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Effects of chronic exposure to a pharmaceutical mixture on the three-spined stickleback (*Gasterosteus aculeatus*) population dynamics in lotic mesocosms

Viviane David[1], Sandrine Joachim[2], Audrey Catteau[2], Katherine Nott[3], Sébastien Ronkart[3], Christelle Robert[4], Nathalie Gillard[4], Anne Bado-Nilles[2], Edith Chadili[2], Olivier Palluel[2], Cyril Turies[2], Naïs Julian[1,2], Julie Castiglione[2], Odile Dedourge-Geffard[5], Younes Hani[5], Alain Geffard[5], Jean-Marc Porcher[2], Rémy Beaudouin[1]

[1] Unité METO (Modèles pour l'Ecotoxicologie et la Toxicologie), UMR-I 02 SEBIO, INERIS, 60550 Verneuil en Halatte, France. Tel: +33344618238; e-mail: remy.beaudouin@ineris.fr

[2] Unité ECOT (Ecotoxicologie in vitro et in vivo), UMR-I 02 SEBIO, INERIS, Parc ALATA, BP2, 60550 Verneuil-en-Halatte, France

[3] Société wallonne des eaux (SWDE), 6220 Fleurus, Belgique.

[4] CER groupe, Health Department, 6900 Marloie, Belgique.

[5] Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO, Moulin de la Housse, Reims, France

Abstract. Pharmaceutical substances are ubiquitous in the aquatic environment and their concentration levels typically range from ng/L up to several µg/L. Furthermore, as those compounds are designed to be highly biologically active, assessing their impacts on non-target organisms is important. Here, we conducted a mesocosm experiment testing a mixture of five pharmaceuticals (diclofenac, carbamazepine, irbesartan, acetaminophen and naproxen) on fish, three-spined stickleback (*Gasterosteus aculeatus*). The mixture concentration levels were chosen on the basis of the contamination of the Meuse river in Belgium which had been measured previously during a monitoring campaign undertaken in 2015 and 2016. Three nominal mixture concentration levels were tested: the lowest concentration level mixture was composed by environmentally-relevant concentrations that approximate average realistic values for each pharmaceuticals (Mx1); the two other levels were 10 and 100 times these concentrations. Although no impact on stickleback prey was observed, the mixture significantly impaired the survival of female fish introduced in the mesocosms at the highest treatment level without causing other major differences on fish population structure. Impacts on condition factors of adults and juveniles were also observed at both individual and population levels. Using a modeling approach with an individual-based model coupled to a bioenergetic model (DEB-IBM), we concluded that chronic exposure to environmentally-relevant concentrations of five pharmaceuticals often detected

in the rivers did not appear to strongly affect the three-spined stickleback populations. Mechanisms of population regulation may have counteracted the mixture impacts in the mesocosms.

Key-words. Mesocosms, Pharmaceuticals, Mixture, Three-spined stickleback, Individual-based model

1. Introduction

Pharmaceuticals are intensively used in human and veterinary medicine (Backhaus 2014). Numerous pharmaceuticals have been detected in the aquatic environment: in sewage treatment plant (STP) effluents, surface water, sea water, groundwater and some drinking waters (Fent et al. 2006, Zhang et al. 2008). In both wastewater treatment plant effluents and surface waters, pharmaceuticals have been found at concentrations ranging from a few ng/L to several µg/L (Corcoran et al. 2010, Loos et al. 2013). A great diversity of pharmaceutical compounds presenting different modes of action have been identified in freshwaters, including nonsteroidal anti-inflammatory drugs, anti-depressants, estrogens and antibiotics (Cleuvers 2003, Corcoran et al. 2010). Because pharmaceutical compounds are designed to be biologically active, they may also impact non-target organisms; thus, analyzing effects of pharmaceuticals on aquatic non-target organisms is of major importance.

Several studies have explored the impacts of pharmaceuticals on aquatic organisms. These molecules have a wide variety of modes of action. Indeed, Cleuvers (2003) analyzed the toxicity of nine pharmaceuticals alone on *Daphnia magna,* algae (*Desmodesmus subspicatus*) and plants (*Lemna minor*), revealing very heterogeneous toxicity. Furthermore, pharmaceuticals were shown to impact several life history traits of fish such as growth (Corcoran et al. 2010), feeding rate (Corcoran et al. 2010, Brodin et al. 2014), behavior (activity, sociality, aggression, reproductive behavior) (Brodin et al. 2014) at concentration levels ranging from a few µg/L to up to several mg/L. Effects of pharmaceuticals have also been shown at the fish population level. For example, Kidd et al. (2007) showed that an estrogen, ethinylestradiol, impacted the sustainability of a wild fish population at very low concentrations (5-6 ng/L).

However, ecotoxicological studies have mainly focused on the effects of pharmaceuticals at the individual level and are generally conducted under standardized conditions. Hence, there is a lack of information on the impacts of pharmaceutical compounds at the population level (Corcoran et al. 2010). Furthermore, pharmaceuticals are generally tested as single compounds, leading to an underestimation of the environmental risks, as these compounds are typically present as complex mixtures in aquatic ecosystems (Backhaus and Faust 2012). Studies focusing on the effects of mixtures of pharmaceuticals present in aquatic ecosystems are thus required (Backhaus and Faust 2012, EFSA 2013). Furthermore, compared to studies in the laboratory, mesocosm experiments provide more realistic ecological conditions for the assessment of the effects of chemicals at different levels of biological organization. These experiments allow to simultaneously identify direct and indirect effects of toxicants and provide a better understanding of environmentally relevant effects of chemicals (Caquet et al. 2000). Therefore, the study in mesocosms of a mixture of pharmaceuticals commonly found in rivers is highly relevant to better evaluate the ecotoxicological risks caused by pharmaceuticals.

Biomarkers are observable or measurable indicators of morphological, behavioral, functional or structural changes in organisms in response to changes in its environment (van der Oost et al. 2003). In risk assessment, biomarkers have been extensively used to provide the connection between contaminant exposure and effects in organisms (van der Oost et al. 2003, Kroon et al. 2017). Furthermore, using a population dynamics model to determine the relationship between the responses of biomarkers to a toxicant and the adverse impact at the population level could be a powerful approach to support environmental risk assessments (Conolly et al. 2017).

Among pharmaceuticals, naproxen (NPX), acetaminophen (ACE), diclofenac (DIC), carbamazepine (CBZ) and irbesartan (IRB) were the most frequently detected pharmaceutical residues in surface waters of the Meuse River in Belgium between February 2015 and July 2016 (Imhotep 2013–2017). These chemicals are also frequently found in drinking, ground, waste and river waters throughout Europe (Loos et al., 2013) and worldwide (Godoy et al. 2015, Patel et al. 2019). These pharmaceuticals exhibit different modes of action: NPX, ACE and DIC belong to the family of analgesics, anti-pyretic and anti-inflammatory compounds, respectively, whereas IRB is an antihypertensive and CBZ an

antiepileptic. A mixture composed of these 5 pharmaceuticals has been shown to cause endocrine disrupting effects in fish according to a study of reproductive traits of female rainbow trout over a 42 day-exposure period in aquaria contaminated at three different concentration levels (Schmitz et al. 2018).

In this work, long-term effects of a mixture of NPX, ACE, DIC, IRB and CBZ on individual and population responses of the three-spined stickleback were studied in aquatic mesocosms using a well-tried experimental design (Roussel et al. 2007, de Kermoysan et al. 2013, David et al. 2019). We combined different approaches to understand the potential impacts of the mixture on the test organisms, by analyzing biomarkers and population endpoints, and by modeling population dynamics with or without integrating potential mixture impacts. We analyzed several biomarkers related to the reproduction, stress level and immunity. The individual-based model developed by David et al. (2019) was used to better understand the fish population dynamics in the mesocosms when exposed to the mixture. To that end, we tested several potential impacts on key processes, considering the effects observed in the mesocosms. Those effects (impacts on survival and condition factors) were integrated in the model to simulate the mesocosm experiment and help to better understand the mixture toxicity. Long-term impacts of the mixture were also assessed in order to evaluate population resilience.

2. Material and methods

2.1. Mesocosm experiment

The experiment was performed using 12 lotic artificial streams (mesocosms) located in the North of France (INERIS, Verneuil-en-Halatte, France). A detailed description of the mesocosms is provided in de Kermoysan et al. (2013). Briefly, each mesocosm had a length of 20 m and is 1 m wide. Each mesocosm was divided into three sections: the upper section (0 – 9 m), the slope (9 – 10 m) and the lower section (11 – 20 m). Each mesocosm received denitrified and dechlorinated tap water at a flow rate of 0.8 m^3/h. A 0.25-mm mesh was placed at the outlet of the mesocosms to avoid any drift of macroinvertebrates and fish. Before the experiment, mesocosms were set up with sediments composed

of 80% sand and 20% clay (See Supplementray Information (S1, Figure S1) for a schematic view of the installation).

In October 2016, three species of macrophytes were introduced; water starwort (*Callitriche platycarpa*), dense pondweed (*Groenlandia densa*) and watercress (*Nasturtium officinale*). In November 2016, zooplankton and periphyton were obtained by sieving 36 L of water collected from an unpolluted artificial pond located in the vicinity of the mesocosm platform using a 50 µm mesh, and then introduced into each mesocosm. Finally, three gastropod species (*Radix perega*, *Planorbis planorbis* and *P. antipodarum*), two crustacean species (*Asellus aquaticus* and *Gammarus pulex*), two species of leeches (*Glossiphonia* sp. and *Erpobdella* sp.) and one species of Heteroptera (*Notonecta* sp.*)* were also introduced in each mesocosm. For each macroinvertebrate, the quantity introduced per mesocosm is given in Supplementary Information (SI) (Table S1). In addition, during the experiment, aquatic insects were allowed to naturally colonize the mesocosms. The mesocosms were then left to equilibrate until March 2017.

On March 5th 2017, the three-spined sticklebacks were introduced into the mesocosms. In August 2016, juvenile fish under 25 mm long coming from our breeding facilities were reared in an artificial pond supplied with tap water at a flow rate of 1L/h and located in the vicinity of the mesocosms and fed ad-libitium every day with frozen *Chironomidae* larve until February 2017. For each mesocosm, the initial populations were composed of 15 mature females and 10 mature males (hereafter referred to as the founders). Fish with similar lengths were selected and their sex was determined according to the imaging method developed by de Kermoysan et al. (2013). Mean female length was 47.3 ± 5.9 mm, ranging from 34.3 to 61.5 mm (n = 180). Mean male length was 46.0 ± 4.4 mm, ranging from 37.2 to 56.9 mm (n = 120). Fish were randomly assigned to the mesocosms and the length of the founders at the beginning of the experiment did not significantly differ among treatments (ANOVA, $p > 0.05$). The experiment lasted until October 3rd, 2017. At the end of the experiment, all the fish from each mesocosm were recovered and killed by an overdose of MS-222. Then, 20 fish per mesocosm were used directly for physiological and chemical analyses after being measured, weighted and sexed. The other fish were stored in 4 % formalin before being measured, weighed and sexed.

From March to October, temperature was monitored every 10 min with two temperature sensors in each mesocosm (HOBO0257, Prosensor, Amanvilliers, France) at 5 and 15 m from the inlet of water, respectively, at the surface and at 70-cm depth. Other routine water parameters such as pH, conductivity and dissolved oxygen were measured every week at 10 m from the water inlet and 35-cm depth in each mesocosm by using a WTW multi-parameter instrument equipped with digital sensors (MultiLine® IDS, Multi3430, WTW, Germany). Biological measurements were also made during the experiment: zooplankton and macroinvertebrates were sampled every 4 weeks. The sampling methodology is described in de Kermoysan et al. (2013).

2.2. Exposure to the mixture

The mixture was composed of carbamazepine (CAS: 298-46-4, purity 98 %, Acros Organics), 4-acetamidophenol (CAS: 103-90-2, purity 98 %, Acros Organics), diclofenac sodium salt (CAS: 15307-79-6, purity 98 % Interchim), naproxen sodium (CAS: 26159-34-2, purity 98 %, Alfa Aesar) and irbesartan (CAS: 138402-11-6, purity 98 %, Sigma Aldrich). The concentration levels in the environmentally-relevant mixture (Mx1) were the median measured concentrations in the Walloon part of the Meuse river (Nott et al. 2018) (see also SI, Table S2). This mixture was composed of 25 ng/L of CBZ, 25 ng/L of DIC, 25 ng/L of NPX, 50 ng/L of IRB and 100 ng/L of ACE. Two other mixtures (Mx10 and Mx100) represented 10 and 100 times the concentrations of Mx1. The mesocosm water inlet was contaminated continuously as the environmental measurements show that the Meuse river is chronically contaminated, which is due to continuous release of the target compounds by water treatment plants.

Treatment began on the 20th of April 2017 (46 days after introduction of the founders) and ended on the 2nd of October 2017 (211 days after introduction of the founders), thus the exposure lasted 165 days. Exposure was carried out by an automatic dosing system which continuously delivered the according test mixture to each mesocosm. The dosing system is fully described in SI (see Table S3, Figure S2).

Mixture concentrations in the water of each of the contaminated mesocosms were monitored every month at different locations (0, 5 and 19 m from the inlet of the water) to measure actual exposure concentrations. The concentration of a metabolite of CBZ, the carbamazepine-10,11-epoxide (CBZ-EP), was also monitored. In addition, samples were also taken from the control mesocosms at 10 m. The 250 mL samples obtained in the control, the Mx1 and Mx10 treatments were stabilized with sodium thiosulphate and filtered through fiberglass filters with 1-µm porosity. An internal standard was added to each sample for each studied molecule (homologues labeled with heavy isotopes, deuterium or carbon-13) prior to solid phase extraction using an automatic extractor (Smartprep from Horizon Technologies). Mx100 treatment samples were processed by adding internal standards directly to the sample without extraction prior to their injection into the LC-MS / MS, given the high concentrations of these samples. All samples were then extracted using OASIS HLB cartridges that were conditioned beforehand with methanol and water. After sample loading, the cartridge was rinsed with water, and then the molecules were eluted with acetonitrile. After addition of ultra pure water (LC-MS/MS grade), the extract was evaporated to 1 mL (80/20; water/acetonitrile) and then analyzed by high performance liquid chromatography coupled with tandem mass spectrometry (Agilent Technologies LC-MS / MS 6490). In this analysis, the molecules were separated on a reverse phase chromatography column (Zorbax Eclipse Plus C18 rapid resolution HD, 2,1*50 mm, 1.8 µm from Agilent Technologies), and then detected and identified by tandem mass spectrometry after ionization by electrospray in the positive mode. Finally, concentrations were determined using an external calibration curve corrected by internal standards. The recovery of the internal standards for the method including a solid phase extraction step prior to the LC-MS/MS analysis was approximately of 90% for CBZ, DIC and NPX, 80% for IRB and 65% for ACE. The recovery of the internal standards were approximately of 98% for the five pharmaceuticals for the direct LC-MS/MS injection method. Blanks (extraction, solvents, LC-MS / MS) were performed as well as quality controls (extraction of reconstituted samples at 1, 4, 12 and 50 ng/L).

2.3. Stickleback endpoints

2.3.1. Bioaccumulation

At the end of the experiment, between 4 and 13 individuals (49.71 ± 8.31 mm, corresponding to the larger individuals in mesocosms) were taken from each mesocosm to obtain at least 5 g of fish to determine the internal concentration of each pharmaceutical of the mixture in whole fish tissue. Several relevant metabolites were also quantified and were listed in the SI. After being homogenized (IKA universal grinder M20), the sample was placed in a 50-mL polypropylene centrifuge tube and spiked with 50 µL IS solution. The mixture was allowed to stand for 15 min. Twenty mL of acetonitrile was used to extract the pharmaceuticals and precipitate proteins. The sample was then thoroughly shaken at room temperature for 15 min and centrifuged at 4,650 x g for 5 min. The extract was collected and loaded on an Oasis HLB cartridge (preconditioned with 3 mL of methanol and 3 mL of water) under vacuum to obtain a flow rate of about 1 mL/min. The cartridge was then rinsed with 3 mL ultra-pure water and vacuum-dried to remove excess water. Finally, the retained components were eluted with 3 mL of methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen in a water bath set at 40°C. Then, the pellet was resuspended in 1 mL ACN/water (10:90, v/v). The extract was centrifuged at 11,500 x g for 5 min at 20°C. The clear supernatant was transferred into a vial prior to UHPLC-MS/MS analysis.

Analyses were performed using an Acquity UHPLC system (Waters, Milford, MA, USA) and chromatographic separations were done by injecting 20 µL of reconstituted extract on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.7 µm particle size, Waters). Detection was carried out with a Waters Acquity TQ mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source operating in the positive (ESI+) ionization mode. More details on the analytical method can be found in SI.

2.3.2. Biomarkers: individual endpoints

At the end of the mesocosm experiment, 10 males and 10 females (32.93 ± 3.03 mm) per mesocosm were used for analyzing biomarker responses. This range of length was selected in order to have enough fish sample to conduct all the biomarker analyses. Each fish was sacrificed by cervical dislocation after anesthesia with tricaine methanesulfonate MS222 (70 mg. L-1, SIGMA- ALDRICH, France), then measured and weighed. As described by Janssen et al. (1995), the condition factor (CF) was calculated

as [100 x body weight (g)]/(length)^3 (cm)]. The liver somatic index (LSI) was calculated as [100 x liver wet weight (g)]/total body weight (g)] (Slooff et al. 1983). The gonadal somatic index (GSI) was calculated as [100 x gonad weight (g)]/fish weight (g)] (Lofts et al. 1966). Then, for digestive enzyme activities, the whole digestive tract was removed on ice, rinsed with cold Tris-HCl buffer (0.01 M, pH 7, SIGMA-ALDRICH, France) and stored at -80°C until further analysis. Finally, the spleen was sampled for immunomarker analysis.

Each whole digestive tract was homogenized with ceramic (3 mm Ø) and glass (1 mm Ø) beads in cold Tris-HCl buffer (0.01 M, pH7), using a PRECELLYS241 homogenizer (BERTIN TECHNOLOGIES, France), at 5,500 rpm (2x10 sec), and centrifuged at 15,000 x g for 30 min at 4°C (Hani et al. 2018). Measurements of amylase and intestinal alkaline phosphatase (IAP) activity levels were performed according to Junge et al. (2001) and Panteghini and Bais (2008) using Thermo-Scientific Gallery ready-to-use reagents. Trypsin activity measurements were performed according to the García-Carreño and Haard (1993) method, using N-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, 3 mM) as substrate. All enzymatic assays were adapted on the Gallery™ Automated Photometric Analyzer (Thermo Fisher Scientific Oy) and performed at 37°C, by kinetic colorimetric assay at 405 nm. Results are reported in U.g^{-1} of gut tissue.

Immunomarkers were measured in leucocytes; splenic leucocyte isolation was conducted following protocols described previously by Bado-Nilles et al. (2013). Then analyses were carried out on whole leucocytes, using a Cyan™ADP flow cytometer (Beckman Coulter). For each leucocyte sample, 10,000 cells were counted. Leucocyte distribution, cellular mortality (apoptotic and necrotic leucocytes), leucocyte respiratory burst (Bado-Nilles et al. 2014), lysosomal presence (Bado-Nilles et al. 2013) and phagocytosis activity (Bado-Nilles et al. 2011) were measured.

Vitellogenin (VTG) concentration was measured in blood as described by Sanchez (2005) and expressed in ng.mL^{-1}. This assay is based on an indirect competitive ELISA assay using anti-sticklebacks VTG antibodies at 2000-fold final concentration (GA-306, Biosense Laboratories, Bergen, Norway) and purified stickleback VTG as standard. Blood was assayed on 12 serial dilutions with 3-

fold dilution steps. The method was adapted to microplates on a TECAN Freedom EVO® automated platform (Tecan, Switzerland).

2.3.3. Populational endpoints

During the mesocosm experiment, all drifting larvae were recovered every day in acrylic glass containers placed at the outlet of each mesocosm. The total number of larvae was recorded with a distinction between dead and alive individuals. All the larvae were then placed back in their respective mesocosm. Results were expressed as total number of larvae per day and per mesocosm. At the end of the experiment, fish were measured and weighed and individuals with a length smaller than 26 mm were assumed to be immature, and thus classified as juveniles (de Kermoysan et al. 2013). Sex of fish with a length greater than 26 mm was determined. From these measurements, descriptive variables of the populations were determined: the population abundance (N.tot), the number of female and male founders at the end of the mesocosm experiment (N.F.0, N.M.0), female and juvenile frequencies (F.F and F.J) and the mean lengths and the coefficients of variation of the lengths of five categories of individuals: male and female founders (L.M.0, L.F.0, CV.M.0, CV.F.0), males and females born in the mesocosms (L.M.X, L.F.X, CV.M.X, CV.F.X) and juveniles (L.J, CV.J). Finally, observations of gonad maturity of males (immature vs mature) were also made and the frequency of mature males among the total number of males (F.M.m) was calculated as well as the mean length of mature males and the coefficient of variation of their lengths (L.M.m and CV.M.m). Mean weight and condition factors were also determined as described by Janssen et al. (1995) for each category of individuals.

During this project, all experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes.

2.4. Analysis based on population dynamics modelling

The DEB-IBM used in the present study was developed for predicting the three-spined stickleback population dynamics in mesocosms without toxicant stress (David et al. 2019). The entire ODD (Overview, Design concepts, and Details) description (Grimm et al. 2010) of the model is detailed in

David et al. (2019). Briefly, the model includes relevant information of the environment such as temperature, photoperiod and prey density over time. The stickleback prey organisms considered in the model are zooplankton, higher crustacea (*Gammarus pulex* and *Asellus aquaticus*) and a diptera family (*Chironomidae*). The model was calibrated with two datasets from previous mesocosm experiments and was successfully evaluated on three others (David et al. 2019). The accuracy of the distributions of each population endpoint without toxicant stress was validated using data from the control mesocosms made in this experiment. Parameter values and descriptions can be found in SI Table S5. The model inputs for the simulations were the daily mean water temperature, photoperiod and food density in mesocosms as well as the lengths of the 15 female and 10 male founders that were introduced in the mesocosm experiments. For each mixture treatment, the temperature and food scenarios (food density per day) were calculated based on the samplings of macroinvertebrates and zooplankton made in the mesocosms as described in David et al. (2018) (Figures S5 and S6). The time step of the model was set to 1 day and the simulation runs lasted as long as the real experiment (212 days). The model outputs were the endpoints which were monitored in the real populations.

To account for direct treatment effects on populations, a chronic excess mortality induced by the mixture was introduced in the model to reproduce the effects observed in the mesocosms for the Mx100 treatment. First, the basal mortality (M. n) was calculated for each fish with Eq.1 (e.g. normal mortality rate in control conditions) as in the DEB-IBM without toxicant stress. Then, the effect of the mixture was modelled as causing an excess mortality M. ex (Eq. 2).

$$\mathrm{M.n} = \mathrm{M.u} \times W_{(t)}^{b} \qquad \text{Eq. 1}$$

$$\mathrm{M.tox} = \mathrm{M.n} + \mathrm{M.ex}_{(t)} \qquad \text{Eq. 2}$$

Excess mortality was modelled using a dose-response relationship defined by a Hill equation (Eq. 3). Hill model coefficients were estimated using all population data observed in mesocosms using the DEB-IBM and a genetic algorithm as in David et al. (2019) (See SI for more details).

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$$M.ex_{(t)} = \frac{C_{(t)}{}^{n_{tox}}}{EC_{50}{}^{n_{tox}} + C_{(t)}{}^{n_{tox}}} \quad \text{Eq. 3}$$

With $C_{(t)}$ the exposure concentration (given as a proportionally factor x M) at time t, EC_{50} the concentration leading to 50 % of effect and n_{tox} the Hill coefficient.

In order to model impacts on the condition factors due to changing individual weights, we increased the individual weight of the adults and decreased the individual weight of the juveniles using the same magnitude using the stress level $s(t)$ (Eqs. 4 and 5). The stress level was calibrated with the condition factors observed in the population data (See SI for more details). The condition factors were then calculated for each fish as [100 x body weight (g)]/(length)^3 (cm)].

$$W_{adult}(t) = W_{adult}(t) \times (1 + s(t)) \quad \text{Eq. 4}$$

$$W_{juvenile}(t) = W_{juvenile}(t) \times (1 - s(t)) \quad \text{Eq. 5}$$

With $W_{adult}(t)$ and $W_{juvenile}(t)$ the individual weight of the adults or juveniles at time t and $s(t)$ the stress level induced by the mixture.

Simulated endpoints of the populations were compared to the observations made in the mesocosms for each mixture treatment. To that end, 1000 simulations of the DEB-IBM were performed to account for the stochasticity of the model (inter-mesocosm environmental variability, inter-individual performance variability and stochastic processes). Simulations were made with or without integration of the dose-response curve of mortality to assess the improvement of the predictions. Goodness-of-fit was calculated as relative errors (RE) between the model simulations and the observations for the control and each mixture treatment.

Finally, the population resilience to the mixture treatment was assessed by extrapolating the impacts of the mixture for a treatment between Mx100 and Mx10^7 while keeping the temperature and food scenarios identical to those in the control. 1000 simulations were made at each concentration level. We

then calculated the extrapolated dose-response curve and we highlighted the mixture treatment leading to 80% of the fish abundance distribution shifted towards the lower length. We also used the method of EFSA et al. (2017) suggesting that the benchmark response could be defined in terms of a difference in the control mean equal to 1 standard deviation (SD).

2.5. Statistical analysis

For the observations made in the mesocosms, all statistical analyses were performed using R version 3.4.3 software. The level of significance for all the analyses was 5 %. Firstly, to assess the mixture impacts for each endpoint, ANOVA was performed to identify significant effects on the observed endpoints and a Dunnett's post hoc test was then used to compare each treatment to the control. For analyzing the larvae drift, repeated measures ANOVA (RM-ANOVA) were performed which take into account the correlation between sampling dates. Furthermore, the minimal detectable differences (MDD) (EFSA 2013, Brock et al. 2015) were calculated for each population endpoint. The MDD defines the difference between the mean of a treatment and the control that must exist to detect a statistically significant effect. Second, the Low Effect Concentrations (LOECs) were re-calculated comparing the distribution of the control endpoints estimated by the DEB-IBM to the observations made in the contaminated mesocosms using a Kolmogorov-Smirnov test and the methodology described in David et al. (2019).

3. Results

3.1. Concentrations of pharmaceuticals in water

Overall, the measured concentrations at each sampling point for each pharmaceutical in the mesocosms were consistent with the nominal concentration, except for ACE, which was only found at approximately 50 % of the nominal concentration in the three treatments (Table 1 and Figure S3). For ACE, DIC and NPX, the measured concentrations significantly decreased with the distance from the water inlet (RM-ANOVA or p-value < 0.05) (Figure S4). Time also had a significant impact on the concentrations of CBZ, ACE and NPX (RM-ANOVA). Finallt, for CBZ-EP, concentrations in water were under the quantification limit or always inferior at 5% of CBZ (data not shown).

3.2. Physico-chemical characteristics of water

No significant difference was observed between the various mixture treatments for temperature, pH, conductivity and dissolved oxygen (figure S5) (RM-ANOVA, p-value > 0.05). In addition, the statistical analyses showed no impact of the mixture on the different taxonomic groups of macroinvertebrates and zooplankton (Figures S6, S7 and S8).

3.3. Three-spined stickleback endpoints

3.3.1. Concentrations of pharmaceuticals in fish

None of the chemicals present in the mixture bioaccumulated in fish subjected to the Mx1 treatment (results below limit of detection; Table 2). In fish exposed to Mx10 and Mx100 treatments, IRB, DIC and a metabolite of CBZ, CBZ-EP were detected. No CBZ was detected in fish tissues from the Mx10 treatment whereas it was found in fish in the Mx100 treatment. Neither ACE nor NPX were detected in fish in the Mx10 and Mx100 treatments (Table 2). The bioaccumulation factors (BCFs) were < 1 for the fish exposed to the Mx10 treatment for the five substances. Inversely, in the Mx100 treatment, the BCF was equaled to 3.63 ± 2.20 for DIC and < 1 for the four other substances.

3.3.2. Individual responses

In the mesocosms, all mixture treatments had significant impacts on the individual weight and the condition factor of the fish born in mesocosms and sampled at the end of the experiment (ANOVA, p-value < 0.05) (Figure 1). The highest treatment also significantly impacted the digestive enzyme alkaline phosphatase (ANOVA, p-value < 0.05) (Figure S9). For GSI, the oxidative burst, phagocytosis capacity and efficacy as well as the length, significant effects were observed in the Mx1 or Mx10 mixture treatments but without any clear dose-response relationship suggesting the result on alkaline phosphatase may have been a false positive result (Figure S9). Indeed, 21 biomarkers were analyzed with an alpha risk equaled of 5 %, thus, assuming a binomial distribution, from 0 to 3 false positive results can be expected.

3.3.3. Populational responses

Overall, the coefficient of variation (CV) of the outputs ranged from 0.17% for the mean length of immature males to 59.6% for the frequency of immature males (Table S4). Accordingly, the MDD ranged between 1.2 % for the mean length of immature males to 57.9 % for the number of male founders. In parallel, we calibrated the parameters related to the mixture's potential impacts on fish mortality and condition factors. The parameters of the dose-response curve of the fish mortality were calibrated to -0.194 ± 0.004 and 38.33 ± 0.289 for n_{tox} and EC_{50} respectively (Eq. 3). Similarly, the stress level for the condition factors was calibrated to 0.096 ± 0.005.

3.3.3.1. Fish abundance

No significant difference in fish abundance between the control and the treatments was found on the total number (dead and alive) of drifted fry during the experiment (Figure 2) (RM-ANOVA, p-value > 0.05).

Furthermore, using a classical statistical test comparing the mesocosm observations, the mixture treatments had no effect on the total abundance of fish at the end of the experiment (ANOVA, p-value > 0.05). The mean total abundance was 989.83 ± 192.96 individuals per population (Figure 3). However, using our DEB-IBM, we showed that when integrating the dose-responses curve for fish mortality and condition factors, total abundance was simulated to be slightly lower in the Mx100 treatment (725.5 fish were predicted in median in the Mx100 treatment against 828.5 for the control).

3.3.3.2. Number of founders

A significant decrease of the number of female founders was observed in the highest treatment (Mx100) by a classical statistical analysis comparing only the mesocosm observations (ANOVA, p-value < 0.05). This was confirmed by our analysis based on the DEB-IBM simulations (*i.e.* simulated distributions in control conditions, see Table S6). On the contrary, no statistical difference was found on the number of founder males (Figure 3).

Adding the dose-response curves for the fish mortality and condition factors in the DEB-IBM clearly improved the predictions of the number of female and male founders especially for the Mx100 treatment (Figure 4). Indeed, the REs were 3.40 % and 0.43 %, respectively, when integrating the dose-response

curves against 29.11 % and 26.21 % when no impact was integrated in the DEB-IBM. Especially, the number of male founders was also predicted to be impacted by the Mx100 treatment as a median of 4 males was predicted in the Mx100 treatment against 5 in the control.

3.3.3.3. Condition factors

Using a classical statistical test comparing the mesocosm observations, the mean condition factor of females was significantly higher in the Mx1 treatment and significantly lower for the juveniles for all the mixture treatments (ANOVA, p-value < 0.05) (Figure 3). Integrating a thinning effect of the mixture for juveniles and an obesogenic impact of the same magnitude for the adults gave good predictions of the condition factors (Figure 5). The model reproduced especially well the difference of impacts on the condition factors for juveniles and adults (Figures 5 and S14).

3.3.3.4. Other population endpoints

No significant differences were found for the other population endpoints when assessed either with a classical statistical methodology using only the mesocosm observations or with the modelling approach: the mean length frequencies, the mean lengths, the mean weights and the CV of lengths of the different categories of individuals were not significantly affected (Figure 3 and S10). Similarly, no impact of the mixture treatments was observed on frequencies, mean length, CV of lengths, weight and condition factors of the mature and immature males as well as for the total fish biomass at the end of the experiment (Figures S10 to S13).

3.3.3.5. Length frequency

A major part of the control populations (between 75 % and 81 %) had standard lengths between 5 and 26 mm (Figure 6a). Above 45 mm, only founders were observed. The same population structure was observed for the mean length frequency distributions in the Mx1 and Mx10 mixture treatments. Indeed, populations in the controls or exposed to the Mx1 and Mx10 mixture treatments presented a maximum frequency at 19-20 mm (5.6 to 13 % of the population) (Figure 6a). For the Mx100 treatment, populations presented a maximum frequency at 22-23 mm (11 % of the populations), consequently the population structure was slightly shifted to the right (Figure 6a).

Using the modeling approach, the length frequencies of the control and Mx100 treatment were also well predicted when integrating the dose-response curves for the mortality and the condition factors. All the points were inside the prediction interval (Figures 6b,c).

3.3.3.6. Long-term predictions

Without integrating an effect of the mixture on the stickleback prey abundance, the fish population was predicted to be highly resilient. Indeed, the model extrapolation showed that at least 8000 times the environmental mixture (Mx1) would be required to have 80 % of the distribution of the total abundance shifted towards lower abundances compared to the control distribution (Figure 7). The benchmark response as defined by EFSA et al. (2017) was also 8000 times that of the environmental mixture (Mx1).

4. Discussion

It is widely recognized that mesocosm experiments have an increased ecological realism compared to laboratory experiments as they allow to assess both direct and indirect impacts of toxicants on the populations (Caquet 2002). Furthermore, ecological modelling has been suggested to be a useful solution to increase the understanding of population dynamics (Forbes et al. 2010, Beaudouin et al. 2012, Forbes et al. 2017). In this study, we showed that different approaches currently existing in ecotoxicology could be combined to better characterize the hazard of environmental mixtures of pharmaceuticals. Especially, combining mesocosm observations at different level of biological organization and a population dynamics model helped us to analyze the experimental data in depth.

4.1. Occurrence of pharmaceutics in water and fish

The five pharmaceuticals (ACE, CBZ, DIC, IRB and NPX) studied were chosen as they are among the most abundant pharmaceutical residues in the Walloon part of the Meuse river (Nott et al. 2018). The order of magnitude of these concentrations was relevant compared to the concentrations (from few ng/L to 1 µg/L) found in drinking, ground, waste and river waters throughout Europe and worldwide (Godoy et al. 2015, Loos et al., 2013, Patel et al. 2019).

Kim and Tanaka (2009) classified NPX, ACE and CBZ as slowly-degrading pharmaceuticals whereas DIC quickly degraded by photolysis. Indeed, phototransformation has been identified as the main elimination process of DIC (Poiger et al. 2001, Tixier et al. 2003, Zhang et al. 2008, Yan and Song 2014). Bayer et al. (2014) suggested that IRB could be degraded due to its structure even though few studies have focused on its fate in the aquatic environment. In the mesocosms, the measured concentrations of IRB, DIC, NPZ and CBZ were close to their nominal concentrations, suggesting that the possible degradation may be countered by the continuous contamination of the mesocosms. However, ACE was less persistent in the mesocosms as concentrations were half the nominal concentrations for all mixture treatments. One explanation is that this molecule could have been photolyzed as its life time is low (56 h) (Yamamoto et al. 2009). It may also have been biodegraded by microorganisms (Wu et al. 2012). Indeed, Yu et al. (2006) showed that ACE was quickly biotransformed and a nearly complete biotransformation occurred within 14 days of incubation in a wastewater treatment plant. Furthermore, its percentage of degradation reported in the literature for classical sludge activated waste water treatment plants is close to 99% (Kasprzyk-Hordern et al. 2009, Soulier et al. 2011, Gao et al. 2012).

Concerning the internal concentrations of the mixture components in sticklebacks, CBZ and its metabolite CBZ-EP were detected in the whole fish tissue for the Mx10 and Mx100 treatments. The presence of CBZ-EP could be due to its metabolization in the liver (Kerr et al. 1994, Zhang et al. 2008). It could also come from several biotic and abiotic degradation reactions of CBZ in water possibly leading to CBZ-EP (Daniele et al. 2017), but as CBZ-EP concentrations in water were low, it did not seem to be the main pathway. Here, the bioconcentration factors for CBZ and its metabolite were very low (BCF < 1). Valdés et al. (2016) and Gasca-Pérez et al. (2019) measured CBZ in several fish tissues and showed that CBZ was preferentially concentrated in the brain and liver, confirming a low propensity to bioconcentrate in the whole body compared to specific organ tissues. Concerning DIC, Naslund et al. (2017) reviewed several studies and concluded that the bioconcentration factor of DIC in fish varies from 0.1 up to 2732 depending on the organ analyzed. For three-spined stickleback, the authors showed that the bioconcentration factor was between 0.2 and 0.4. In our study, the BCF were higher for

stickleback (equal to 3.63 ± 2.20), which may be consistent with the large variability found for fish in Naslund et al. (2017). IRB was also detected in fish in the Mx10 and Mx100 treatments while the two other mixture components (ACE and NPX) were not found in any of the fish tissues analyzed regardless of treatment. Finally, in a study by Schmitz et al. (2018) that tested the same mixture assessed by us on female rainbow trout in laboratory over a 42-day exposure period, none of the pharmaceuticals (IRB, ACE, NPX, CBZ and DIC) showed any bioconcentrative properties. In contrast, we found that some mixture components were bioaccumulated. This could result from more realistic biomagnification processes as sticklebacks in our study were not artificially fed with uncontaminated food but ate prey that was also exposed to the mixture during the mesocosm experiment. Consequently, compared to the study of Schmitz et al. (2018), the pharmaceuticals could have been taken up from the prey. Furthermore, the time of exposure was longer increasing the occurrence of potential bioaccumulation processes

4.2. Mixture impacts on three-spined sticklebacks

During our study, none of the three treatment levels led to impacts on the physico-chemical characteristics of the mesocosms nor on prey abundance. Consequently, indirect impacts of the pharmaceutical mixture on sticklebacks were likely to be negligible compared to its direct impacts. This hypothesis seemed to be consistent with the predictions of the DEB-IBM. Indeed, by integrating mixture impacts only on the stickleback physiological processes, their population dynamics in the mesocosms were well reproduced.

The toxicity of the whole mixture on sticklebacks was assessed at both individual and population levels in the mesocosms. Furthermore, the two impacts that we considered in the model (effects on fish mortality and on the condition factors) led to good predictions for the population endpoints in the Mx100 treatment. These hypotheses were based on the mesocosm observations as we found a significant decrease of the observed number of the female founders as well as observed impacts on condition factors for the highest treatment. This increased mortality of the female founders could play a major role on the stickleback population dynamics (Figure 8). Furthermore, as no significant number of dead female founders was found at the beginning of the exposure, the mixture may have induced a chronical mortality rather than an acute mortality of the female founders.

The simulations also showed a decrease of the number of male founders in the Mx100 treatment at the end of the experiment which was not picked up with a simple statistical analysis (parametric or non-parametric tests on the mesocosm observations): this simple statistical analysis could have produced a false negative result. The CVs for this output were high for each mixture treatment (> 50%) as well as the MDD (> 50%) (Table S4), and thus a basic statistical evaluation would be difficult to perform (EFSA 2013, Brock et al. 2015). Consequently, the use of our DEB-IBM improved the statistical analysis and showed that male founders were also impacted by the mixture in the Mx100 treatment leading to 4 males in median at the end of the experiment against 5 in the control. This result seems logical as female founders were also significantly impacted. Furthermore, even a slight decrease of the number of male founders, if they die before the effective reproduction period (the firsts nests were built from April to June), could have a non-negligible influence on the overall population dynamics. Indeed, male sticklebacks have a strong investment in the reproduction (Clutton-Brock and Vincent 1991, Wootton 1984). Nevertheless, the design of the mesocosm experiment could not allowed us to know the time of the death of the founders, and no strong impacts were observed on the population structure at the end of the experiment.

The impact on survival could be due to the several of the pharmaceutical components of our mixture. Indeed, direct mortality was observed in several earlier laboratory studies at high DIC concentrations, for juvenile trouts and zebrafish (Praskova et al. 2011, Schwarz et al. 2017) and 80 µg/L of DIC for juvenile three-spined sticklebacks (Naslund et al. 2017). The viability of *Danio rerio* eggs were also significantly impaired by CBZ at 10 and 10,000 µg/L (da Silva Santos et al. 2018) and 10 µg/L of ACE induced a high mortality rate and developmental abnormalities in *D. rerio* embryos (Galus et al. 2013). Finally, a high concentration of NPX (500 µg/L) significantly impacted the survival of juveniles of medaka (Kwak et al. 2018). CBZ-EP, which we detected in whole fish tissue, could also induce mortality as it was suggested to be potentially more toxic than CBZ itself because of its anticonvulsant and neurotoxic properties (Donner et al. 2013). However, to our knowledge, laboratory studies on the impacts on fish of the single compounds of the mixture at environmental concentrations, especially for IRB and NPX, are still scarce. Furthermore, interactions between the pharmaceuticals in the mixture

could lead to either antagonistic and synergistic toxicities, modulating the impact of the whole mixture (Backhaus and Faust 2012). Consequently, testing the whole mixture in mesocosms resulted in a more realistic assessment of the impact of the contamination of the surface waters of the Meuse River on fish, but additional studies should be conducted to understand the toxicities of the pharmaceuticals alone and the interactions between them.

It should also be noted that the exposure conditions were not the same for all fish in the mesocosms. Indeed, founders were exposed from April to the end of the experiment, whereas exposure duration was shorter for fish that were born in the mesocosms. In addition, founders were only exposed at adult stage whereas fish born in the mesocosms were exposed during all their life cycle. Consequently, the responses may differ between individuals, thus an interpretation was not straightforward. For example, the impacts for the fish born in the mesocosms could have been minimized due to a lower time of exposure. Indeed, fish which are chronically exposed to pharmaceuticals could accumulate them in their bodies and thus, be more impacted than fish with a shorter exposure (Corcoran et al. 2010). However, it has been shown that impacts of pharmaceutics could also depend on the fish stage. For example, Praskova et al. (2011) showed that zebrafish embryos could be more sensitive than juveniles at high concentration of DIC. Consequently, at lower concentrations, the mixture could have induced a pressure selection and adaptive responses (Figure 8) as fish born in mesocosms were exposed directly at the embryo stage and during all its development and growth.

Furthermore, we extrapolated the potential impacts on survival to higher concentrations of the mixture by modelling. We found that the population was modelled to be highly resistant and resilient to the mixture up to a theoretical Mx8000 treatment, which would be required to have 80 % of the distribution of the total abundance shifted toward lower abundances compared to the control distribution. With this Mx8000 treatment, the nominal concentrations of the mixture would be 0.2 mg/L for CBZ, DIC, NPX, 0.4 mg/L for IRB and 0.8 mg/L for ACEs which is way above the environmental concentrations of these pharmaceuticals. However, these results are based on the strong hypothesis that the mixture would have no impacts on macrophytes, zooplankton and macroinvertebrates in the

mesocosms between Mx100 and Mx8000. Consequently, the fish population may collapse at a lower mixture concentration if the stickleback preys or the habitat become impacted by the mixture.

Other significant impacts were observed on fish condition factors. Indeed, the individual weight and condition factors of the adult fish sampled in the mesocosms at the end of the experiment were significantly higher for all treatments (i.e. individual responses). For the mean condition factors for all juveniles, male and female adults at the end of the mesocosm experiment (i.e. population responses), the results were significative only for the Mx1 treatment for the females born in the mesocosms. However, the inverse pattern was observed for juveniles as their condition factors significantly decreased for all treatments. From the data at the individual level, this increase of the condition factors for the adults could be explained by a significant increase of the fish weight with the three mixture treatments.

To better understand the experimental results, we introduced a thinning impact on the juveniles and an obesogenic impact on the adults in the DEB-IBM. However, in case of substances that impact the weight but not the length, our DEB model presents some limits. Indeed, the allometric relationship between the weight and the length, and consequently the assumption of isomorphism, was no longer valid. Thus, we did not change the DEB model but introduced one parameter which modulate the fish weight independently of the length to consider the impact of the mixture. Doing this, the model reproduced well the observations made in the mesocosms. Furthermore, we considered that the dose-response curves of the condition factors already reached the asymptotic value of the stress level at the Mx1 treatment which seemed to be a relevant assumption. Globally, these results suggested a direct impact on adult bioenergetics and a subsequent impact on the condition factors of the juveniles may be linked to inter-cohort competition rather than a direct effect of the mixture. Indeed, mixture treatments could have disrupted food competition by lowering access to food for juveniles compared to adults, and by, for the adults, increased their energetic reserve at the expense of the juveniles (Figure 8). Indeed, several studies have shown unequal competition between sticklebacks when foraging, as larger individuals are dominant compared to smaller sticklebacks (Foster et al. 1988, Gill and Hart 1996)

At the individual level, we did not observe strong impacts on the measured biomarkers, except for an effect on the levels of a digestive enzyme, the alkaline phosphatase (PAL), at the highest mixture treatment. The same result was found in a complementary laboratory experiment testing the same mixture on sticklebacks over a 21-day period (data shown in SI). The PAL is an enzyme of the intestinal brush border involved in the last phase of digestion, including dephosphorylation (Moss 1992) and nutrient uptake (i.e. calcium and phosphate) (Eguchi 1995). Here, we observed a decrease of PAL in both laboratory and mesocosm experiments. Several hypotheses could explain this result. Indeed, this could be linked to a difference in food assimilation or be representative of a disruption of the absorption of certain nutrients such as lipids (Hani et al. 2018). This result should also be considered in context with the impacts on the condition factors of the adults as it could suggest a difference in the lipid storage (Figure 8).

Finally, no significant impact was found on larvae drift suggesting that there was no impact on the quantity of juveniles born in the mesocosms. Furthermore, contrary to the literature, we did not find impacts on reproductive biomarkers. Indeed, several studies have shown reprotoxic impacts for some mixture components alone (Hong et al. 2007, Galus et al. 2013, Gröner et al. 2017) and for all the components in mixture on fish (Schmitz et al. 2018). Especially, an experiment that was conducted in the laboratory where fish were exposed during a shorter period of time (42 days compared to 212 days in our study) to the same mixture and with the same concentrations showed impacts on biomarkers related to several reproductive traits of female rainbow trout such as plasma levels of sex-steroid hormones, especially 11-ketotestosterone, and the expression of genes encoding key proteins involved in ovarian development and in the maintenance of the ovary (Schmitz et al. 2018). However, such impacts were not observed in our mesocosm study or in laboratory testing the same mixture on sticklebacks over 21 days (data shown in SI). Consequently, our results do not support an impact of the tested pharmaceuticals on the reproductive traits of sticklebacks. It is nevertheless possible that the complexity of the artificial ecosystems could have modulate the impacts of the mixture.

At the population level, our study showed that populations were resilient to the pharmaceutical mixture. Indeed, despite the fact that mixture impacts were found at the individual level, no strong

impacts of the mixture was observed on the population dynamics in mesocosms. Consequently, compensation mechanisms may have occurred in the mesocosms (Rose et al. 2001) counteracting the impacts of the mixture at the population level. For example, the effects of density-dependence on the mortality have been widely studied in fish and are assumed to be one of the main factors regulating population dynamics (Lorenzen 2005, Hazlerigg et al. 2012).

5. Conclusion

In this study, the effects of a pharmaceutical mixture at environmentally-realistic concentration levels were assessed in an aquatic mesocosm experiment. Individual and populational impacts were found for the three-spined sticklebacks in the highest treatment level, which corresponds to 100 times the environmental concentrations of the pharmaceuticals, although no impact was observed on the other trophic levels. Especially, significant mortalities of the females and males introduced in the mesocosms was detected in this treatment without strong disturbance of the overall population dynamics. Furthermore, individual and populational impacts on the condition factors of adults and juveniles were probably due to varying intensity of inter-cohort competition for food. Thus, this study highlights the importance of assessing toxicant impacts at high level of biological organization as complex interactions may occur between the individuals and their environment. Here, compensatory mechanisms seemed to have taken place increasing the resilience of the fish population to the pharmaceutical mixture. Adaptative responses to the mixture toxicity could also have happened. Consequently, our mesocosm experiment as well as the use of our DEB-IBM allowed us to increase the ecological realism of our risk assessment of pharmaceutical mixture.

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8. Tables and figures

Table 1: Nominal concentrations and average measured concentrations *in situ* for each pharmaceutical and mixture treatment (CBZ: carbamazepine, IRB: irbesartan, DIC: diclofenac, NPX: naproxen et ACE: acetaminophen) averaged on the whole exposure period.

Mixture	**Mx1**			**Mx10**			**Mx100**		
Substances	[Nominal] ($ng.L^{-1}$)	[Measured] ($ng.L^{-1}$)	%	[Nominal] ($ng.L^{-1}$)	[Measured] ($ng.L^{-1}$)	%	[Nominal] ($\mu g.L^{-1}$)	[Measured] ($\mu g.L^{-1}$)	%
CBZ	25	26.5	105.9	250	229.5	91.8	2.5	2.2	88.1
IRB	50	50.4	100.7	500	470.5	94.1	5	4.3	85.1
DIC	25	20.6	82.3	250	179.1	71.6	2.5	1.7	69.2
NPX	25	21.1	84.4	250	191.9	76.8	2.5	1.9	78.0
ACE	100	51.6	51.6	1000	497.5	49.7	10	4.9	49.2

LOQs: 1 ng/l for CBZ, DIC and IRB and 4 ng/l for NPX and ACE. LODs are the third of these LOQ values.

Table 2: Measured concentrations of the mixture components in whole fish tissue (CBZ: carbamazepine, CBZ-EP: carbamazepine-10,11-epoxide, IRB: irbesartan, DIC: diclofenac, NPX: naproxen et ACE: acetaminophen).

Substances	**Mx1**	**Mx10**	**Mx100**
Mixture	**[measured] in µg/kg**	**[measured] in µg/kg**	**[measured] in µg/kg**
CBZ	LOD	LOD	1.25 ± 0.36
CBZ-EP	LOD	0.48 ± 0.32	6.02 ± 0.25
IRB	LOD	0.42 ± 0.28	3.47 ± 0.41
DIC	LOD	0.87 ± 1.23	6.17 ± 3.74
ACE	LOD	LOD	LOD
NPX	LOD	LOD	LOD

LOQs: 0.15 µg/kg for CBZ-EP and IRB, 0.3 µg/kg for CBZ and 1 µg/kg for DIC, ACE and NPZ. LODs are equal to one third of the LOQ values.

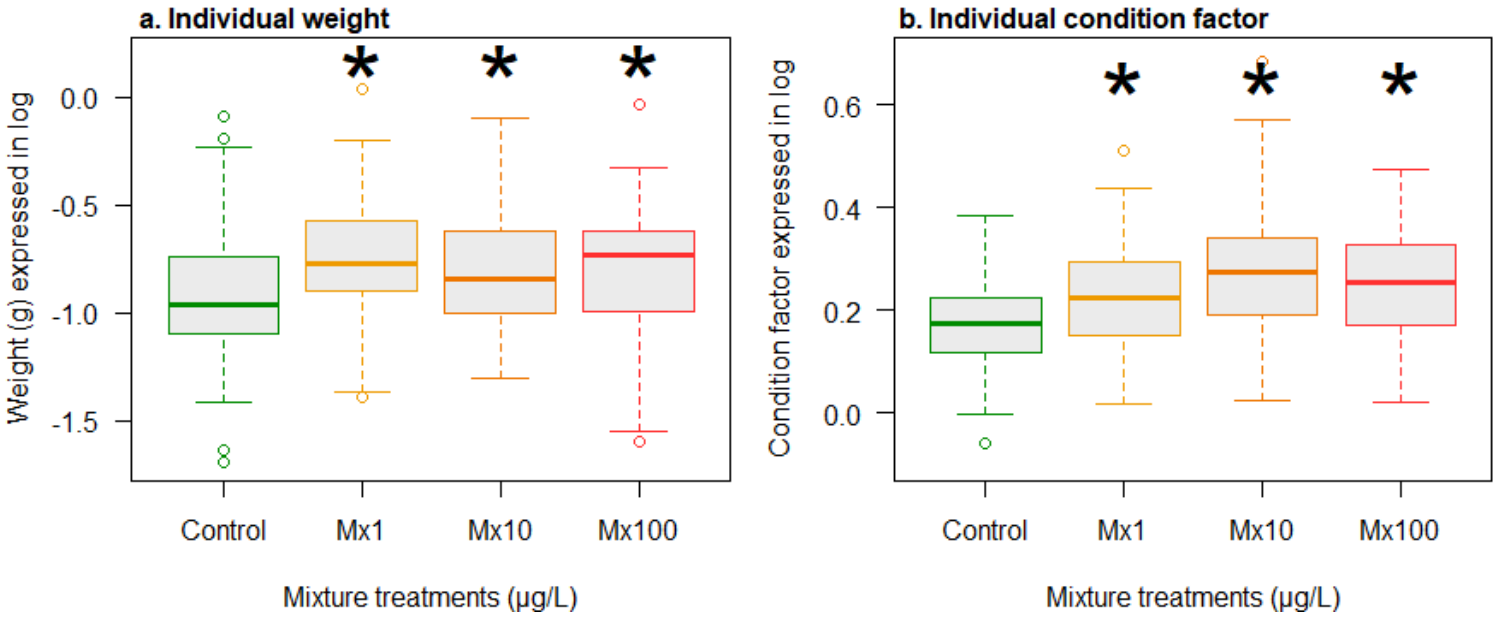

Figure 1: Individual weight (a) and condition factor (b) of the sticklebacks sampled at the end of the experiment in the mesocosms for each treatment (Control, Mx1, Mx10 and Mx100, expressed in log). Asterisks indicate statistically-significant differences between mixture treatments and control (p-value < 0.05).

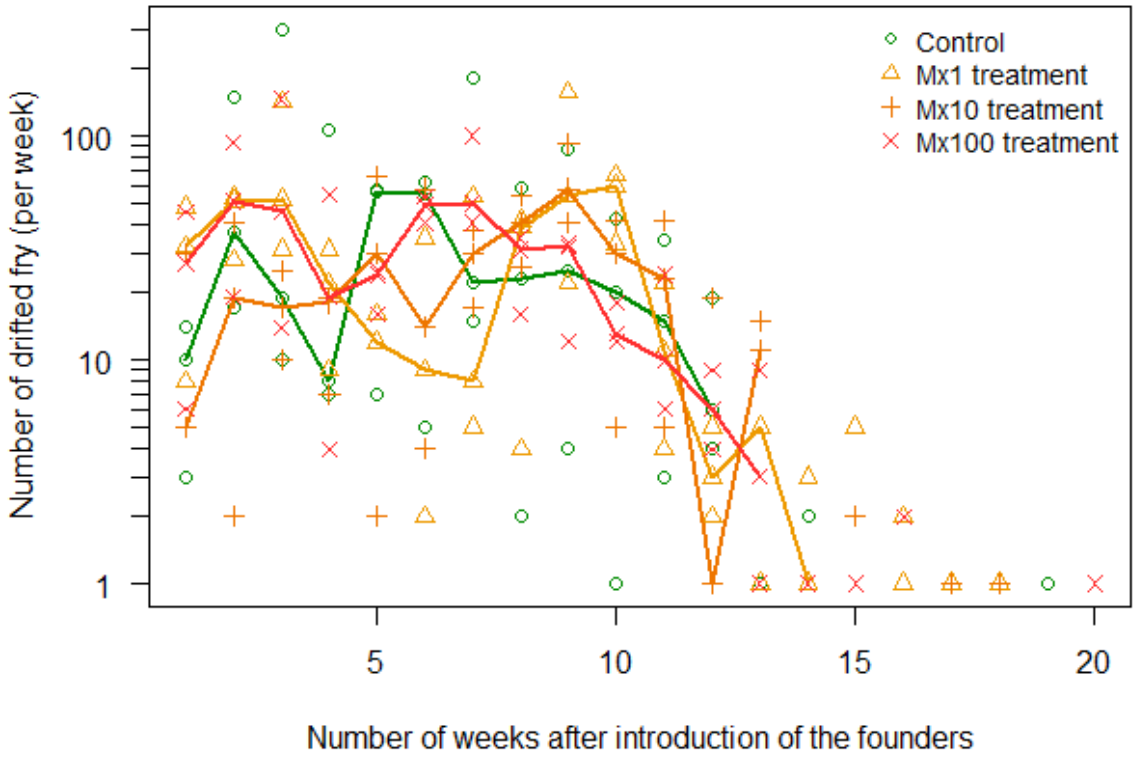

Figure 2: Cumulated number of drifted fry per week observed in mesocosms for each mixture treatment level (control, Mx1, Mx10 and Mx100). Points represent the observations made in mesocosms, and the lines represent the median per treatment.

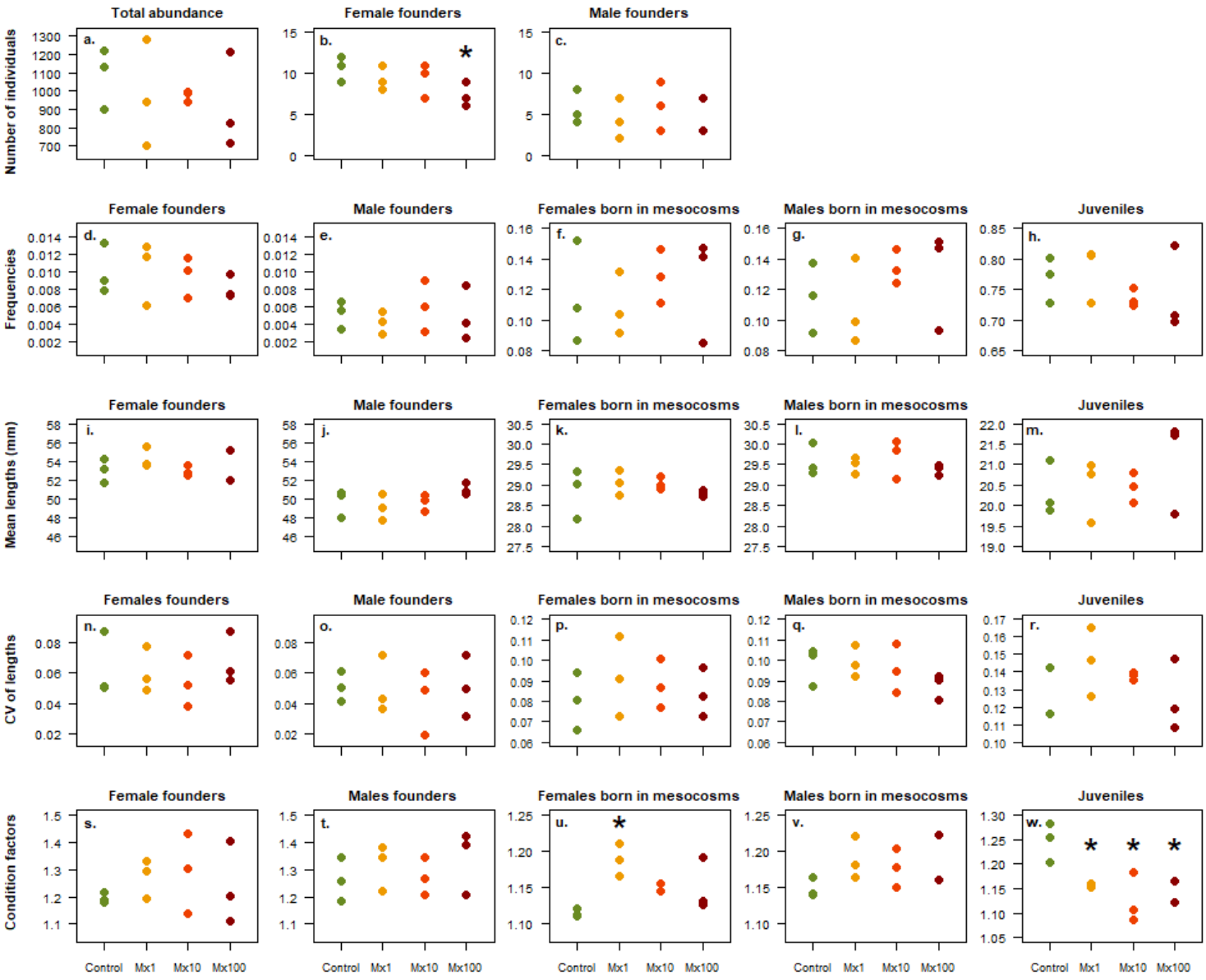

Figure 3: Observations of the descriptive variables of the population for each mixture treatment level (control, Mx1, Mx10 and Mx100). Asterisks indicate significant difference between treatments and control (p-value < 0.05).

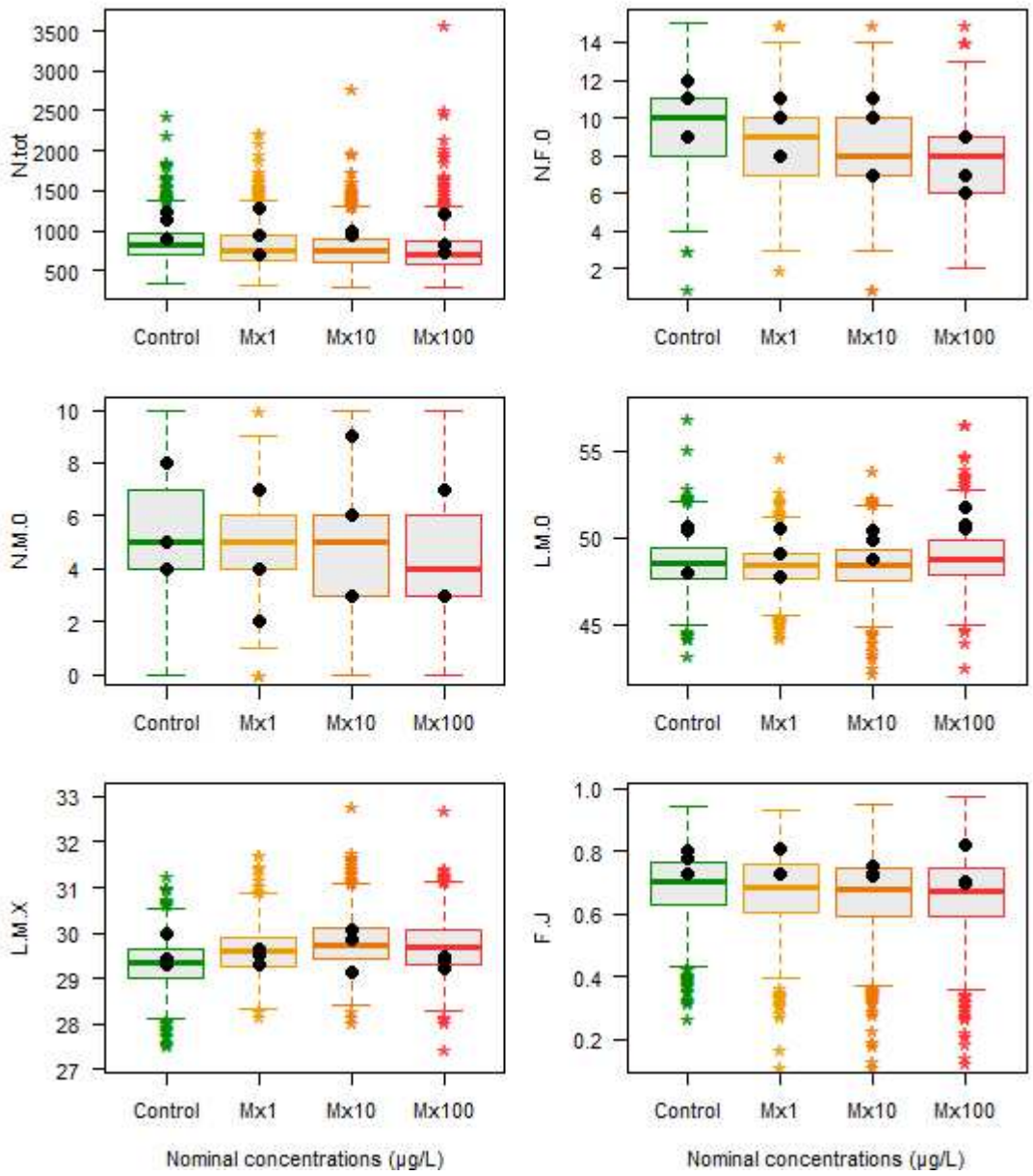

Figure 4: Simulated and observed population endpoints for each the mixture treatment level (Mx0, Mx1, Mx10 and Mx100) assuming a chronic mortality effect on all fish. Boxplots represent the simulations of the model (n=1,000 per treatment) whereas the points represent the observations made in the mesocosms. Population endpoints were observed or simulated at the end of the experiment (N.tot: total abundance of fish, N.F.0: number of the female founders, N.M.0: number of the male founders, L.M.0: mean length of the male founders, L.M.X: mean length of the males born in mesocosms and F.J: frequency of juveniles).

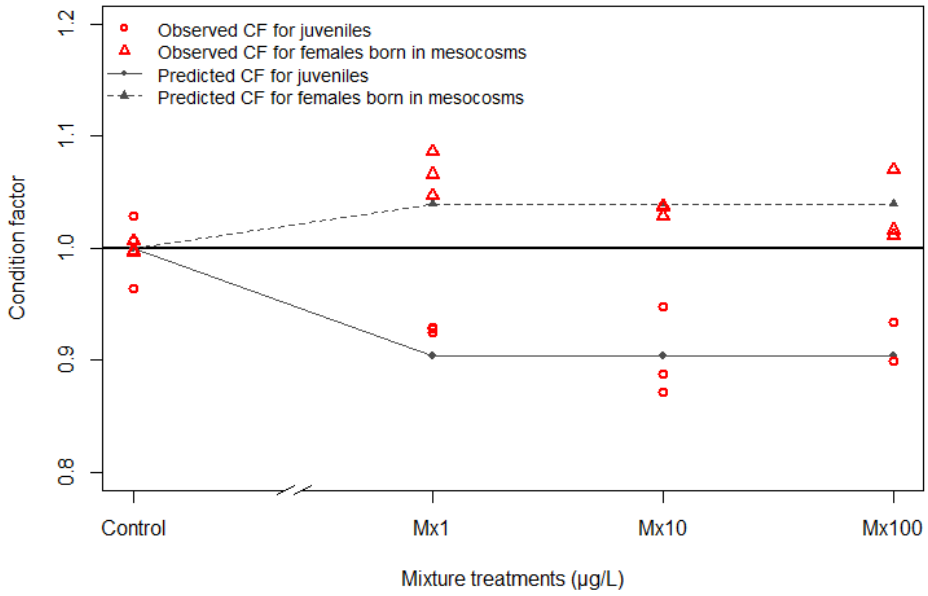

Figure 5: Simulated and observed mean condition factors for the juveniles and females born in mesocosms for the different mixture treatment levels. The lines represent the predictions for the juveniles (full line) and females born in mesocosms (dashed line) whereas the red points represent the observations (circles for juveniles and triangles for females born in mesocosms). CF: Condition factor.

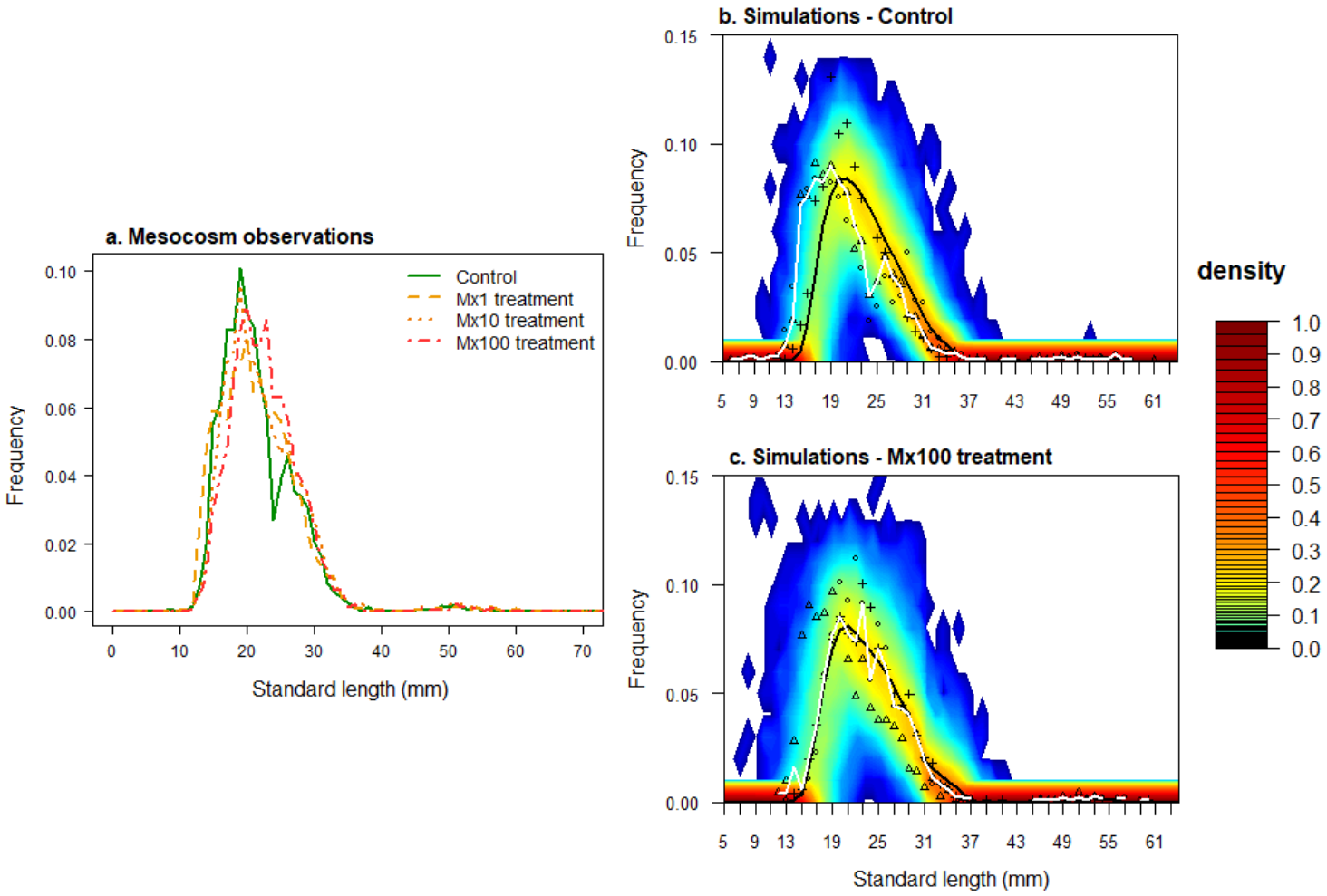

Figure 6: Observed probabilistic distributions of the length frequency in the mesocosms (a) and predicted by the model in the control (b) and Mx100 (c) mixture treatment. On figure a, length frequency distributions are the mean of the 3 populations observed in each treatment. The green full line represents the mean length frequency in the control whereas the Mx1, Mx10 and M100 treatments are represented by a yellow dashed line, orange dotted line and red dot-dashed line respectively. For the figures b and c, different symbols represent the observed length frequency distributions in mesocosms. Full black and white lines represent the median length frequency distributions of the simulated and observed populations respectively. Color level represents the frequency of simulated populations (n = 1000) having a given percentage of individuals for a given class length. Frequencies smaller than 1e-04 are represented in white.

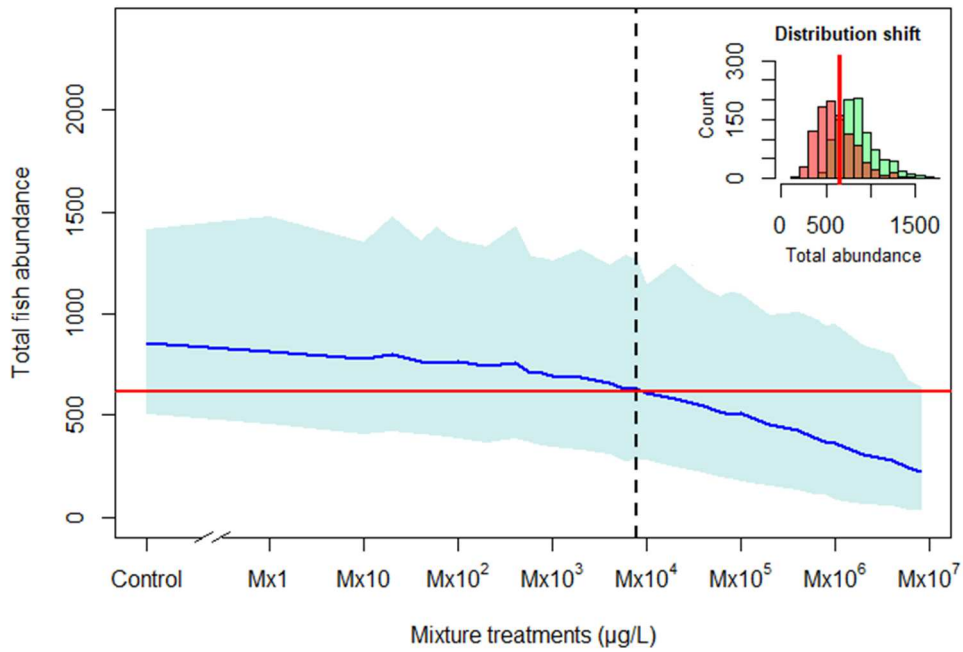

Figure 7: Population abundance with concentrations extrapolated from $Mx10^2$ to $Mx10^7$. The blue line represents the median of the simulations and the blue light zone represents the 95% prediction interval. The dotted black lines represent the first extrapolated mixture concentration where 80% of the distribution of the total abundance shifted towards to the lower abundances compared to the control distribution. The red lines represents a difference in means equal to 1 SD (EFSA et al. 2017). On the top right panel, the green distribution represents the control distribution whereas the red distribution represents the distribution for the extrapolated Mx8000 treatment. The red line represents 80 % the red distribution shifted towards lower abundances compared to the green distribution.

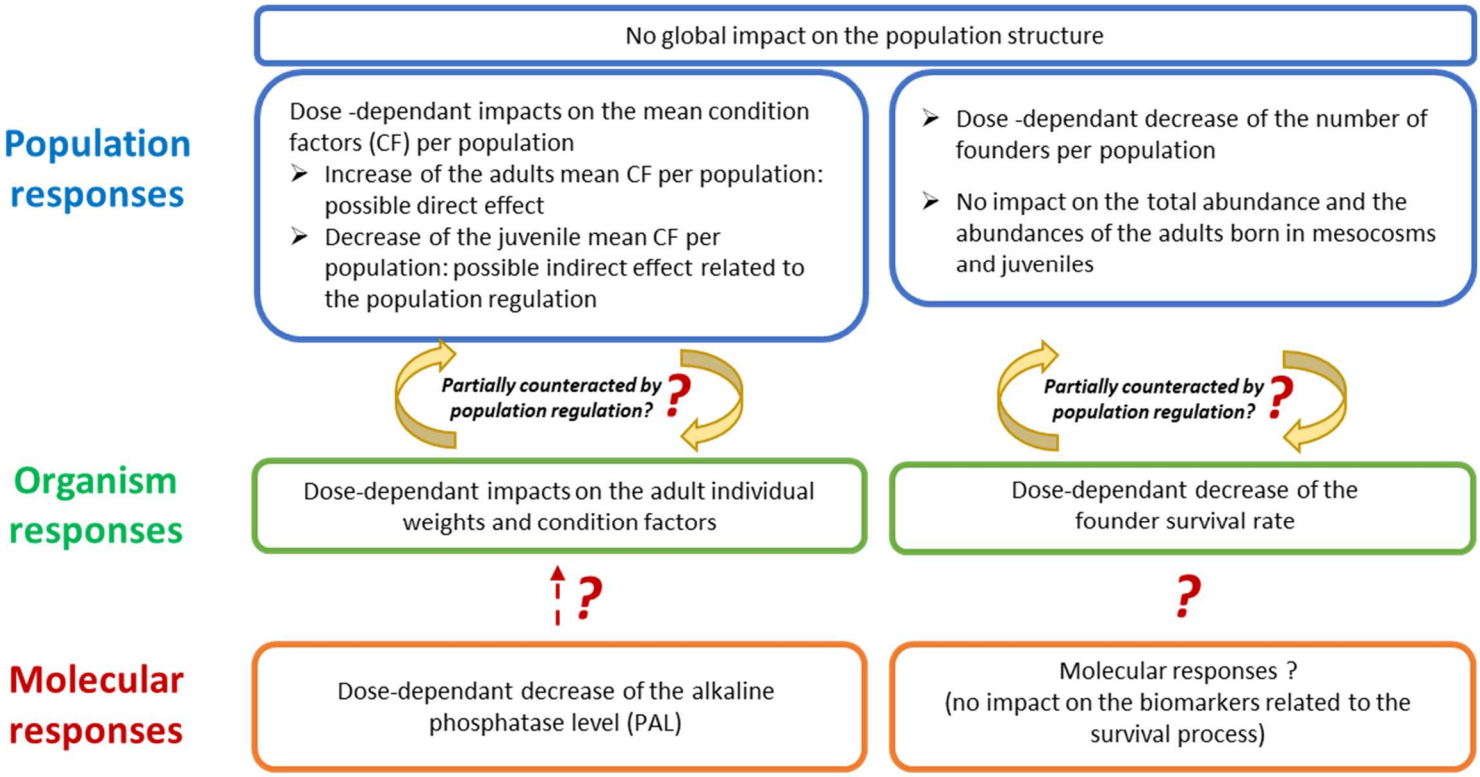

Figure 8: Conceptual representation of the mixture impacts at the different level of biological organization (molecular, organism and population).