO treatments (PBO) and generations (G) were assessed by determining the components of variance of a three way ANOVA, which was conducted using General Linear Models and IV sum of squares, which account for unbalanced designs (since PBO treatments varied across partners and generations). Not all labs used the same clones, which may have affected interlab sensitivity variation across PBO concentrations (V_{LXPBO}). Thus to account for the potential influence of clone, two distinct models were used, including either laboratory or clone as factor crossed with chemical treatment and generation. Indeed, laboratory and clone could not be considered in the same model, because they were not totally independent (some labs used the same clone and some others did not). The two models for variance partitioning were:

1. $VT = V_L + V_{PBO} + V_G + V_{LXPBO} + V_{LxG} + V_{PBOxG} + V_{LXPBOxG} + V_E$,
2. $VT = V_c + V_{PBO} + V_G + V_{CXPBO} + V_{CxG} + V_{PBOxG} + V_{CXPBOxG} + V_E$,

in which “x” terms in sub-indices indicate two and three factor interaction terms for L or C, PBO and G.

One-way ANOVA and Student's *t*-test analyses were performed to compare exposure treatments and controls. LOECs (Lowest observed effect concentrations) and NOECs (No observed effect concentrations) were determined using one side Dunnett's post hoc tests or the equivalent test for non-parametric analyse (Zar, 1996), and using the solvent control as the reference treatment. Regression analyses were used to determine EC10 (10% effect concentration) and median effect concentrations (EC50) of the tested chemical. Regression analyses were limited to cumulative fecundity and population growth rate and determined from fitting responses to the allosteric decay regression model following previous procedures (Barata et al., 2000b). Statistical comparison of population growth rate was based on a jack-knife procedure (Barata et al., 2001). Prior to ANOVAs, assumptions of normality and variance homoscedasticity were assessed and data was log transformed when required. Percentage survival and age at first reproduction were analyzed by non-parametric Kruskal-Wallis and Wilcoxon tests (Zar, 1996). ANOVA and non-linear regression analyses were conducted using IBM SPSS v26 and Sigma plot v13.0, respectively.

3. Results

3.1. Water physical-chemical parameters and PBO concentrations

Measured water quality parameters during the test (pH, oxygen levels and temperature) of participating labs were quite similar and met OECD 211 quality criteria. pH and Oxygen levels (Mean $\pm$ SD) were 7.9 ± 0.5 , 8.6 ± 0.3 mg/L, respectively, and temperature was kept at 20 ± 0.5 °C.

Measured concentrations of PBO in freshly prepared (Tf) and old test solutions (Told) varied little across labs and generations, thus samples were considered together in Table 2. Freshly prepared solutions were from 2 to 20% lower than nominal concentrations. Stability of PBO varied little during the tests and across labs, since measured levels in old test solutions (Told

in Table 2) were from 7 to 20% lower than those of freshly prepared ones (Tf in Table 2). For the sake of clarity, hereafter, our results are referred to nominal concentrations.

3.2. Effects over two generations

A total of 14 different trials were performed since partners 2 and 5 each conducted the two-generation assay twice. In all but one of the assays, non-solvent control treatments were also included. In 13 out of 14 trials, cumulative fecundity in control treatments over 21 days were 60, thus OECD validity of test criteria was met in most trials (Table 3).

The coefficient of variation was <25% in 91% of the control treatments (control and solvent controls) (Table 3). Results of a three way ANOVA indicated that cumulative fecundity varied significantly ($P < 0.05$) between controls and solvent controls ($F_{1,390} = 11.4$), among labs ($F_{11,390} = 115.9$), and between generations ($F_{1,390} = 105.8$). In general cumulative fecundity of controls (Mean $\pm$ SD, 105.8 ± 47.5) was higher than those of solvent controls (100.3 ± 33.6) and decreased from first (110.8 ± 41.7) to the second generation (94.6 ± 39.4). Significant interaction terms involving lab, however, denoted heterogeneity in the performance of controls between generations. Indeed significant differences ($P < 0.05$) were found between control and solvent controls in 2 out of 13 trials in the first generation and in 10 out of 13 trials in the second generation (bold values in Table 3). Nevertheless, only 4 of these differences exceeded 25% (C/CS or CS/C * 100, bold and underlined values, in Table 3), which is the recommended maxima for the OECD TG 211 guideline.

Individual replicated values for cumulative offspring production and population growth rate across labs, PBO concentrations, and generations are depicted in Figs. 1 and 2, respectively, together with fitted regression lines obtained for each lab. The range of variation of individual values for cumulative fecundity was quite large exceeding 3 to 4 fold in most PBO treatments (Fig. 1). The same trend was observed for population growth rate responses, despite that r values were less variable (Fig. 2). Depicted individual regression lines indicated that an important part of this variability was related to inter-lab variation within and across PBO treatments, whereas variation between generations within labs was low. Variance partitioning of cumulative fecundity and population growth rate responses across PBO treatments, labs or clones and generations are depicted in Fig. 3. For both responses all main factors and their interactions were significant ($P < 0.05$) and PBO treatment alone (PBO) and across labs ($PBO \times L$) accounted for most variation (76 and 68% for fecundity and population growth rate, respectively). For cumulative fecundity, generational effects within and across PBO treatments accounted for 6.3% of variability (Fig. 3A), whereas for population growth rate such effects only accounted for 2.6% of variation (Fig. 3B). When clone instead of lab was considered, variance partitioning across most factors remained similar except for the contribution of the environment by clone interaction ($PBO \times L$), which decreased by half (from 20 to 10%) (Fig. 3A1).

Reported effect levels for cumulative fecundity and population growth rate are shown in Tables 4 and 5, respectively. Significant non-linear regression curves ($P < 0.05$) were obtained in 27 and 24 out of 28 cases for cumulative fecundity and population growth rate, respectively. Regression models explained >60% of the total variance ($r^2 > 0.6$) and in most cases allowed to get feasible EC10 and EC50 estimates. The lower success obtained for

population growth rate (24 out of 28 cases) was related to the fact that this parameter is less variable than cumulative fecundity and hence it was not always possible to fit regression curves. For cumulative fecundity, second generation individuals (F1) had greater sensitivity to PBO than first generation ones in only 46, 54 and 28% of the cases for effect levels EC10, EC50 and NOEC, respectively (ratios >1 in Table 4). However, effect concentration endpoints of F0 (EC10, EC50 and NOEC) measured across the 14 trials were not significantly ($P < 0.05$) different than those of F1 (based on paired sample Student's t -tests). For population growth rate and EC50, F1 individuals were significantly more sensitive than F0 ones in 70% of the cases (paired sample Student's t -tests, $t_{10} = 2.34$, $P < 0.05$; Table 5).

Besides fecundity, the life-history traits that most affect population growth rate, namely juvenile and adult survival, age at first reproduction and embryo survival, showed large variation (error bars in Fig. 4). These variations, however, were consistent across PBO treatments and generations showing the greatest effects on the percentage of viable embryos, which was significantly affected at 200 $\mu\text{g/L}$ (based on nonparametric ANOVA tests), followed by age at first reproduction and adult and juvenile survival, which were affected, respectively, at 400, 400 and 800 $\mu\text{g/L}$ of PBO.

4. Discussion

The aim of this inter-laboratory calibration exercise was to test for feasibility and consistency for evidence of adverse effects in the second generation individuals using a 21 days reproduction test protocol similar to the OECD 211. This exercise was successfully performed by 12 different labs across two generations using the pesticide synergist PBO, which was selected as the compound having greatest effects in F1 from a previous study (Campos et al., 2016). The reproduction test performed by these labs met OECD quality criteria in most cases in terms of having acceptable levels of water quality (pH, oxygen levels), reproduction (production of >60 neonates per female in 21 days in control treatments) and for intra-lab variability (coefficient of variation for reproductive responses in controls not exceeding 25%). The stability of the tested chemical was also within acceptable values (20% of nominal concentration), which facilitated interlab and multigenerational comparisons. Differences in reproductive performances between control and solvent controls, however, were quite frequent in second generation trials relative to first generation ones. Differences in cumulative fecundity between solvent and nonsolvent controls, however, rarely exceeded 20%. Interestingly cumulative fecundity in solvent controls was higher than that of controls in 9 out of the 12 significant trials, which indicates that other factors than cumulative toxicity of solvent accounted for such differences (Hallare et al., 2006; Hutchinson et al., 2006). To minimize false positives due to carrier effects, thus, effect concentrations were always estimated using the solvent control treatment.

The analysis of sources of variance in cumulative fecundity, which is the target endpoint for the OECD 211 *D. magna* assay, showed that treatment effects alone and across labs accounted for most variation, which indicates that PBO was the factor affecting most reproduction responses followed by the variability of response to PBO between labs. A large variation observed in the response of participating labs across PBO concentrations (about 20%) has also been reported for other intercalibration exercises. According to Baird et al.

(Baird et al., 1989; Baird et al., 1991) in clonal organisms such as *D. magna* the use of different clones may inflate treatment $\times$ interlab variability (PBO $\times$ L) since different clones may have different sensitivities. Indeed several studies indicated that clonal by environmental variation is one of the factors affecting most *D. magna* performances across chemical pollutants (Barata et al., 2000a; Barata et al., 2002a). In our study, several labs used the same clone, thus it was possible to recalculate variance components using clone instead of participating lab. Obtained results showed a 50% reduction of the variation accounted for by participating labs across PBO concentrations (i.e., 10% variation), which is in line with previous studies (Barata et al., 2000a; Barata et al., 2002a). Thus, taking together the previous percentage and the variation accounted for by labs alone (i.e. 3% variation), makes about 13% of variation across labs explained by other factors than clone. The use of different culture conditions across participating labs are likely to explain observed differences in sensitivities and reproductive outputs. In line with the previous argument, several studies have reported that the use of different algal diets, food ratios, water types and culture volumes may affect reproduction in *Daphnia* and its life cycle performance across pollutants (Barry et al., 1995; Ginjupalli et al., 2015; Hansen et al., 2008; Heugens et al., 2006; Martínez-Jerónimo et al., 2000; Pavlaki et al., 2014; Samel et al., 1999). About 8% of the variation was not explained by any of the studied factors and thus it was associated to intra-lab variability. Having an intra-lab variability $<10\%$ is acceptable in environmental toxicology (OECD, 1997).

Population growth rate responses, which allow integration of other important life history traits such as juvenile and adult survival and the timing of reproduction (Barata et al., 2000a; Barata et al., 2002a) behaved similarly to cumulative fecundity responses. Indeed we found that PBO affected mostly embryo viability and hence cumulative fecundity (which in this study refers to alive offspring production) rather than juvenile or adult survival and age at first reproduction. Piperonyl butoxide (PBO) is a cytochrome P450 (CYP) inhibitor (Hardstone et al., 2015) that inhibits steroid hydroxylases in *Daphnia* (Baldwin and LeBlanc, 1994), and hence is likely to alter the biosynthesis and metabolism of ecdysteroids, which are crucial for embryo development (LeBlanc et al., 2000). This is in line with a previous study that found that effects of PBO on *D. magna* population growth rate was mostly related to PBO effects on embryo survival and hence on cumulative fecundity. Accordingly, variance partitioning across the studied sources were equivalent to those reported for cumulative fecundity.

Multigenerational effects within and across PBO treatments were significant and accounted for about 6% and 2.6% of total variation of cumulative fecundity and population growth rate, respectively. Note that 8.5 and 11.2% of total variance (for cumulative fecundity and population growth rate, respectively) was accounted for by the three interaction terms that involved multigenerational effects across PBO treatments and labs. The occurrence of greater effects of PBO in second generation individuals was heterogeneous and varied across effect levels, endpoints and labs. In some cases toxic effects aggravated in F1 individuals, whereas in others were unchanged or decreased. The greatest differences between F0 and F1 were observed when comparing EC50, probably due to the observed intra-lab variation, which was close to 10%, thus making median effect concentration estimates more accurate and reliable than low effect levels (EC10 or NOEC). In 54 and 70% of cases, second

generation individuals (F1) were more sensitive (had lower EC50) than first generation ones for cumulative fecundity and population growth rate responses, respectively. However, on average EC50s in second generation individuals were only 16.5 and 20.8% lower than those of first generation for cumulative fecundity and population growth rate, respectively. Note, however, that the coordinator lab (i.e. lab1 in Figures and Tables) succeed in obtaining aggravation effects of PBO in F1, which is in line with his previous results (Campos et al., 2016). This means that the proposed protocol is reproducible and consistent within the same lab.

Among the 13 studies reported in Table 6, 10 out of 19 compounds and/or environmental stressors (52%) had greater toxic effects in second generation individuals than in first generation ones. The occurrence of multigenerational effects increased to 55% when the analysis was restricted to hormones or endocrine disruptors. There were also discrepancies across results from different labs for a given contaminant (i.e. 4-nonylphenol, (Brennan et al., 2006; Campos et al., 2016)). The two previous mentioned experiments differed in many respects: *Daphnia* clone, solvent (ethanol vs acetone). It is also not clear in Brennan et al. (2006) how the second generation was set up and if the brood rank was considered. All these differences are potential sources of discrepancy between the two studies.

Contrary to the studies reported in Table 6, ours was performed with a contaminant (PBO), which a priori was previously found to aggravate toxic effects on cumulative fecundity in second generation individuals (Campos et al., 2016). This means that our success in detecting aggravated effects in second generation individuals using the OECD TG211 testing protocol was moderate (54–70%), and not different to the reported occurrence of multigenerational detrimental effects (52–55%; Table 6).

5. Conclusions

The results reported in the present study showed that the proposed two generation *D. magna* reproduction assay was reproducible across the tested labs. Variability in cumulative fecundity among labs across PBO treatments was quite large (20%) but within the range that is often reported in other inter-calibration tests (Baird et al., 1989; Bradley et al., 1993; Samel et al., 1999). About half of this variability was due to the different sensitivities of clones as stated in early work (Baird and Barata, 1998). Nevertheless, differences in cumulative fecundity between control and solvent control treatments varied across labs and across generations. In first generation (F0) cumulative fecundity in controls (Mean $\pm$ SD, 119 $\pm$ 43) was higher than those of solvent controls (106 $\pm$ 27) and decreased in generation F1 (controls; 97 $\pm$ 40; solvent controls; 96 $\pm$ 25). Decreased fecundity in generation F1 was probably related to the fact that distinct culture conditions preceding the initiation of test were used for F0 and F1. The OECD TG 211 guideline and our multigeneration test protocol do not strictly regulate the pre-feeding conditions of the mothers used to generate the experimental progeny to initiate F0. However, during the test, feeding conditions of F0, (i.e. mothers that generated the progeny from which F1 individuals were collected), are highly regulated. *D. magna* reproductive females regulate the quality and quantity of their offspring depending on the feeding conditions that they encounter and require about three generations to adapt their progeny to a given new feeding condition (Barata and Baird, 1998). This

feature is known as maternal effects, which is often an important source of unexplained variance difficult to control and highly neglected in ecotoxicological investigations (Barata and Baird, 1998). Further development of this test will require to minimize maternal effects and setting up density and feeding conditions in daphnid cultures before initiating multigenerational assays. The proposed assay had little success in assessing multigenerational effects across labs. Note, however, that the coordinator lab (i.e. lab1 in Figures and Tables) succeed in obtaining aggravation effects of PBO in F1, which is in line with his previous results (Campos et al., 2016). ANOVA results indicated a small contribution of generation within and across PBO treatments on the total variance of cumulative fecundity (6.3%). This was in contrast with the high contribution of treatments within and across labs (76.6%). Indeed aggravation of toxic effects of PBO in second generation individuals only occurred in 54% of cases when EC50 and cumulative fecundity was considered and in 70% of cases when EC50 and population growth rate response were accounted for. This low occurrence was in line with the rather small increase of sensitivity to PBO measured in second generation individuals: EC50s in second generation individuals were for cumulative fecundity and population growth rate, respectively, only 16.5 and 20.8% lower than those of first generation. PBO affected embryo viability and hence the number of live offspring produced at the highest tested doses, which prevented from initiating F1 experiments. This resulted in fewer PBO treatments in F1, which may have diminished the power of ANOVA analyses to detect effect concentrations in F1. The lower magnitude of aggravation accounted by the NOEC (28%) relative to the EC50 (54%) for cumulative fecundity supports this argument. This means that further developments of the OECD TG 211 are needed to better detect cumulative toxicity across generations.

Acknowledgments

This work was supported by the Spanish MEC grant CTM2014-51985-R, and by the SETAC working group EVOGENERATE.

References

- Baird, DJ., Barata, C. Genetics and ecotoxicology. Taylor and Francis; Ann Arbor: 1998. Genetic variation in the response of *Daphnia* to toxic substances: implications for risk assessment; p. 207-221.
- Baird DJ, Barber I, Bradley M, Calow P, Soares AMVM. The *Daphnia* bioassay: a critique. *Hydrobiologia*. 1989; 188–189:403–406.
- Baird DJ, Barber I, Bradley M, Soares AMVM, Calow P. A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* straus. *Ecotoxicol Environ Saf*. 1991; 21:257–265. [PubMed: 1868782]
- Baldwin WS, LeBlanc GA. Identification of multiple steroid hydroxylases in *Daphnia magna* and their modulation by xenobiotics. *Environ Toxicol Chem*. 1994; 13:1013–1021.
- Baldwin WS, Bailey R, Long KE, Klaine S. Incomplete ecdysis is an indicator of ecdysteroid exposure in *Daphnia magna*. *Environ Toxicol Chem*. 2001; 20:1564–1569. [PubMed: 11434299]
- Barata C, Baird DJ. Phenotypic plasticity and constancy of life — history traits in laboratory clones of *Daphnia magna* Straus: effects of neonatal length. *Funct Ecol*. 1998; 12:412–419.
- Barata C, Baird DJ, Amat F, Soares AMVM. Comparing population response to contaminants between laboratory and field: an approach using *Daphnia magna* e-phippial egg banks. *Funct Ecol*. 2000a; 14:513–523.

- Barata C, Baird DJ, Minarro A, Soares A. Do genotype responses always converge from lethal to nonlethal toxicant exposure levels? Hypothesis tested using clones of *Daphnia magna* straus. *Environ Toxicol Chem.* 2000b; 19:2314–2322.
- Barata C, Baird DJ, Soares A. Phenotypic plasticity in *Daphnia magna* Straus: variable maturation instar as an adaptive response to predation pressure. *Oecologia.* 2001; 129:220–227. [PubMed: 28547600]
- Barata C, Baird DJ, Soares AMVM. Determining genetic variability in the distribution of sensitivities to toxic stress among and within field populations of *Daphnia magna*. *Environ Sci Technol.* 2002a; 36:3045–3049. [PubMed: 12141480]
- Barata C, Baird DJ, Soares AMVM. Phenotypic plasticity in *Daphnia magna* Straus: variable maturation instar as an adaptive response to predation pressure. *Oecologia.* 2002b; 129:220–227.
- Barry MJ, Logan DC, Ahokas JT, Holdway DA. Effect of algal food concentration on toxicity of two agricultural pesticides to *Daphnia carinata*. *Ecotoxicol Environ Saf.* 1995; 32:273–279. [PubMed: 8964255]
- Bradley, MC., Naylor, C., Calow, P., Baird, DJ., Barber, I., Soares, A. Progress in Standardization of Aquatic Toxicity Tests. Lewis; Boca Raton, FL, USA: 1993. Reducing Variability in *Daphnia* Toxicity Tests—A Case for Further Standardization; p. 57-70.
- Brennan SJ, Brougham CA, Roche JJ, Fogarty AM. Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*. *Chemosphere.* 2006; 64:49–55. [PubMed: 16405951]
- Campos B, Jordão R, Rivetti C, Lemos MFL, Soares AMVM, Tauler R, et al. Two-generational effects of contaminants in *Daphnia magna*: effects of offspring quality. *Environ Toxicol Chem.* 2016; 35:1470–1477. [PubMed: 26505489]
- Colborn T, Dumanoski D, Myers JP. Our Stolen Future: Are We Threatening our Fertility, Intelligence and Survival?—A Scientific Detective Story. 1996
- Dalla Bona M, Zouneková R, Merlanti R, Blaha L, De Liguoro M. Effects of enrofloxacin, ciprofloxacin, and trimethoprim on two generations of *Daphnia magna*. *Ecotoxicol Environ Saf.* 2015; 113:152–158. [PubMed: 25497771]
- Dietrich S, Ploessl F, Bracher F, Laforsch C. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna* — a multi-generational study. *Chemosphere.* 2010; 79:60–66. [PubMed: 20116828]
- Faassen EJ, García-Altares M, Mendes e Mello MML. Trans generational effects of the neurotoxin BMAA on the aquatic grazer *Daphnia magna*. *Aquat Toxicol.* 2015; 168:98–107. [PubMed: 26465128]
- Ginjupalli GK, Gerard PD, Baldwin WS. Arachidonic acid enhances reproduction in *Daphnia magna* and mitigates changes in sex ratios induced by pyriproxyfen. *Environ Toxicol Chem.* 2015; 34:527–535. [PubMed: 25393616]
- Hallare A, Nagel K, Köhler HR, Triebkorn R. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. *Ecotoxicol Environ Saf.* 2006; 63:378–388. [PubMed: 16125774]
- Hammers-Wirtz M, Ratte HT. Offspring fitness in *Daphnia*: is the *Daphnia* reproduction test appropriate for extrapolating effects on the population level? *Environ Toxicol Chem.* 2000; 19:1856–1866.
- Hansen LK, Frost PC, Larson JH, Metcalfe CD. Poor elemental food quality reduces the toxicity of fluoxetine on *Daphnia magna*. *Aquat Toxicol.* 2008; 86:99–103. [PubMed: 18037510]
- Hardstone MC, Strycharz JP, Kim J, Park IK, Yoon KS, Ahn YJ, et al. Development of multifunctional metabolic synergists to suppress the evolution of resistance against pyrethroids in insects that blood feed on humans. *Pest Manag Sci.* 2015; 71:842–849. [PubMed: 25044360]
- Heugens EHW, Tokkie LTB, Kraak MHS, Hendriks AJ, Van Straalen NM, Admiraal W. Population growth of *Daphnia magna* under multiple stress conditions: joint effects of temperature, food, and cadmium. *Environ Toxicol Chem.* 2006; 25:1399–1407. [PubMed: 16704075]
- Huebner JD, Loadman NL, Wiegand MD, Young DLW, Warszycki LA. The effect of chronic exposure to artificial UVB radiation on the survival and reproduction of *Daphnia magna* across two generations. *Photochem Photobiol.* 2009; 85:374–378. [PubMed: 19161403]

- Hutchinson T, Shillabeer N, Winter M, Pickford D. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. *Aquat Toxicol.* 2006; 76:69–92. [PubMed: 16290221]
- Jacobasch C, Völker C, Giebner S, Völker J, Alsenz H, Potouridis T, et al. Long-term effects of nanoscaled titanium dioxide on the cladoceran *Daphnia magna* over six generations. *Environ Pollut.* 2014; 186:180–186. [PubMed: 24378815]
- Janer G, Hakkert BC, Slob W, Vermeire T, Piersma AH. A retrospective analysis of the two-generation study: what is the added value of the second generation? *Reprod Toxicol.* 2007; 24:97–102. [PubMed: 17572063]
- Jeong TY, Kim HY, Kim SD. Multi-generational effects of propranolol on *Daphnia magna* at different environmental concentrations. *Environ Pollut.* 2015; 206:188–194. [PubMed: 26176633]
- Kim HY, Lee MJ, Yu SH, Kim SD. The individual and population effects of tetracycline on *Daphnia magna* in multigenerational exposure. *Ecotoxicology.* 2012; 21:993–1002. [PubMed: 22252291]
- Kim HY, Yu S, Jeong TY, Kim SD. Relationship between trans-generational effects of tetracycline on *Daphnia magna* at the physiological and whole organism level. *Environ Pollut.* 2014; 191:111–118. [PubMed: 24832921]
- LeBlanc GA, Mu X, Rider CV. Embryotoxicity of the alkylphenol degradation product 4-nonylphenol to the crustacean *Daphnia magna*. *Environ Health Perspect.* 2000; 108:1133–1138. [PubMed: 11133392]
- LeBlanc GA, Wang YH, Holmes CN, Kwon G, Medlock EK. A transgenerational endocrine signaling pathway in Crustacea. *PLoS One.* 2013;8.
- Li S, Xu J, Sheng L. The trans-generation effect during pulsed cadmium exposure: tolerance and induction of hsp70. *Ecotoxicol Environ Saf.* 2014; 107:300–305. [PubMed: 25050534]
- Li S, Sheng L, Xu J, Tong H, Jiang H. The induction of metallothioneins during pulsed cadmium exposure to *Daphnia magna*: recovery and trans-generational effect. *Ecotoxicol Environ Saf.* 2016; 126:71–77. [PubMed: 26720811]
- Marczylo EL, Jacobs MN, Gant TW. Environmentally induced epigenetic toxicity: potential public health concerns. *Crit Rev Toxicol.* 2016; 46:676–700. [PubMed: 27278298]
- Martínez-Jerónimo F, Espinosa-Chávez F, Villaseñor R. Effect of culture volume and adult density on the neonate production of *Daphnia magna*, as a test organism for aquatic toxicity tests. *Environ Toxicol.* 2000; 15:155–159.
- Massarin S, Alonzo F, Garcia-Sanchez L, Gilbin R, Garnier-Laplace J, Poggiale JC. Effects of chronic uranium exposure on life history and physiology of *Daphnia magna* over three successive generations. *Aquat Toxicol.* 2010; 99:309–319. [PubMed: 20646767]
- Mayer-Helm B, Hofbauer L, Müller J. Method development for the determination of selected pesticides on tobacco by high-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry. *Talanta.* 2008; 74:1184–1190. [PubMed: 18371768]
- Muyssen BTA, Janssen CR. Multi-generation cadmium acclimation and tolerance in *Daphnia magna* Straus. *Environ Pollut.* 2004; 130:309–316. [PubMed: 15182964]
- Nakamura A, Tamura I, Takanobu H, Yamamuro M, Iguchi T, Tatarazako N. Fish multigeneration test with preliminary short-term reproduction assay for estrone using Japanese medaka (*Oryzias latipes*). *J Appl Toxicol.* 2015; 35:11–23. [PubMed: 25580481]
- OECD. Report of the Final Ring Test of the *Daphnia magna* Reproduction Test, Series on Testing, and Assessment n°6. Organization for Economic Cooperation and development; Paris: 1997. p. 190
- OECD. Assessment OEHaSPSoTa. Detailed review paper on aquatic arthropods in life cycle toxicity tests with an emphasis on developmental, reproductive and endocrine disruptive effects. Paris: 2006. p. 125
- OECD. *Daphnia magna* Reproduction Test. Organization for Economic Cooperation and development; Paris: 2012. Guideline for the Testing of Chemicals No 211; p. 202
- Oliveira-Filho EC, Grisolia CK, Paumgartten FJR. Effects of endosulfan and ethanol on the reproduction of the snail *Biomphalaria tenagophila*: a multigeneration study. *Chemosphere.* 2009a; 75:398–404. [PubMed: 19138786]
- Oliveira-Filho EC, Grisolia CK, Paumgartten FJR. Trans-generation study of the effects of nonylphenol ethoxylate on the reproduction of the snail *Biomphalaria tenagophila*. *Ecotoxicol Environ Saf.* 2009b; 72:458–465. [PubMed: 18061673]

- Papchenkova GA, Golovanova IL, Ushakova NV. The parameters of reproduction, sizes, and activities of hydrolases in *Daphnia magna* Straus of successive generations affected by roundup herbicide. *Inland Water Biology*. 2009; 2:286–291.
- Parisot F, Bourdineaud JP, Plaire D, Adam-Guillermin C, Alonzo F. DNA alterations and effects on growth and reproduction in *Daphnia magna* during chronic exposure to gamma radiation over three successive generations. *Aquat Toxicol*. 2015; 163:27–36. [PubMed: 25840277]
- Pavlaki MD, Ferreira ALG, Soares AMVM, Loureiro S. Changes of chemical chronic toxicity to *Daphnia magna* under different food regimes. *Ecotoxicol Environ Saf*. 2014; 109:48–55. [PubMed: 25164202]
- Plaie D, Bourdineaud JP, Alonzo A, Camilleri V, Garcia-Sanchez L, Adam-Guillermin C, et al. Transmission of DNA damage and increasing reprotoxic effects over two generations of *Daphnia magna* exposed to uranium. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*. 2013; 158:231–243. [PubMed: 24035969]
- Samel A, Ziegenfuss M, Goulden CE, Banks S, Baer KN. Culturing and bioassay testing of *Daphnia magna* using Elendt M4, Elendt M7, and COMBO media. *Ecotoxicol Environ Saf*. 1999; 43:103–110. [PubMed: 10330328]
- Sarapultseva EI, Dubrova YE. The long-term effects of acute exposure to ionising radiation on survival and fertility in *Daphnia magna*. *Environ Res*. 2016; 150:138–143. [PubMed: 27288911]
- Verslycke T, Ghekiere A, Raimondo S, Janssen C. Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. *Ecotoxicology*. 2007; 16:205–219. [PubMed: 17235667]
- Völker C, Boedicker C, Daubenthaler J, Oetken M, Oehlmann J. Comparative toxicity assessment of nanosilver on three *Daphnia* species in acute, chronic and multi-generation experiments. *PLoS One*. 2013;8.
- Wang J, Lu J, Mook RA, Zhang M, Zhao S, Barak LS, et al. The insecticide synergist piperonyl butoxide inhibits hedgehog signaling: assessing chemical risks. *Toxicol Sci*. 2012; 128:517–523. [PubMed: 22552772]
- Ward TJ, Robinson WE. Evolution of cadmium resistance in *Daphnia magna*. *Environ Toxicol Chem*. 2005; 24:2341–2349. [PubMed: 16193764]
- Yang XF, Lu GH, Liu JC, Yan ZH. Multigenerational chronic effects of pharmaceuticals on *Daphnia magna* at environmentally relevant concentrations. *Zhongguo Huanjing Kexue/China Environmental Science*. 2013; 33:538–545.
- Zar, JH. *Biostatistical Analysis*. Bioestatistical Analysis Prentice-Hall International, Inc; New Jersey: 1996.

HIGHLIGHTS

- A two generation *Daphnia magna* reproduction test inter-laboratory calibration exercise was conducted.
- We tested the robustness and consistency of the assay detecting cumulative toxicity of pyperonyl butoxide.
- The proposed assay was reproducible across participant labs.
- Cumulative toxicity in the second generation could not be detected with this assay.

Fig. 1. Cumulative fecundity of *D. magna* individuals exposed to piperonyl butoxide (PBO) during two successive generations across 14 different assays performed by 12 different labs. Each symbol corresponds to a single observation. Curves are fits to the allosteric decay regression model. White and black symbols and black and grey lines correspond to F0 and F1 generations, respectively. Axis X is in log scale.

Fig. 2. Population growth rate responses of *D. magna* individuals exposed to piperonyl butoxide (PBO) by two generations across 14 different assays performed by 12 different labs. Each symbol corresponds to a single observation. Lines are fits to the allosteric decay regression model. White and black symbols and black and grey lines correspond to F0 and F1 generations, respectively. Axis X is in log scale. SC, solvent control.

Fig. 3.

Variance explained by treatment (PBO), Laboratory (L), Generation (G), treatment by laboratory ($PBO \times L$), treatment by generation ($PBO \times G$), laboratory by generation ($L \times G$), and the three factor interaction ($PBO \times L \times G$) terms. Unexplained residual variance is also included. Results for cumulative fecundity and population growth rate using ANOVA model 1 are presented in graphs A and B, respectively. Graph A1 shows the results obtained for cumulative fecundity when lab was substituted by clone and model 2 was used (see Methods, Data analyses for further details). In graph A1 green, purple, grey and pink areas corresponds to clone, $PBO \times$ clone, clone $\times$ Generation and $PBO \times$ clone $\times$ Generation, respectively.

Fig. 4.

Survival probabilities (juvenile: Sj; adult: Sa), age at first reproduction, and percentage of non-viable offspring (Mean $\pm$ SD, N = 9–138) as function of PBO concentration across all trials. White and black bars correspond to F0 and F1 generations, respectively. Within each generation, * indicates significant differences from solvent control (SC) following Kruskal-Wallis and multi-comparison Wilcoxon and Wilcox tests (Zar, 1996). Death or the absence of reproduction make sample size (N) to differ largely across treatments.

Table 1

Distinctive culture conditions of each participant lab. FDTW, filtered de-chlorinated tap water.

Lab	Photoperiod	Clone	Water	Medium renewal	Food regime
1	16 h:8 h L:D	F	100 ml ASTM	2 d	<i>Chlorella vulgaris</i> , 0.1 mg C/animal/day (25×10^6 cells/animal/day)
2	16 h:8 h L:D	Own	40 ml ASTM	2 d	<i>Raphidocelis subcapitata</i> (7×10^6 cells/daphnia/day), Tetrafin (0.2 mg dry wt.)
3	16 h:8 h L:D	Own	50 ml M7	2 d	<i>C. pyrenoidosa</i> daily (0.2 mg C/daphnia/day)
4	16 h:8 h L:D	Own	100 ml M4	2 d	<i>C. vulgaris</i> , daily (0.15 mg C/daphnia/day)
5	12 h:12 h L:D	Own	50 ml FDTW	2 d	<i>C. vulgaris</i> , 0.09–0.1 C/daphnia/day (1.87 mg C/ml, 4×10^5 cells/ml)
6	16 h:8 h L:D	F	50 ml M4	2 d	<i>C. vulgaris</i> (0.1–0.2 mg C/daphnia/working day)
7	16 h:8 h L:D	F	100 ml M4	2 d	<i>R. subcapitata</i> (29.6×10^6 cells/daphnia/day)
8	16 h:8 h L:D	Own	100 ml ASTM	2 d	<i>R. subcapitata</i> (40×10^6 cells/daphnia/day)
9	16 h:8 h L:D	F	50 ml FDTW	2 d	<i>C. vulgaris</i> (10×10^6 cells/daphnia), <i>Desmodesmus subspicatus</i> (7×10^6 cells/daphnia)
10	16 h:8 h L:D	NIES	50 ml M4	2 d	<i>C. vulgaris</i> , 0.2 mg C/animal/day (50×10^6 cells/animal/day) + yeast, Cerophile, Trout choow additive
11	16 h:8 h L:D	F	50 ml ASTM	2 d	<i>R. subcapitata</i> (30×10^6 cells/daphnia/day)
12	16 h:8 h L:D	F	40 ml COMBO	2 d	<i>R. subcapitata</i> (2.5–5 mg C/L/daphnia/day)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Nominal and measured PBO concentrations of freshly prepared (Tf) and old (Told) test solutions.

Table 2

Nominal	Tf		Told			
	Mean	SD	N	Mean	SD	N
50	40.2	11.8	56	37.3	6.6	55
100	89.0	9.7	53	70.0	5.7	51
200	163.7	9.7	51	133.4	5.0	52
400	393.4	23.2	54	322.4	47.3	52

Table 3

Cumulative offspring production in first (F0) and second (F1) generation in control (C) and solvent control (SC) treatments and associated coefficient of variation (CV). Sample size varied between 8 and 10. CV values exceeding 25% and significant ($P < 0.05$) distinct mean values of C and SC are in bold. Mean values for C and SC differing $>25\%$ are also underlined. Empty spaces are missing values.

Lab	F0				F1			
	C		SC		C		SC	
	Mean $\pm$ SD	CV	Mean $\pm$ SD	CV	Mean $\pm$ SD	CV	Mean $\pm$ SD	CV
1	98.5 $\pm$ 8.0	8.1	94.1 $\pm$ 7.1	7.5	94.4 $\pm$ 8.2	8.7	108 $\pm$ 3.6	3.4
2	102.6 $\pm$ 11.9	11.6	107.8 $\pm$ 8.7	8.1	77.8 $\pm$ 6.3	8.1	90.2 $\pm$ 8.7	9.6
	129 $\pm$ 27.5	21.3	105.6 $\pm$ 21	19.9	98.8 $\pm$ 9.2	9.3	126.2 $\pm$ 15.2	12
3	138.4 $\pm$ 22.2	16.0	134.3 $\pm$ 26.5	19.7	79.3 $\pm$ 15.7	19.8	120.3 $\pm$ 9.8	8.1
4	84 $\pm$ 10.2	12.2	92.2 $\pm$ 25.5	27.7	68 $\pm$ 10.9	16.1	74.1 $\pm$ 16.3	22.1
5			70.1 $\pm$ 7.0	10.1			73.9 $\pm$ 15.7	21.2
	61.7 $\pm$ 7.6	12.2	63.1 $\pm$ 8.9	14.0	63.8 $\pm$ 5.9	9.3	58.8 $\pm$ 12.7	21.6
6	106.2 $\pm$ 6.7	6.3	84 $\pm$ 16.3	19.4	94.3 $\pm$ 4.8	5.1	76.4 $\pm$ 19.1	25
7	138 $\pm$ 13.2	9.6	138 $\pm$ 19.9	14.4	90.9 $\pm$ 8.9	9.8	118.4 $\pm$ 12.8	10.8
8	168.8 $\pm$ 22.7	13.4	142.8 $\pm$ 17.5	12.3	177.4 $\pm$ 20.8	11.7	141.7 $\pm$ 26.9	19.0
9	100.8 $\pm$ 26.6	26.4	118.3 $\pm$ 37.3	31.5	76.2 $\pm$ 11.1	14.5	109.1 $\pm$ 20.5	18.8
10	226.5 $\pm$ 9.9	4.4	142.4 $\pm$ 43.4	30.5	187.8 $\pm$ 20.7	11	95.6 $\pm$ 23.5	24.6
11	122.1 $\pm$ 10	8.2	119.7 $\pm$ 7.1	6	99.1 $\pm$ 10.3	10.4	99.8 $\pm$ 12.2	12.2
12	75.9 $\pm$ 10.6	13.9	74.7 $\pm$ 8.6	11.4	53.9 $\pm$ 7.0	12.9	60.5 $\pm$ 5.5	9.1

Table 4

Low (EC10, NOEC) and median (EC50) effect concentrations of PBO for cumulative fecundity across participant labs and generations (F0, F1). Ratios between F0/F1 endpoints are also reported. Values are in µg/L. *, ANOVA analyses were not significant ($P > 0.05$) so NOEC was the highest tested concentration. <, effects were observed at the lowest tested concentration, thus it was not possible to estimate a proper NOEC.

Lab	F0		F1		F0		F1		F0		F1		Ratio F0/F1			
	EC10		EC10		EC50		EC50		NOEC		NOEC		EC10		EC50	
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	EC10	EC50	NOEC	NOEC
1	196.6 ± 26	104.4 ± 9.5	408.8 ± 18.9	219.8 ± 8.1	100	25	1.9	1.9	4							
2	179.3 ± 23.6	240.3 ± 37.1	363.4 ± 15.5	336.1 ± 19.1	100	200	0.7	1.1	0.5							
2	323.6 ± 56		451.4 ± 51.7		200	400*										
3	225.2 ± 34.1	220.7 ± 19	318.7 ± 21	279 ± 18.2	200	200	1	1.1	1							
4	74 ± 10.1	80.1 ± 12.7	129.8 ± 8.4	119.9 ± 11.4	50	50	0.9	1.1	1							
5	354.4 ± 120.1	133.4 ± 59.3	434.1 ± 122.5	583.8 ± 93.5	200	100	2.7	0.7	2							
5	180.5 ± 25.8	36.9 ± 10.5	430.1 ± 18	229.3 ± 25.4	50	50	4.9	1.9	1							
6	207.8 ± 31	205.1 ± 24.4	274.5 ± 50.6	284.5 ± 62	100	50	1	1	2							
7	101.4 ± 9.4	91.3 ± 7.3	135.8 ± 4.1	141.6 ± 6.7	200	200*	1.1	1	1							
8	75.9 ± 16.6	58.8 ± 12.4	171.3 ± 15.8	149.7 ± 11.7	50	<25	1.3	1.1	2							
9	69.7 ± 20.4	72.7 ± 19.9	273.5 ± 29.1	151.8 ± 16.7	<25	100	1	1.8	0.25							
10	176.9 ± 23.1	190.1 ± 21.9	257.6 ± 25.4	246.1 ± 61.9	200	200	0.9	1	1							
11	88.5 ± 6.7	95.5 ± 6	150.8 ± 5.8	149.2 ± 4.1	50	50	0.9	1	1							
12	107.8 ± 11.5	101.8 ± 14.1	166.4 ± 6.5	184.8 ± 7.5	100	100	1.1	0.9	1							

Table 5

Low (EC10, NOEC) and median (EC50) effect concentrations of PBO for population growth rates across participant labs and generations (F0, F1). Ratios between F0/F1 endpoints are also reported. Values are in $\mu\text{g/L}$. *, ANOVA analyses were not significant ($P > 0.05$) so NOEC was the highest tested concentration. <, effects were observed at the lowest tested concentration, thus it was not possible to estimate a proper NOEC.

Lab	F0		F1		F0		F1		F0		F1		Ratio F0/F1			
	EC10		EC10		EC50		EC50		NOEC		NOEC		EC10			
	Mean $\pm$ SE		Mean $\pm$ SE		Mean $\pm$ SE		Mean $\pm$ SE									
1	192.7 $\pm$ 24.9		198.5 $\pm$ 22.5		821 $\pm$ 11.3		547.7 $\pm$ 37.9		100		100		1	1.5		1
2	329.2 $\pm$ 28.3				424.3 $\pm$ 18.9		460.4 $\pm$ 10.7		200		200		0.9			1
2					484.8 $\pm$ 22.7				200		200					1
3	321.9 $\pm$ 59.1		258.6 $\pm$ 18		494.8 $\pm$ 44.1		380.2 $\pm$ 73.6		200		200		1.2	1.3		1
4	172.7 $\pm$ 16.7				225.4 $\pm$ 15.8		172.6 $\pm$ 76.2		100		100					1
5	391.5 $\pm$ 27.6				535.4 $\pm$ 47.2				400		100					4
5	346.5 $\pm$ 22.9		159.5 $\pm$ 19		492.8 $\pm$ 29.3		265.7 $\pm$ 19.8		200		50		2.2	1.9		4
6	226.6 $\pm$ 25.3				285.1 $\pm$ 30.6				200		400*					0.5
7	85.7 $\pm$ 17.4		154.3 $\pm$ 66.9		220.2 $\pm$ 30.5		199.2 $\pm$ 6.9		100		100		0.6	1.1		1
8	163.3 $\pm$ 10.9		126.6 $\pm$ 19		225.36 $\pm$ 12.3		280.6 $\pm$ 31.5		100		50		1.3	0.8		2
9	173.5 $\pm$ 20.2		114.4 $\pm$ 34.8		310.9 $\pm$ 18.1		326.5 $\pm$ 86.9		100		<25		1.5	1		4
10	232.2 $\pm$ 29.1		239.3 $\pm$ 27.5		394.3 $\pm$ 10.7		345.2 $\pm$ 20.2		200		200		1	1.1		1
11	104.6 $\pm$ 10.9		134.8 $\pm$ 17.6		307.4 $\pm$ 12.5		247.8 $\pm$ 19.7		100		100		0.8	1.2		1
12	93.2 $\pm$ 17.8		142.2 $\pm$ 25.9		368.6 $\pm$ 27		254.8 $\pm$ 50.8		100		100		0.7	1.4		1

Table 6
Reported effect concentrations in selected multigenerational studies conducted in *D. magna*.

Compounds	F0	F1	F > F1	F0/F1	F0/F > F1	Studies
Diethylstilbestrol, mg/L	LOEC >0.5	LOEC 0.2		2.5		Brennan et al. (2006)
Nonylphenol, µg/L	LOEC, 80	LOEC, 40		2		
Enrofloxacin, mg/L	LOEC, 6.3	LOEC, 3.1		2		Dalla Bona et al. (2015)
	EC50, 6.5 (4.5–9.4)	EC50, 3.9 (2.5–5.9)		1.7		
Ciprofloxacin, mg/L	LOEC, 30	LOEC, 30		1		
	EC50, 24 (16–38)	EC50, 36 (23–58)		0.7		
Trimethoprim, mg/L	LOEC, 13	LOEC, 13		1		
	EC50, 15 (13–18)	EC50, nd				
nTiO ₂ , mg/L	LOEC, 2.67	LOEC, 4	LOEC, 1.78, 2.67, 1.78, 1.19 (F2-F5)	0.7		Jacobasch et al. (2014)
Propanolol, µg/L	LOEC >26	LOEC >26	LOEC >26 (F2–6,F8), F7, 26	1	1	Jeong et al. (2015)
Tetracycline	LOEC, 10	LOEC, 0.5,5	LOEC, 0.1, 0.1 (F2,F3)	2	100	Kim et al. (2014)
Uranium, µg/L	LOEC, 25	LOEC, 25	LOEC, 10 (F2)	1	2.5	Massarin et al. (2010)
Glyphosate (Roundup)	LOEC, 25	LOEC, 25	LOEC, 25, 50 (F2,F3)	1	0.7	Papchenkova et al. (2009)
Gamma radiation, mGy/h	LOEC, 35.4	LOEC, 35.4	LOEC, >35.4 (F2)	1	1	Parisot et al. (2015)
Uranium, µg/L	LOEC, 9.9	LOEC, 2		5		Plaire et al. (2013)
NanoAg, µg/L	LOEC, 1.25	LOEC, 2.5	LOEC, 1.25 (F2–F4)	0.5	1	Völker et al. (2013)
Ethinyl estradiol, ng/L	LOEC, 10	LOEC > 10	LOEC >10(F2–F4,F6), LOEC, 10 (F5)	1	1	Dietrich et al. (2010)
Metoprolol, µg/L	LOEC, 1.2	LOEC > 1.2	LOEC >1.2 (F2–F4,F6), LOEC, 1.2 (F5)	1	1	
4-Nonylphenol, µg/L	LOEC, 40	LOEC, 40		1		Campos et al. (2016)
	EC50, 52.3 (35.3–69.5)	EC50, 49.9 (42.1–58.4)		1		
Tributyltin, µg/L	LOEC, 0.5	LOEC, 0.2		2.5		
Pyperonyl butoxide, µg/L	LOEC, 200	LOEC, 50		4		
	EC50, 514 (486–542)	EC50, 339 (220–358)		1.5		
20-hydroxyecdysone, µg/L	LOEC, 125	LOEC > 62.5		2		Baldwin et al. (2001)
Ponasterone A, µg/L	LOEC, 12.5	LOEC, 3.1		4		