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Corentin Mit, Rémy Beaudouin, Olivier Palluel, Cyril Turiès, Gaëlle Daniele, et al.. Exposure and hazard of bisphenol A, S and F: a multi-biomarker approach in three-spined stickleback. *Environmental Science and Pollution Research*, 2023, 10.1007/s11356-023-28462-4 . ineris-04208928

**HAL Id: ineris-04208928**

**<https://ineris.hal.science/ineris-04208928>**

Submitted on 7 Nov 2023

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# Exposure and hazard of bisphenol A, S and F: a multi-biomarker approach in three-spined stickleback

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## Abstract

Due to the oestrogenic behaviour of bisphenol (BP) A, industries have developed many substitutes, such as BPS and BPF. However, due to their structural similarities, adverse effects on reproduction are currently observed in various organisms, including fish. Even if new results have shown impacts of these bisphenols on many other physiological functions, their mode of action remains unclear. In this context, we proposed to better understand the impact of BPA, BPS and BPF on immune responses (leucocyte sub-populations, cell death, respiratory burst, lysosomal presence and phagocytic activity) and on biomarkers of metabolic detoxification (ethoxyresorufin-O-deethylase, EROD, and glutathione S-transferase, GST) and oxidative stress (glutathione peroxidase, GPx, and lipid peroxidation with thiobarbituric acid reactive substance method, TBARS) in an adult sentinel fish species, the three-spined stickleback. In order to enhance our understanding of how biomarkers change over time, it is essential to determine the internal concentration responsible for the observed responses. Therefore, it is necessary to explore the toxicokinetics of bisphenols. Thus, sticklebacks were exposed either to 100 µg/L of BPA, BPF or BPS for 21 days, or for seven days to 10 and 100 µg/L of BPA or BPS followed by seven days of depuration. Although BPS has very different TK, due to its lower bioaccumulation compared to BPA and BPF, BPS affect oxidative stress and phagocytic activity in the same way. For those reasons, the replacement of BPA by any substitute should be made carefully in terms of risk assessment on aquatic ecosystems.

**Keywords** : Bioaccumulation, Bisphenols, three-spined stickleback, Immune responses, Metabolic detoxification, Oxidative stress

# 1. Introduction

Bisphenol A (BPA) was the first bisphenol synthesized in 1891 (Dodds and Lawson 1936). Its estrogenic properties were described from the 1930s onwards (Dodds *et al.* 1938). However, its use as a synthetic oestrogen was not pursued because its oestrogenic potency was too low compared to that of diethylstilbesterol (DES). The production of plastics from BPA started in the 1950s (Faheem and Bhandari 2021) and until the 2010s, BPA was found in many everyday products like cans and tins (inside coating made of BPA protected the metal from corrosion) or in thermal papers. Due to its progressive ban, BPA is gradually replaced by its substitutes (ECHA 2022). Currently, the bisphenol family counts 148 substances including 17 bisphenols sharing the same generic structure and structurally related bisphenol derivatives (ECHA/NR/22/08) (ECHA 2022). Among them, the BPF and BPS are widely used (Faheem and Bhandari 2021, Wang *et al.* 2022, Wu *et al.* 2018). In order to reduce the risk for humans and the environment, ECHA highlighted the need to collect more data to characterize their physiological effects and determine the associated risks.

One of the environmental risks concerns aquatic ecosystems, where BPs are ubiquitous due to their very high production (Flint *et al.* 2012). For example, BPA has been detected worldwide in various types of water from concentrations ranging from a few ng/L to hundreds of µg/L (Huang *et al.* 2012), with an average value inferior to 12 µg/L in aquatic ecosystems (Flint *et al.* 2012). BPA substitutes have also been detected in waterways at slightly lower concentrations, in the order of a few ng/L (Faheem and Bhandari 2021). This risk is also linked to the bioaccumulation capacities of BPs, demonstrated in fish sampled in the field (Lv *et al.* 2019, Miège *et al.* 2012, Zhao *et al.* 2021 777) or from controlled laboratory exposure. Some recent publications have shown that substitutes, such as BPAF, BPB and BPZ, have a higher bioaccumulation potential than BPA (Wang 2020). Currently, few bisphenols breaks this rule, e.g. BPS, this certainly due to its lowest logKow among bisphenols (Moreman *et al.* 2017, Wang *et al.* 2020).

Furthermore, environmental risks are also linked to the impact of BPs on physiological functions. Due to the endocrine disrupting properties of BPs, reproductive function has been extensively studied, especially regarding the induction of vitellogenin (Andersson *et al.* 2007, Le Fol *et al.* 2017, Lindholst *et al.* 2000, Mihaich *et al.* 2012, Schultz *et al.* 2001, Sun *et al.* 2014). Nevertheless, the impact on other physiological functions, such as oxidative stress, metabolic detoxification or immune responses, has also been studied (Faheem and Bhandari 2021, Qiu *et al.* 2018a, Qiu *et al.* 2018b, Qiu *et al.* 2018c). Currently, there is a flagrant lack of information in terms of overall physiological impact.

Thus, the present study aimed to better understand the impact of three different BPs (BPA, BPS, BPF), on oxidative stress, metabolic detoxification and innate immune responses, in adult three-spined stickleback (*Gasterosteus aculeatus*), a species often used to investigate the effects of EDCs (Bado-

Nilles *et al.* 2014, Jolly *et al.* 2009, Porseryd *et al.* 2019, Prokkola *et al.* 2016). Two types of exposure scenario (21-day exposure and seven-day exposure followed by seven-day depuration) were performed to better understand the physiological responses of sticklebacks. During the first scenario (21 days of exposure, which correspond to the standard duration of a regulatory test), the sticklebacks were exposed to 100 µg/L of BPA, BPF or BPS. In the second scenario, sticklebacks were exposed for seven days, achieving the steady state of internal concentration (Lindholst *et al.* 2001a, Lindholst *et al.* 2003), to 10 and 100 µg/L of BPA or BPS followed by seven days of depuration. The concentration of 10 µg/L is based on the average BPA concentration found in the field (Flint *et al.* 2012) and the 100 µg/L, even if observed in the field (Huang *et al.* 2012), were mainly used to obtain effects on biomarkers used. In addition, the BP kinetic of accumulation were analyzed to better understand the dynamics of biomarker responses. In order to complete the mass balance of the substance, which involves understanding the proportion of the substance that is internalized versus the proportion that is metabolized or excreted, the liver, the blood and the carcass, including the contains the skin, the muscle and the skeleton, were analysed.

## 2. Materials and Methods

Experimental protocols were conducted following the European directive 2010/63/UE for the protection of animals used for scientific purposes at INERIS, registration number E60–769–02. The experimental protocols were submitted and reviewed by a French nationally recognized ethical committee, CREMEAPS number 96. The approved project has the registration number #15757.

### 2.1. Model species: the stickleback

The 1020 adult three-spined sticklebacks used in this study come from the breeding facilities of INERIS and were selected according to their size ( $> 35\text{mm}$ ) and the absence of signs indicating an active reproduction phase. Concerning this last point, all the experiments took place between mid-October and the end of December, which is out of breeding period (Wootton 1984). The sex of sticklebacks was determined using head morphology model (de Kermoisan *et al.* 2013).

During each experiment, fish were monitored daily for survival, aeration maintenance, and waste cleanup. A scoring list covering a wide range of clinical aspects was used to provide an overview of the health status of animals over time. Thresholds of severity were used for fish presenting clinical signs to end test procedure earlier to avoid pain or prolonged distress. Fish were fed *ad libitum* with bloodworms every day except the day before sampling.

### 2.2. 21-day exposure

Each BP (99 %, Sigma Aldrich) was diluted in dimethylsulfoxide (DMSO at  $1/2000^{\text{th}}$ ) as they have poor water solubility. A flow-through exposure was carried out over 21 days, using the following nominal concentrations of  $0\ \mu\text{g/L}$  (control DMSO) and  $100\ \mu\text{g/L}$  of BPA, BPS or BPF. The 260 fish ( $52.7 \pm 5.6\ \text{mm}$ ,  $1.8 \pm 0.6\ \text{g}$ ) were exposed into 26 aquaria divided into two identical benches (one bench by sex to avoid aggressive behaviour) containing each four aquaria for controls and three aquaria by BPs. Ten individuals by sex were randomly placed in each 8-L aquarium four days before exposure. To reduce the stress due to “mirror induced aggression”, grey opaque plastic plates and pictures of aquatic decoration were placed on all sides. The temperature ( $16 \pm 1\ ^\circ\text{C}$ ), the oxygen saturation ( $90 \pm 5\ \%$ ), the pH ( $7.0 \pm 0.1$ ), and the conductivity ( $348 \pm 5\ \mu\text{S/cm}$ ) were stable along the experiment regardless of the BP used. Exposure concentrations were measured in the aquarium water at 1, 3, 8, 15 and 21 days after the beginning of the exposure. Ten males and ten females were sampled by condition at four dates: prior to the exposure (Day 0), and at seven (Day 7), 14 (Day 14) and 21 days (Day 21) post-exposure.

### 2.3. Seven-day exposure and seven-day depuration

As for the 21 days of exposure, BPA and BPS (99 %, Sigma Aldrich) were diluted in dimethylsulfoxide (DMSO at 1/2000<sup>th</sup>). A flow-through exposure was carried out over 7 days, using the following nominal concentrations of 0 µg/L (control DMSO), 10 µg/L and 100 µg/L of BPA or BPS. The week of exposure was followed by 7 days of depuration. The 380 fish by BP (BPA: 48.8 ± 4.4 mm, 1.7 ± 0.4 g; BPS: 41.0 ± 3.5 mm, 0.9 ± 0.3) were exposed into 38 aquaria divided into two identical benches (one bench by sex to avoid aggressive behaviour) containing each seven aquaria for controls and six aquaria by BP concentrations. Ten individuals by sex were randomly placed in each 8-L aquarium four days before exposure. To reduce the stress due to “mirror induced aggression”, grey opaque plastic plates and pictures of aquatic decoration were placed on all sides. The temperature (16 ± 1 °C for both BP), the oxygen saturation (95 ± 5 % for BPA; 89 ± 4 % for BPS), the pH (7.0 ± 0.1 for both BP), and the conductivity (360 ± 2 µS/cm for BPA; 347 ± 3 µS/cm for BPA) were stable along the experiment. Ten males and ten females were sampled by condition at six dates. The first four dates relate to the seven days of exposure and correspond to 5 h, (Day 0,25), 24 h (Day 1), 96 h (Day 4) and 168 h (Day 7). The last two dates relate to the seven days of depuration and correspond to 24 h (Day 8) and 168 h (Day 14) post-exposure. Exposure concentrations were measured at 2, 4, 8, 24, 48, 72, and 168 h in the aquarium water.

## **2.4. Tissue sampling**

At each sampling date, fish were anaesthetized by immersion in MS222 (tricaine methanesulfonate, 100 mg/L, Tricaine Pharmaq, Overhalla), once unconscious animals were sacrificed by fracture of the spine at the base of the brain, measured, weighed and sampled. Blood, liver and the carcass of the fish (the whole fish minus the GIT, gonads, spleen and liver) were sampled to measure BPA and BPS bioaccumulation. In parallel, blood, liver, kidney and spleen were also sampled for biomarker measurements. Except for the blood and the spleen of the fish, each organ was weighted prior to sampling. All the samples were stored at -80°C before analysis except the spleen (analyses on fresh tissue).

## **2.5. Bisphenol quantification**

The Table 1 to 3 provide information on the limit of quantification for each BP and each matrix.

### **2.5.1. Water analysis**

Bisphenol water concentrations were monitored at each sampling time to measure the actual exposure concentrations in the tank sampled. After 10 min in an ultrasonic bath, an aliquot (900 µl) of the water sample was added to 100 µl of MeOH containing the internal standards for BPA, BPS and BPF. After homogenization, 10 µl of this mixture were injected and analyzed in LC-MS/MS.

### **2.5.2. Tissues analysis**

Quantification methods were optimized for each of the three compartments and for each BP.

A liquid-liquid microextraction was performed onto 20 mg of fresh liver or 20  $\mu$ L of blood in 2 mL-screw tubes using 1 mL ACN/MeOH (50:50, v/v). The tube was vortexed, shaken with a Beadbug homogenizer (320 rpm), and then placed for five minutes in an ultrasonic bath before centrifugation at 4°C for 10 minutes at 10 000 rpm. Then, 800  $\mu$ l of supernatant was transferred to a new 2 mL-tube before being evaporated to dryness in a miVac Centrifugal Concentrator (Genevac TM) for 45 min at 35°C. The dry residue was dissolved in 800  $\mu$ l H<sub>2</sub>O/MeOH (90:10, v/v), except for the analysis of BPS in blood where it was dissolved in 200  $\mu$ l H<sub>2</sub>O/MeOH (90:10, v/v) to be concentrated four times, and 5  $\mu$ L of this mixture was injected in LC-MS/MS for analysis of BPA or BPS.

Stickleback carcasses were freeze-dried for 48h, subsequently ground with a mortar. Then, the same microextraction as described above for BPS analysis in blood was performed on 25 mg of the freeze-dried carcass for BPS analysis. For BPA, the microextraction was performed with 1.5 mL ACN/MeOH (50:50, v/v) and a purification of the supernatant (600  $\mu$ L) was realized by dSPE on a PSA/C18 phase. Then, after being shaking and centrifugation, 250  $\mu$ L of the extract was evaporated to dryness and reconstituted in 125  $\mu$ L H<sub>2</sub>O/MeOH (90:10, v/v) before LC-MS/MS injection.

## **2.6. Biomarker analysis**

### **2.6.1. Biometric indices**

Gonad, liver, and kidney weights were recorded to calculate physiological biometric indices. The gonado-somatic (GSI), hepato-somatic (HSI) and nephro-somatic (KSI) indexes (Catteau *et al.* 2019) were calculated using the following formula:  $\text{Index} = (\text{Organ mass}/\text{Total Body Mass}) \times 100$ .

### **2.6.2. Biomarkers of metabolic detoxification and oxidative stress**

Biomarkers of metabolic detoxification as well as biomarkers of oxidative stress were measured using the S9 protein fraction of the liver (Sanchez *et al.* 2007). Briefly, livers were grinded with glass beads and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant of each sample (post-mitochondrial fraction, S9) was placed in 1 mL microtubes. Protein concentration was assessed using the Bradford method (Bradford 1976) with bovine serum albumin (Sigma-Aldrich, USA) adapted for microplates.

Two detoxication enzymes, the ethoxyresorufin-O-deethylase (EROD) and the glutathione-S-transferase (GST) were chosen. The activity of those enzymes (EROD, pmol/min/mg and GST, U/g of protein) was measured on S9 liver fractions using a liquid handling automaton (Freedom EVO®, Tecan, Switzerland) and a microplate reader (Synergy™ H4 Hybrid, BioTek, USA) following Flammarion *et al.* (2002) and Habig *et al.* (1974) methods. Hepatic biomarkers linked to oxidative

stress were also measured namely glutathione peroxidase (GPx, U/g of protein) and lipid peroxidation with thiobarbituric acid reactive substance method (TBARS, nmol/g protein) (Ohkawa *et al.* 1979).

### 2.6.3. Immunomarkers

Immune parameters were measured using the fish spleen which was pressed against 40  $\mu\text{m}$  sterilized nylon mesh with 5 mL Leibvotitz 15 (L15) medium (Sigma) containing lithium heparin (100 mg/L, Sigma), penicillin (100 mg/L, Sigma), and streptomycin (100 mg/L, Sigma) in order to keep only the leucocytes in suspension (Bado-Nilles *et al.* 2014). This solution was then stored at  $-4^{\circ}\text{C}$  and analysed the following day. Innate immune biomarkers were measured from the leucocyte suspension using a MacsquantX flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). To compare each sample, the leucocyte concentration was normalized to  $10^6$  cells/ mL of culture medium.

Leucocyte sub-populations (granulocytes-macrophages), as a percentage of the total leucocyte population (or number of cells), was identified by their size and complexity using forward scatter (FSC) and size scatter (SSC) parameters.

Cell death was characterized by the percentage of cells in apoptosis and necrosis using a double labelling with Yo-PRO®-1 (Thermo Fisher Scientific, USA, final concentration: 3.14 mg/L) and propidium iodure (Thermo Fisher Scientific, USA, final concentration: 5.01 mg/L) probes to obtain fluorescence of apoptotic (FL1, green fluorescence) and necrotic (FL3, red fluorescence) cells.

Leucocytes respiratory burst was also characterized (Chilmonczyk and Monge 1999). In short, 2',7'- dichlorodihydrofluorescein diacetate acetyl ester ( $\text{H}_2\text{DCF-DA}$ , Thermo Fisher Scientific, USA, final concentration: 29.30 mg/L), a stable non-fluorescent molecule was hydrolysed to dichlorodihydro-fluorescein diacetate (DCFH) by cytosolic enzymes. DCFH was then oxidized by ROS to the fluorescent dichlorofluorescein (DCF) to quantify unstimulated and cells stimulated by phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA, final concentration: 9.25 mg/L) in FL1. The index of respiratory burst was determined as the ratio of fluorescence of PMA stimulated cells ( $\text{H}_2\text{DCF-DA}$  plus PMA) to that of unstimulated cells ( $\text{H}_2\text{DCF-DA}$ ).

Lysosomal presence in leucocytes were assessed using acridine orange (AO, Sigma, USA), a lysosomotropic weak base, and fluorescence measurement in FL3 (in MFI, fluorescence unit) (Bado-Nilles *et al.* 2013).

Finally, phagocytic activity was assessed through two parameters, leucocyte adhesion capacity and internalization efficiency, measured in % of total cells was evaluated with fluorescent microsphere at a concentration of  $2.7 \times 10^7$  particles/mL (Fluorospheres® carboxylate-modified microsphere, diameter 1  $\mu\text{m}$ , Thermo Fisher Scientific, USA) (Gagnaire *et al.* 2004). Phagocytic capacity corresponded to the fluorescence of at least one bead and phagocytic efficiency to the fluorescence of at least three beads.



## **2.7. Statistical analysis**

Statistical analysis was performed on each immunomarker response over time. All responses were log-transformed. At each date, a one-way ANOVA followed by a post-hoc Dunnett test was used to determine if a true difference existed between conditions and control. When normality and homoscedasticity were rejected, a non-parametric post-hoc many to one comparison test was performed.

## 3. Results

### 3.1. Water concentration

The chemical monitoring of the different exposures was carried out throughout the whole duration of each exposure.

Regarding the long-term exposure (Figure 1), even if concentration was randomly assessed only in one tank for each condition, stable concentrations were measured starting the first day of exposure for both female and male fish except male fish exposed to BPS. The measured concentrations (mean  $\pm$  SD, calculated between day 1 and day 21) were  $56.1 \pm 6.7$   $\mu\text{g/L}$  for the BPA,  $74.3 \pm 10.1$   $\mu\text{g/L}$  for BPF and around  $92.8 \pm 2.7$   $\mu\text{g/L}$  for female fish exposed to BPS. For aquaria with male fish, BPS concentration was not stable ( $58.9 \pm 44.4$   $\mu\text{g/L}$ ). The analysis of water samples showed a malfunction of the exposure system and that the fish were not exposed correctly after the seventh day of exposure (actual exposure  $< 20\%$  of the nominal, raw measures presented in SI).

In the case of BPA short-term exposure (Figure 2), a stable concentration of around 50 % of the nominal value of 10  $\mu\text{g/L}$  was reached within the first hours (mean  $\pm$  SD, calculated between day 1 and day 7;  $5.2 \pm 1.2$   $\mu\text{g/L}$ ). For the nominal dose of 100  $\mu\text{g/L}$  (Figure 3), a stable and similar concentration for both sexes was reached from the 2<sup>nd</sup> day of exposure and was of  $64.6 \pm 3.0$   $\mu\text{g/L}$ .

Regarding BPS short-term exposure (Figure 2), for the dose of 10  $\mu\text{g/L}$ , a stable concentration is reached within the first day of exposure and reached roughly its nominal value ( $9.5 \pm 0.4$   $\mu\text{g/L}$ ). Stable concentrations were also reached within the first day for the 100  $\mu\text{g/L}$  condition:  $95.6 \pm 4.5$   $\mu\text{g/L}$  (Figure 3).

### 3.2. Organ concentrations

#### 3.2.1. BPA kinetics

The Table 1 concerned the BPA bioaccumulation data obtain for the two sex in in liver (Figure S1, A and B), in blood (Figure S2, A and B) and in carcass (Figure S3, A and B) In addition, BCFs were calculated for each organ based on the ratio of the concentrations measured in organs and measured in the water (see Table 2).

BPA was quickly absorbed and already measurable in the organs for the highest nominal dose after five hours of exposure. For the 10  $\mu\text{g/L}$  dose, BPA was only measurable from the first days. The organ which bioaccumulated BPA the most was the liver, then the carcass and finally the blood. On Table 2, it can be noticed that the BCF calculated in female liver (concentration of 928 ng/g) for the

seventh day of exposure was twice the one calculated in male liver (concentration of 428 ng/g). Roughly, the concentrations measured in fish exposed to the highest nominal dose were higher than the lowest dose. In most cases, there was not consistent trend regarding sex bioaccumulation difference in any organ.

Regarding excretion, BPA were hardly measurable starting the first day of depuration (concentrations below the LQ). In most cases, the concentrations were below the limit of quantification at day 14. Nevertheless, BPA was still measured in female fish liver the seventh day of depuration and reached 20.2 ng/g (Figure S1B).

### **3.2.2. BPS kinetics**

The bioaccumulation of BPS was also assessed in liver, blood and fish carcass (Table 3 and Figure S4). For the lowest nominal dose (10 µg/L) of BPS, no bioaccumulation was detected in any organ. Moreover, BPS was not detected in blood for the nominal dose of 100 µg/L of BPS. For this reason, only the bioaccumulation of BPS in liver is shown on Figure S4A and in carcass on Figure S4B. Bioaccumulation in liver was higher than bioaccumulation in carcass (no bioaccumulation in blood). In female liver, BPS was found within the first hours of exposure and reached 4.1 ng/g. The highest concentration was measured in female liver and reached 30 ng/g when the highest concentration in carcass was only 5.4 ng/g for the female. Overall, as it can be seen on the Table 2, all BCF that could be calculated for BPS were equal or below 0.1.

## **3.3. Biomarker analysis**

Data shown on figures 3, 4, 5 and 6 correspond to biomarker responses measured in male and female fish together as no statistical difference was found between sex. In addition, biological indices, granulocyte population percentage and GPx measured in male and female fish are presented in SI. As analytical issues were detected, the response of GST and TBARS for BPA short-term exposure are not shown on Figure 6.

### **3.3.1. Common effects of bisphenols on the biomarker responses**

Among all biomarkers, only ROS B (Figure 3A, 3B and 3C), respiratory burst index (Figure 3D to F) and phagocytic activity (Figure 4A to 4G) seemed to be clearly affected by the three bisphenols.

In details, during the long-term exposure, BPA, BPS and BPF were inducing ROS B from day 7 to day 21 included. Considering the short-term exposures, induction was found to be significant for

both BPA and BPS and both doses. In addition, this effect of induction was also measurable during the depuration phase at day 8 and day 14 for both doses.

Bisphenols had also an effect of inhibition on respiratory burst index at day 7. This effect was found to be still significant at day 14 for BPA and only for BPS and BPF at day 21. Significant inductions were measured for both doses of BPA (Figure 3E) during exposure phase and during depuration phase for both doses. Regarding BPS short-term exposure (Figure 3F), both doses were inhibiting respiratory burst index. During depuration phase, the inhibition was still significant for the 10 µg/L dose at day 14. Nevertheless, responses measured in fish during the short-term exposures were rather unstable and shown both induction and inhibition depending on the sampling date.

Phagocytosis activity (Figure 4A and 4D) was induced significantly by BPA, BPS and BPF. Both adhesion capacity and internalization efficiency were induced at day 7. Nevertheless, only BPF and BPS at day 14 were associated with a significant induction of both biomarkers. In contrast, no significant effect was found during 100 µg/L BPA short-term exposure for adhesion capacity. Still, both biomarkers were induced during exposure and depuration for at least one dose. For BPS short-term exposure, both doses were found to inhibit adhesion capacity at day 0.25 and 8. The same effect was observed for internalization efficiency at day 0.25. Induction effects were also measured for both BPS doses.

Considering lysosomal presence (Figure 3G to 3I), fish exposed to the three bisphenols shown only biomarker induction for BPF at day 7 and for BPS and BPF for day 14. However, BPA short-term exposure shown significant induction for both doses and for both exposure and depuration. Only 100 µg/L dose of BPS was found to induce lysosomal presence during exposure but both doses induced lysosomal presence during depuration phase.

EROD activity was inhibited only by BPF at day 21 (Figure 6A). However, BPA was also able to inhibit EROD during the short-term exposure at day 7 and 14 for the highest dose (Figure 6B). On the contrary, a significant induction was measured at day 7 for the highest dose of BPS followed by a significant inhibition at day 14 (Figure 6C).

### **3.3.2. Different biomarker responses between the three bisphenols**

Some biomarkers seemed to be only affected by two of the three bisphenols. Granulocyte-macrophage population in male (Figure S7A to 7C) was inhibited by BPA and BPS. During the long-term exposure, no effect was found significant. BPS short-term exposure shown inhibition of granulocyte-macrophage population for the highest dose. However, effects of induction were also significant at day 1 and 8 for the high dose of BPS in male fish (Figure S7C). Cellular mortality was mostly induced in both long-term and short-term exposure by BPS and BPA. In particular, this induction was measured for both BPA doses and in exposure and depuration phase. For BPA short-term exposure, inhibition was also significant at day 4 and 7 at the highest dose.

TBARS measurement in fish (Figure 6F and 6G) was modulated by BPS and BPF. During the long-term exposure, this parameter seemed to be inhibited (day 14). A significant inhibition was measured within the first hours for both doses of BPS and then significant inductions at day 7 (10 and 100 µg/L).

Finally, some biomarker responses were only significantly modulated by one bisphenol.

BPS was able to modulate GST (Figure S6E and S6G). Indeed, significant inhibitions were measured within the first hours of the short-term exposure. Then, the parameter shown significant induction before depuration phase. GST was significantly inhibited at day 8 at the highest dose.

In female fish, GPx was significantly inhibited by BPA for both doses during the short-term exposure and the depuration (Figure S7F).

## 4. Discussion

In this work, we have performed two types of exposure schedules (a 21-day exposure and seven-day exposure followed by seven-day depuration) to better understand the impact of three different BPs (BPA, BPS, BPF), on oxidative stress, metabolic detoxification and innate immune responses, in the three-spined stickleback. To complete the study of the toxicodynamics (TD), the toxicokinetics (TK) of BPA and BPS were also assessed during the short-term exposure.

During the second exposure scenario (seven-day exposure, seven-day depuration), a significant difference was observed between BPA and BPS TK. Indeed, BPA was measurable in all matrices for both doses whereas BPS was only measurable for the highest nominal dose (100 µg/L), and only in liver and in carcass. Those difference of bioaccumulation were already noted by Wang *et al.* (2020), as BPS was the bisphenol with the lowest BCF for all carp organs. In addition, BPA was quickly found (less than 5 hours) in all organs for both doses when BPS was first measurable in liver, then in carcass after one day of exposure. For BPA, those findings were in accordance with Lindholm *et al.* (2001b) which detected BPA within the first two hours of exposure in trout liver, muscle and blood. It was difficult to assess a difference of bioaccumulation of BPA or BPS between sex. For male, an important variability was measured during the seven-day exposure for both bisphenols. In particular, the large variability in the liver regarding bioaccumulation of BPA was already noted in Chen *et al.* (2017) and for other bisphenols (Chelcea *et al.* 2022). However, due to natural inter-individual variability, chemical analysis of a single fish seems insufficient. Nevertheless, the maximal BCF measured in male stickleback liver the 7<sup>th</sup> day of exposure to BPA reached 13.2 which is in accordance with range of BCFs described in Chelcea *et al.* (2022) (6.46-19.2).

Interestingly, our study highlights the importance of taking into account both TK and TD when comparing bisphenol effects, at least regarding BPA and BPS. Indeed, as noticed, BPA concentrations in organs largely exceeded BPS concentrations. However, effects induced by BPA, such as ROS basal level or phagocytosis activity induction were also induced by BPS with a similar intensity. In addition, remarkably, in fish, endocrine disrupting effects of BPF and BPA were described to be stronger than the effects of BPS (Moreman *et al.* 2017). Indeed, BPS did not trigger the transcription of genes related to *Vtg* and oestrogen receptors whereas BPA and BPF triggered their transcriptions (Mu *et al.* 2018). In this study, we showed that immune modulation triggered by BPS was of the same level as BPA. Moreover, considering the greater stability of the immune disturbance measured with BPS, it could indicate a greater toxicity of this substance regarding this biological function and also a difference in terms of mode of action between BPA and BPS. Such discrepancy was already noticed in Frenzilli *et al.* (2021) regarding thyroid function and glucose balance. All

these results support the need to study various biological functions and not only those related to endocrine disruption. More surprisingly, depending on the bisphenol considered, the dynamic of the effects seemed to be different over time. BPA would trigger an induction less stable than BPF and BPS. BPS was shown to interact with ER $\alpha$  on macrophages and mediates immune response (Qiu *et al.* 2018a), as it was also observed for the BPA (Yang *et al.* 2015). However, BPS mode of action would be slightly different than this of BPA: BPS activates both ER $\alpha$  and ER $\beta$  whereas BPA only interacts with ER $\alpha$  (Qiu *et al.* 2018b). It could partly explain the difference observed in terms of effects between BPA and BPS. Nevertheless, in Qiu *et al.* (2018a), for the nominal dose of 100  $\mu$ g/L, BPS and BPF were showed to have similar immunotoxic effects than BPA whereas some parameters we measured, such as the characterization of macrophage population, shown no modulation for BPF but was significantly inhibited by BPS. However, the differences between the two studies could be due to the fact that Qiu *et al.* (2018a) had performed their study on zebrafish larvae and not on adult fish. Indeed, metabolic and regulation pathway are not fully developed in larvae and could be the reason of this discrepancy (Billat *et al.* 2022).

Overall, biomarkers that seemed to have the most consistent responses to BPA, BPS and BPF were related to the innate immunity, and more specifically to phagocytosis activity. In fact, some parameters of this immune response were modified by BPs. During the 21-days exposure and the seven-days of exposure, the BPs induced phagocytosis activity through adhesion and internalization of particles. This result was confirmed by Yang *et al.* (2015) which report an induction of phagocytic capacity of primary macrophages from head kidney of red common carp (*Cyprinus carpio*) after 6h-exposure to BPA. Nevertheless, this induction of phagocytosis has not been observed by all authors (Bado-Nilles *et al.* 2014, Gushiken *et al.* 2002, Qiu *et al.* 2018b, Rehberger *et al.* 2021). The induction of adhesion and internalization capacities of phagocytes could balance the reduction of respiratory burst index by the three BPs. This reduction of the respiratory burst has already been described by Yin *et al.* (2007). Overall, the decrease of this respiratory burst index was especially due to a significant oxidative burst (ROS B induction). This induction of oxidative burst by BPs was commonly described in the literature (Qiu *et al.* 2018b; Qiu *et al.* 2018c; Aykut & Kaptaner 2021; Wang *et al.* 2022; Yin *et al.* 2007; Yang *et al.* 2015). In fact, some positive synergism between estrogen receptor (ER) and NF- $\kappa$ B signaling pathways enhance the oxidative burst response which disturb the immune response of fish (Yang *et al.* 2015). New data has shown that the impact of BPs on NF- $\kappa$ B signaling pathways could be due to consistent expression changes of long non-coding RNAs (lncRNAs) with impact on their predicted target mRNAs (Liu *et al.* 2020). Furthermore, the oxidative stress generated by ROS and the disturbance of NF- $\kappa$ B signaling pathways could induce a major inflammatory reaction that causes cellular damage (Qiu *et al.* 2019, Yang *et al.* 2015, Yin *et al.* 2007). For the moment, the dysregulation of immune homeostasis remains unclear. In human macrophages, BPs seems to affect

inflammatory responses of macrophages through estrogen receptor  $\alpha/\beta$  (ER $\alpha/\beta$ )-dependent mechanism which modulate cytokine expression (Liu *et al.* 2014). In fish, an increase of pro-inflammatory gene expression (IL-1 $\beta$  and IL-6 like) and of anti-inflammatory gene expression (IL-10) was also observed (Yang *et al.* 2015). To conclude, as shown in the present work, the overproduction of ROS and the inflammatory process could lead to activation of apoptic pathway and cell death (Krumnschnabel *et al.* 2005, Risso-de Faverney *et al.* 2001). For example, BPAF could induce apoptosis by up-regulation of caspase 3 and bax and increasing reactive oxygen species concentrations (Gyimah *et al.* 2022). At term, this reduction of immune cells by apoptosis and/or necrosis could disturb organisms' immune capacities and increase pathology effect.

Even if the overproduction of ROS and inflammatory process were clearly demonstrated, limited studies were carried out on the effect of BPs on fish antioxidant enzymes. Usually, enhancement of superoxide dismutase (SOD), catalase, glutathione reductase (GR), and reduced glutathione (GSH) and reduction of glutathione S-transferase (GST) and glutathione peroxidase (GPx) explain the increase of lipid peroxidation (Afzal *et al.* 2022, Akram *et al.* 2021, Li *et al.* 2016, Shehna Mahim *et al.* 2021). In the present work, the same type of results was obtained for GST and TBARS, especially concerning BPS exposure. This response would be essentially linked to an abnormal process of oxidative phosphorylation, inducing an over production of free radicals, resulting in abnormal functions and disorders of antioxidants mechanisms (Akram *et al.* 2021). Concerning the Phase I system of metabolic transformation of lipophilic xenobiotics, some authors detected a downregulation of expression of *cyp1a1* and *cyp19A1b* genes after BP exposure (Faheem and Bhandari 2021, Maradonna *et al.* 2014). One of the main explanations concerned the binding ability of BPs to P4501A1 protein which inhibit the catalytic activity of this protein (Maradonna *et al.* 2014). In another way, the binding of oestrogenomimetic compounds to ER can directly affect the *cyp1a1* gene or interact by inhibition of AhR regulated pathway (Navas and Segner 2001). In the present work, a small reduction of EROD activity was shown after long term exposure to BPF and short-term exposure to BPA and BPS. Finally, all these disorders can ultimately cause disrupt cellular homeostasis and tissue damage.

## 5. Conclusions

Overall, this study confirmed that along with effects on reproduction parameters, that are not reported here, BPA has multiple direct or indirect effects, on innate immunity, oxidative stress, and metabolic disorder. Interestingly, the study also demonstrated that both substitutes, BPS and BPF cannot be regarded as harmless. They triggered effects similar to BPA and more surprisingly were shown to have more stable effect over time, for example considering innate immunity parameters.



Parameters concerning the discrepancy between female and male immune response must be further investigate, especially in terms of ER type. In addition, the monitoring of the toxicokinetics of BPS made it possible to quantify the bioaccumulations of BPS and BPS metabolites in the stickleback organs. These results confirm the importance of grounding the data with more analyses. Despite its really low BCFs in organs compared to BPA, BPS was able to trigger effects as long-lasting and as strong as those measured for BPA. In addition, those effects seemed to be different, in terms of temporal dynamic for example, implying that the mode of action of BPS could be different from BPA. Taken together, those results showed the importance of better characterizing the substitutes of BPA and the usefulness of studying together the TK and TD of chemicals by using multi-biomarker approach to identify and understand the multiple pathways that they could trigger.

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# Statements & Declarations

## Funding

This work was supported by the French National program EC2CO (Ecosphère Continentale et Côtière) as part of the DERBI project and by the French Ministry of ecological transition (P190).

## Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

## Conflict of interest

The authors declare no competing interests.

## Author Contributions

Corentin MIT: Methodology, Formal analysis, Investigation, Visualization, Project administration, Writing - Original Draft

Rémy Beaudouin: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Supervision, Project administration

Olivier Palluel: Investigation

Cyril Turiès: Investigation, Project administration

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### **Ethics declarations**

Experimental protocols were conducted following the European directive 2010/63/UE for the protection of animals used for scientific purposes at INERIS, registration number E60–769–02. The experimental protocols were submitted and reviewed by a French nationally recognized ethical committee, CREMEAPS, registration number 96.

### **Consent to participate**

All authors read and approved the final manuscript.

### **Consent for publication**

All authors read and approved the final manuscript.

### **Availability of data and materials**

All data and code are available.

## Tables



**Table 1.** BPA bioaccumulation (in ng/g) measured in stickleback liver, blood and carcass by LC-MS/MS during the seven-day exposure seven-day depuration for both doses ( $n = 2$  per date). The limit of quantification for BPA in each matrix is also presented.

	Nominal dose	Day 0.25	Day 1	Day 4	Day 7	Day 8	Day 14
<b>BPA in liver<sup>a</sup></b>	10 µg/L	26.5	185.7	79.1	43.9	16.1	<LQ
	(LQ: 10 ng/g) 100 µg/L	326.7	2605.3	360	677.7	261.9	20.2
<b>BPA in blood<sup>b</sup></b>	10 µg/L	<LQ	12.5	16.3	12.1	<LQ	<LQ
	(LQ: 10 ng/g) 100 µg/L	84.5	117.6	91.3	82.3	<LQ	<LQ
<b>BPA in carcass<sup>c</sup></b>	10 µg/L	<LQ	38.4	39.1	48.2	<LQ	<LQ
	(LQ: 20 ng/g) 100 µg/L	405.8	2230.0	1128.3	504.5	113.9	<LQ

<sup>a</sup> individual liver fresh mass: ~100 mg; <sup>b</sup> blood fresh mass: 5 mL; <sup>c</sup> individual carcass fresh mass: ~1500 mg

**Table 2.** Bioconcentration factors calculated for BPA and BPS (when measurable) in male and female liver, blood and carcass at day seven (steady state assumed) and for the nominal dose of 100 µg/L.

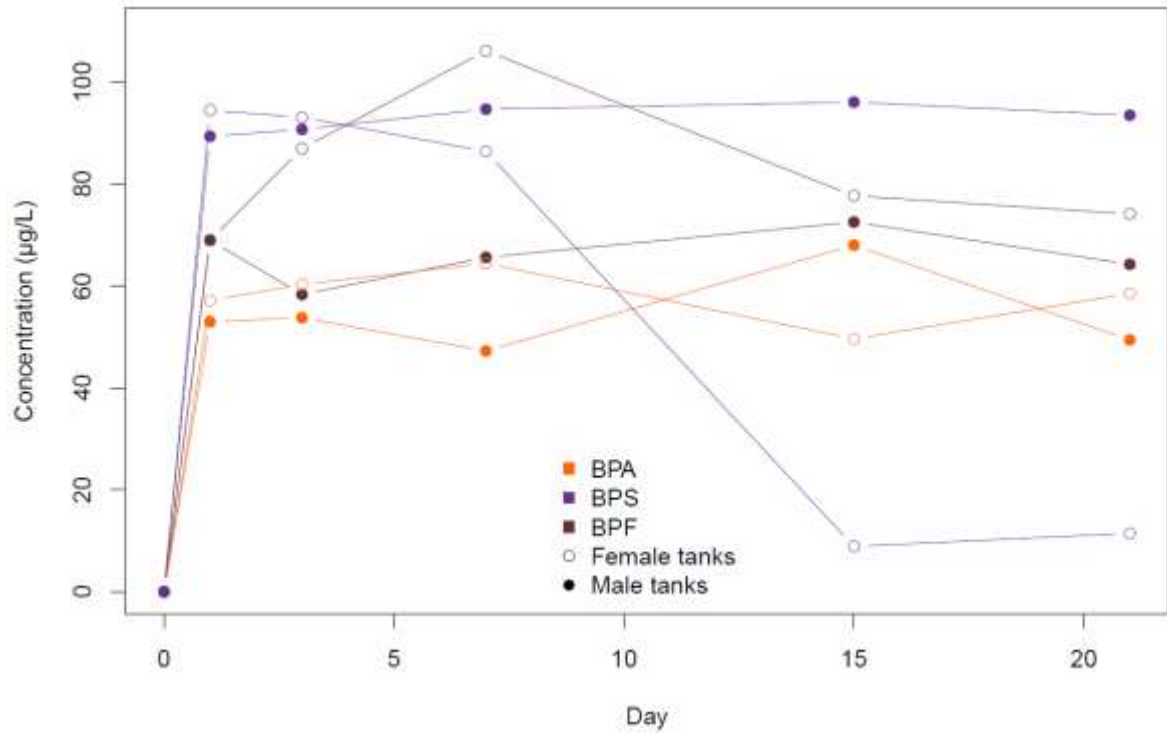
BCF*	BPA		BPS	
	Male	Female	Male	Female
<b>Liver</b>	6.5	13.2	<0.1	
<b>Blood</b>	1.2	1.2	Below LQ (3.5 ng/g)	
<b>Carcass</b>	9.7	5.3	<0.1	

\*BCF was calculated as the mean concentration in organ over the measured concentration in female or male water tanks.

**Table 3.** BPS bioaccumulation (in ng/g) measured in stickleback liver, and carcass by LC-MS/MS during the seven-day exposure seven-day depuration for the nominal dose of 100 µg/L. The limit of quantification for BPS in each matrix is also presented.

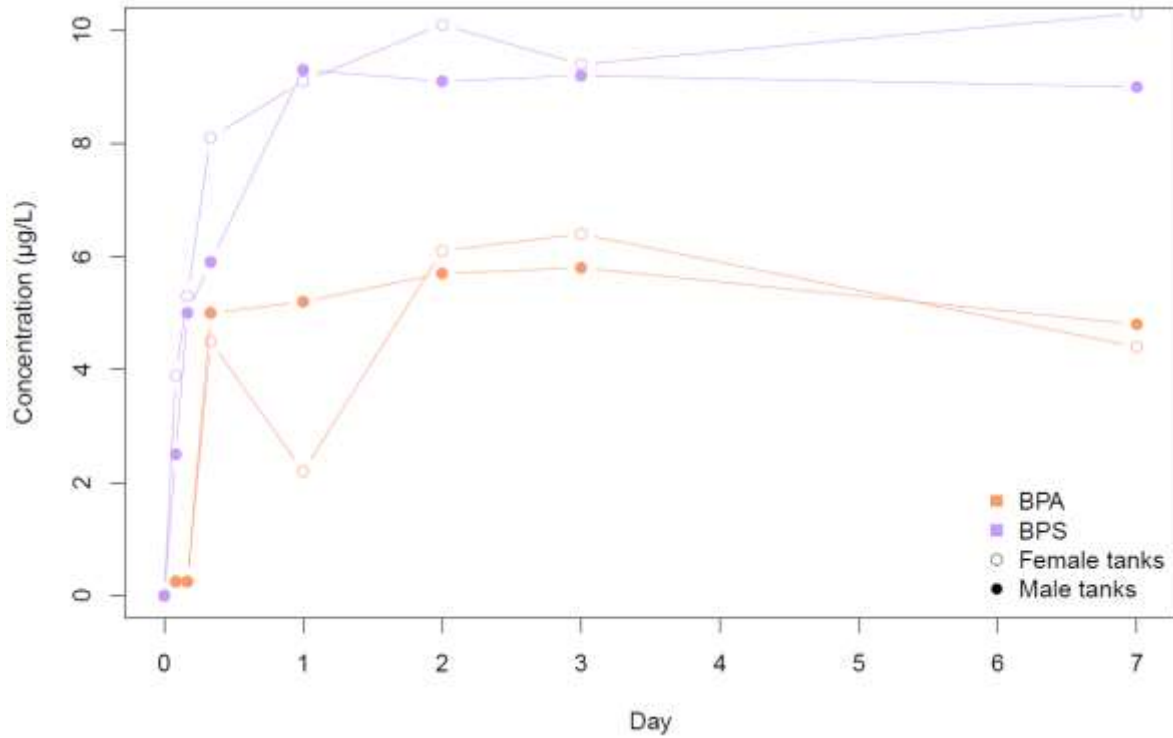
	Nominal dose	Day 0.25	Day 1	Day 4	Day 7	Day 8	Day 14
<b>BPS in liver</b>	100 µg/L	4.1	29.5	6	9.5	<LQ	<LQ
(LQ: 10 ng/g)							
<b>BPS in carcass</b>	100 µg/L	<LQ	5.4	4.2	3.7	<LQ	<LQ
(LQ: 10 ng/g)							

Figures



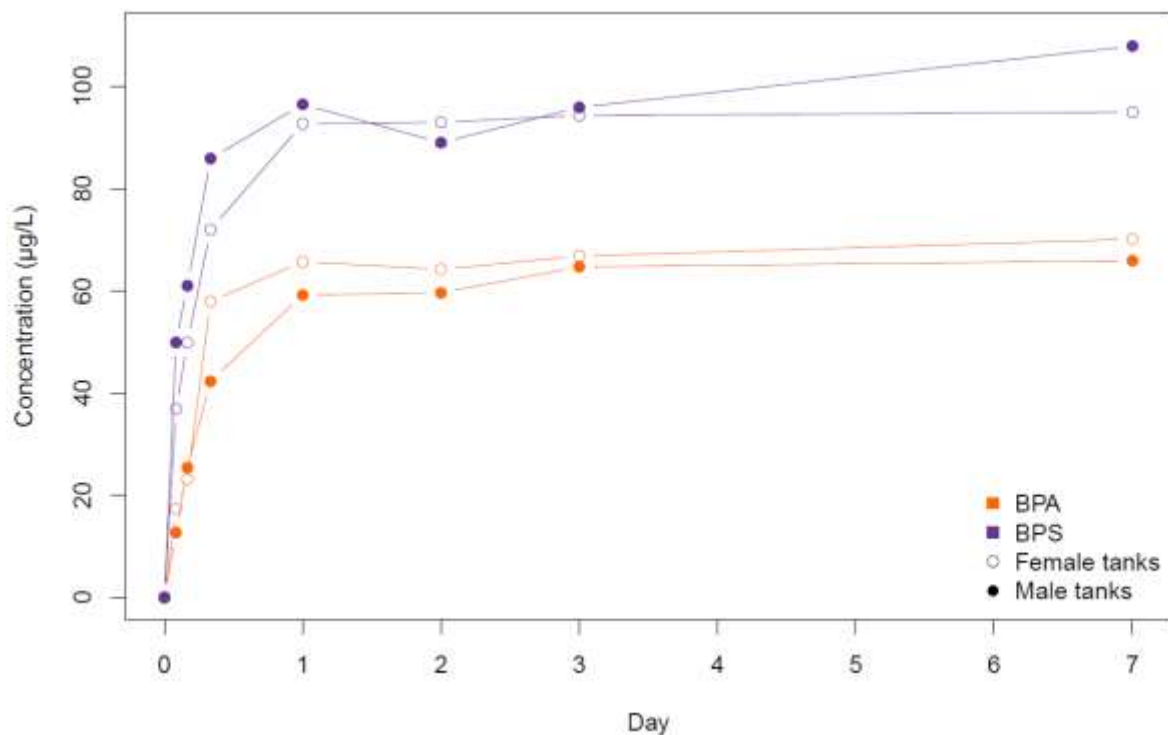
**Figure 1.** Water concentrations of BPA (in orange), BPS (in purple) and BPF (in brown) in water during the long-term exposures.

Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification was evaluated to be 0.25 µg/L for BPA (0.2 µg/L for BPF and 0.1 µg/L for BPS). Concentration was assessed randomly in one tank for each condition and at each sampling time.

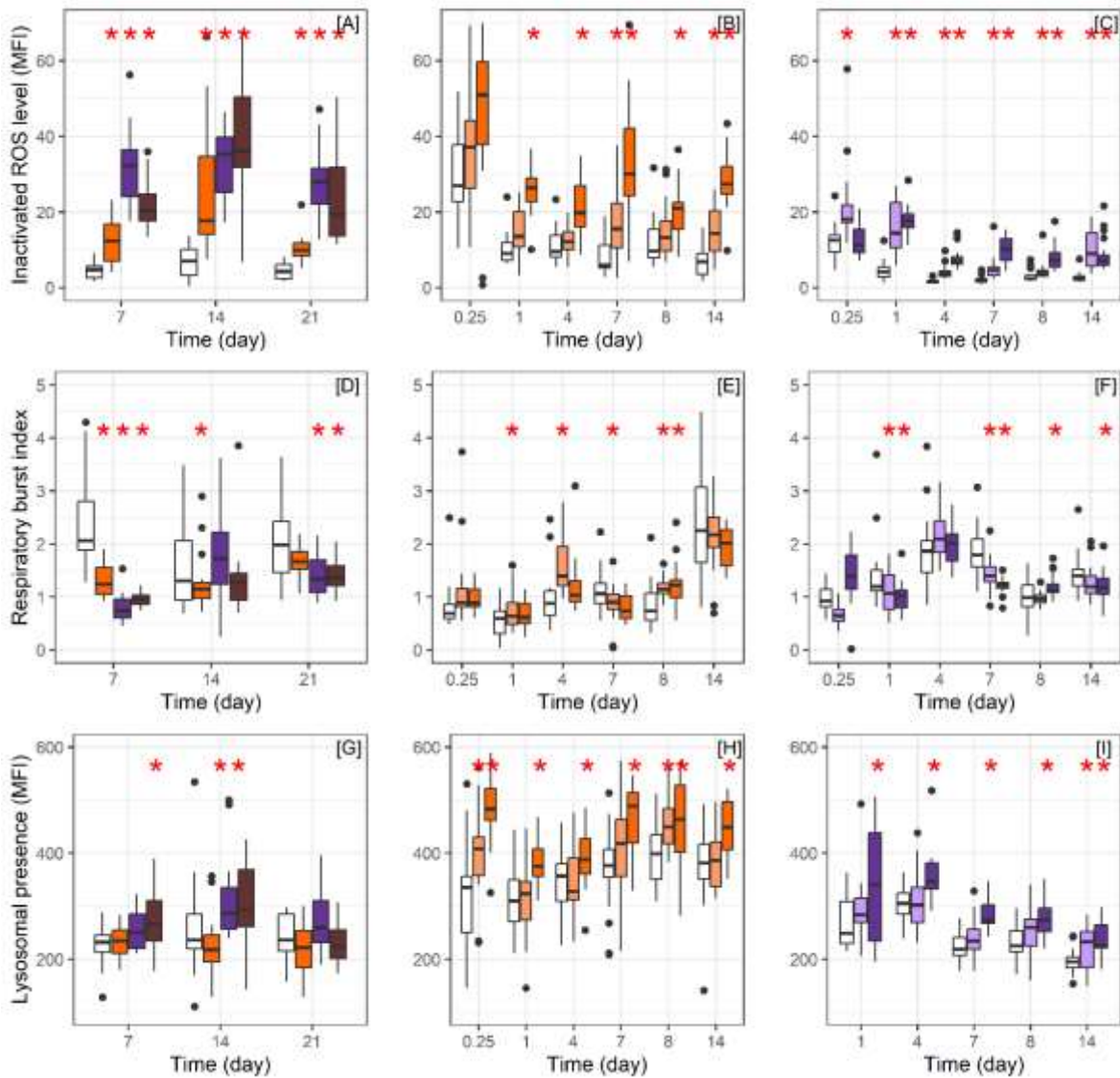


**Figure 2.** Water concentration of BPA (nominal dose, 10 µg/L in light orange) and BPS (nominal dose, 10 µg/L in light purple) during the short-term exposures.

Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification was evaluated to be 0.5 µg/l for BPA and to be 0.2 µg/l for BPS. Concentration was assessed randomly in one tank for each condition at each sampling time.



**Figure 3.** Water concentration of BPA (nominal dose, 100 µg/L in orange) and BPS (nominal dose, 100 µg/L in purple) during the short-term exposures. Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification was evaluated to be 0.5 µg/l for BPA and to be 0.2 µg/l for BPS. Concentration was assessed randomly in one tank for each condition at each sampling time.



**Figure 4.** Respiratory burst (ROS B and Respiratory burst index) and lysosomal presence measured in the long-term exposure to the three bisphenols (first column), in the short-term exposures to BPA (second column) and to BPS (third column).

ROS B (MFI) is represented on the first line (panel A, B and C). Respiratory burst index (no unit) is represented on the second line (panel D, E and F) and Lysosomal presence (MFI) is represented on the third line (panel G, H and I).

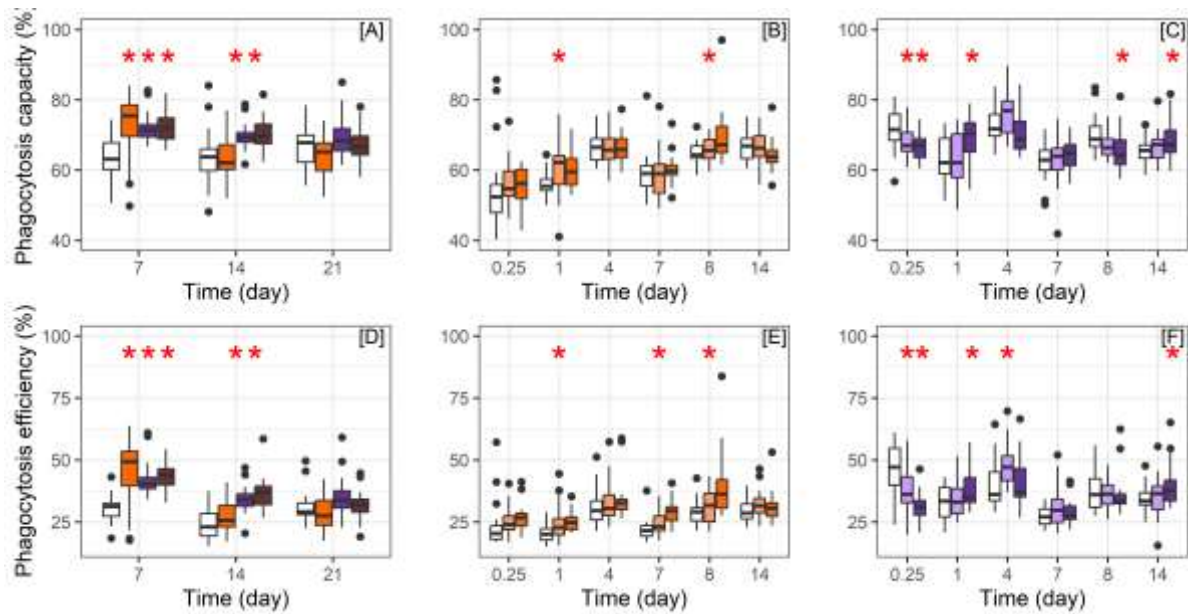
Female and male responses are represented (n=20 per boxplot).

BPA is represented in orange shades: nominal dose, 10  $\mu\text{g/L}$  in light orange and 100  $\mu\text{g/L}$  in orange.

BPS is represented in purple shades: nominal dose, 10  $\mu\text{g/L}$  in light purple and 100  $\mu\text{g/L}$  in purple.

BPF is represented in brown.

Asterisks correspond to a statistical difference (p-value<0.05) between control (blank) and condition.



**Figure 5.** Phagocytic activity (Adhesion capacity and internalization efficiency) measured in the long-term exposure to the three bisphenols (first column), in the short-term exposures to BPA (second column) and to BPS (third column).

Adhesion capacity (%) is represented on panel A, B and C. Internalization efficiency (%) is represented on panel D, E and F.

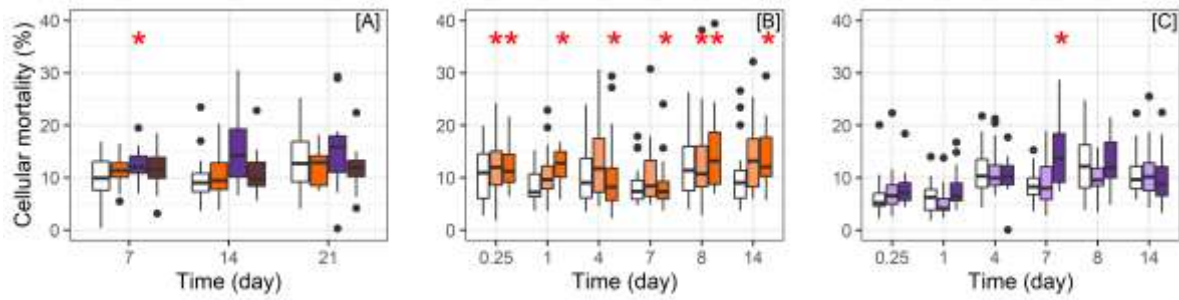
Female and male responses are represented (n=20 per boxplot).

BPA is represented in orange shades: nominal dose, 10 µg/L in light orange and 100 µg/L in orange.

BPS is represented in purple shades: nominal dose, 10 µg/L in light purple and 100 µg/L in purple.

BPF is represented in brown.

Asterisks correspond to a statistical difference (p-value<0.05) between control (blank) and condition.



**Figure 6.** Cellular mortality measured in the long-term exposure to the three bisphenols (first column), in the short-term exposures to BPA (second column) and to BPS (third column).

Female and male responses are represented (n=20 per boxplot).

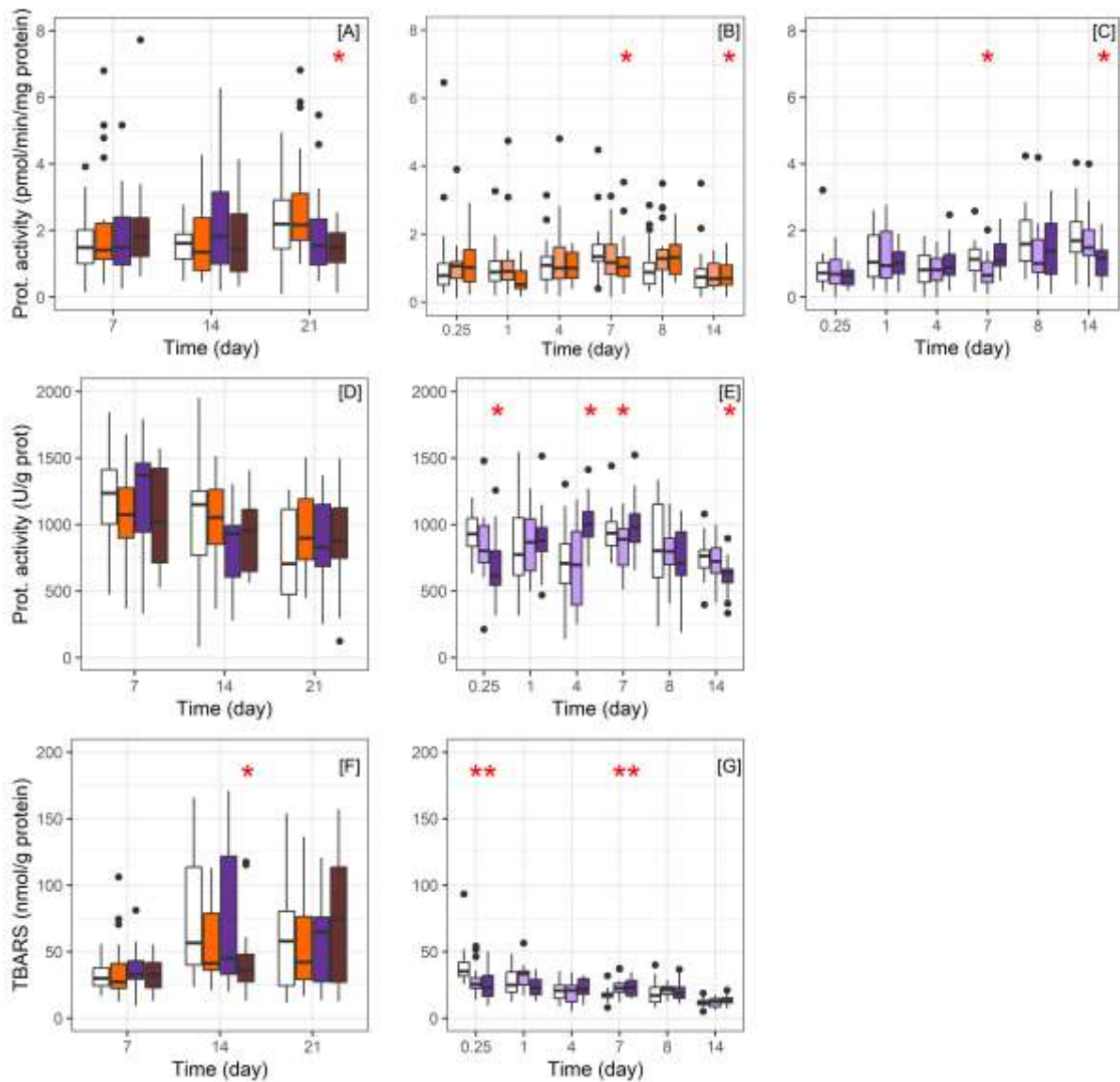
BPA is represented in orange shades: nominal dose, 10 µg/L in light orange and 100 µg/L in orange.

BPS is represented in purple shades: nominal dose, 10 µg/L in light purple and 100 µg/L in purple.

BPF is represented in brown.

Asterisks correspond to a statistical difference (p-value<0.05) between control (blank) and condition.





**Figure 7.** Detoxication enzyme activities (EROD and GST) and TBARS measurements measured in the long-term exposure to the three bisphenols (first column), in the short-term exposures to BPA (second column) and to BPS (third column).

EROD activity (pmol/min/mg protein) is represented on the first line (panel A, B and C). GST activity (U/gprot) is represented on the second line (panel D and E). GST activity for BPA short-term exposure is not shown because of analytical errors. TBARS measurements (nmol/gprot) is represented on the third line (panel F and G). The measurements of EROD for BPA short-term exposure are not shown because of analytical errors. Female and male responses are represented (n=20 per boxplot).

BPA is represented in orange shades: nominal dose, 10  $\mu\text{g/L}$  in light orange and 100  $\mu\text{g/L}$  in orange. BPS is represented in purple shades: nominal dose, 10  $\mu\text{g/L}$  in light purple and 100  $\mu\text{g/L}$  in purple. BPF is represented in brown.

Asterisks correspond to a statistical difference (p-value<0.05) between control (blank) and condition

## Appendix

	BPS (ng/ml)	
	10 µg/L	100 µg/L
2h	< LQ	49,9
4h	5,0	61,0
8h	5,9	85,9
24h	9,3	96,5
48h	9,1	89,0
72h	9,2	95,9
168h	9,0	107,9

Table S1. BPS measured concentrations in water (male fish)

	BPS (ng/ml)	
	10 µg/L	100 µg/L
2h	3,9	36,9
4h	5,3	49,9
8h	8,1	72,0
24h	Analytical error	92,7
48h	10,1	93,0
72h	9,4	94,3
168h	10,3	95,0

Table S2. BPS measured concentrations in water (female fish)

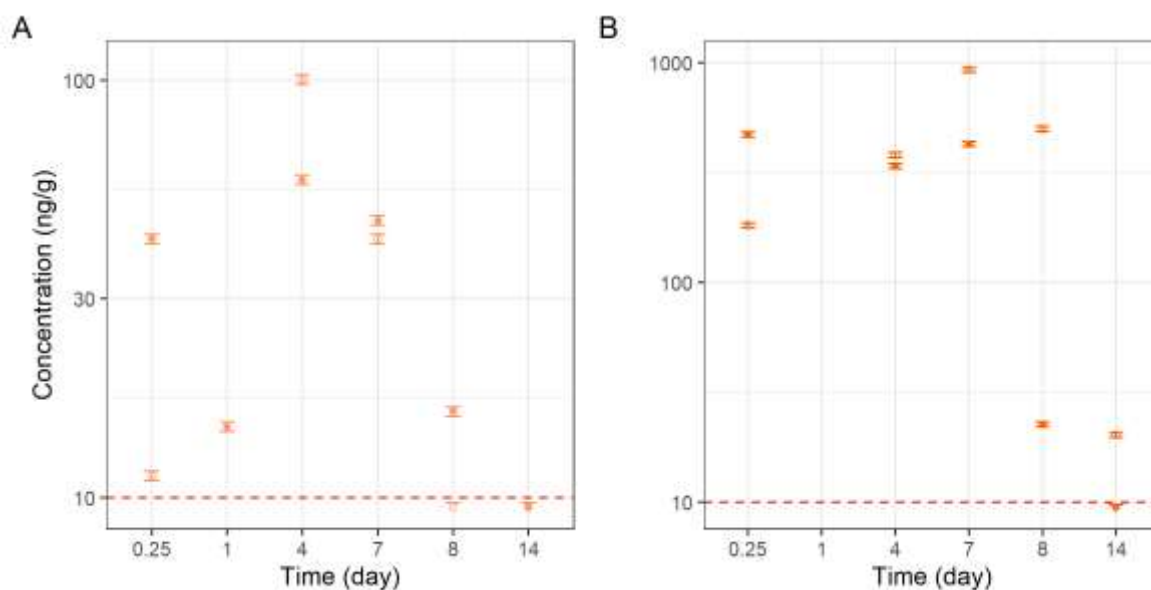
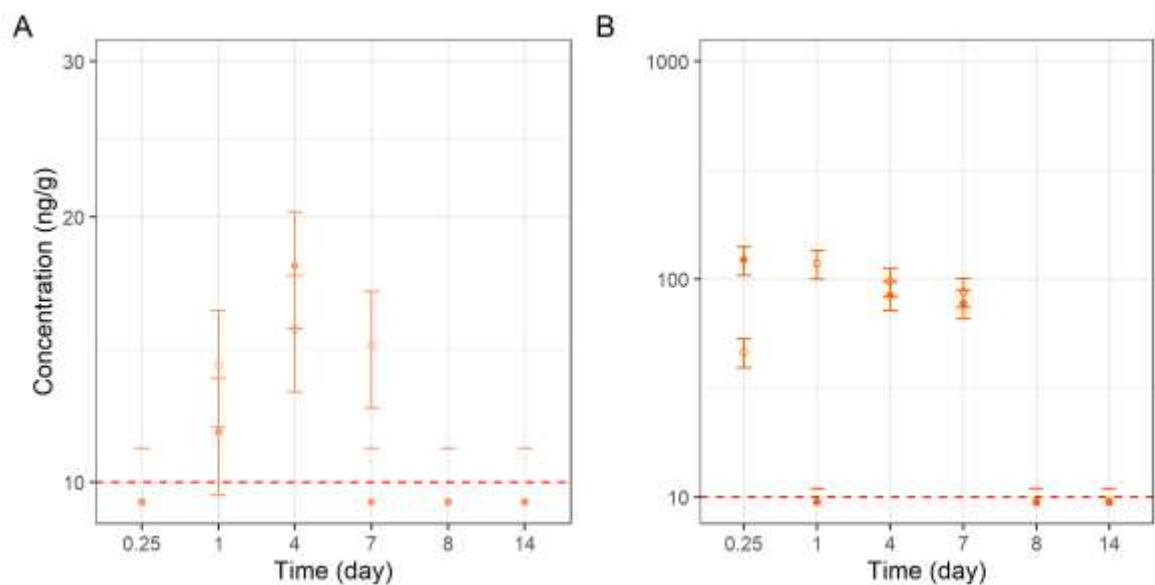


Figure S1. Liver concentrations of BPA measured by LC-MC/MS in stickleback.

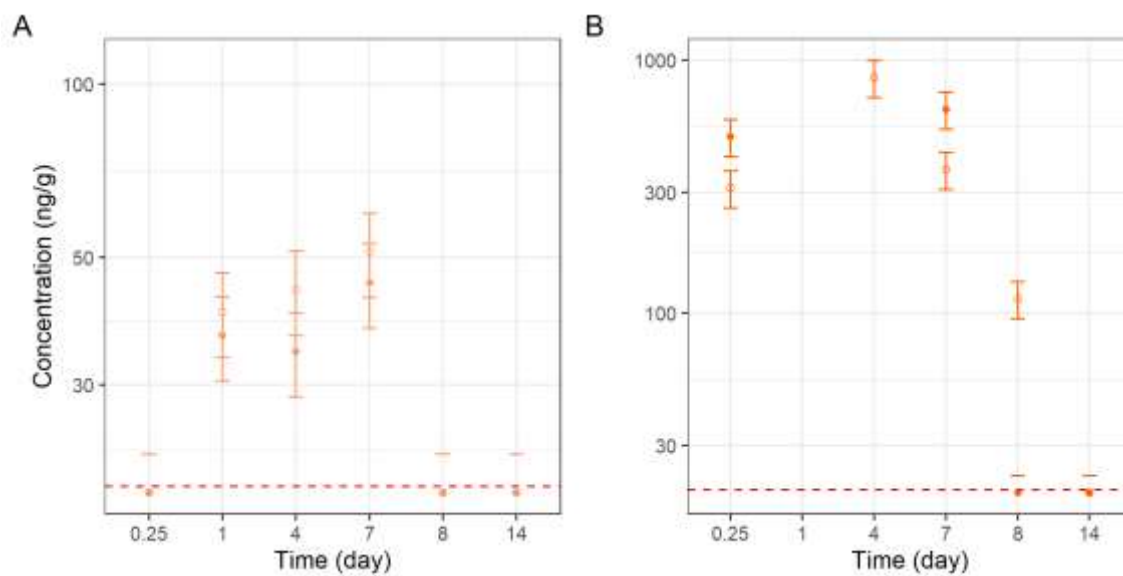
Organ concentrations for the nominal dose of 10 µg/L and 100 µg/L of BPA are represented on panel A and B, respectively. Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification of 10 ng/g is represented by the red dashed line (points below LOQ were represented for the sake of comprehension). The precision of the measure for each organ was estimated to draw the error for each point ( $n = 1$ ).





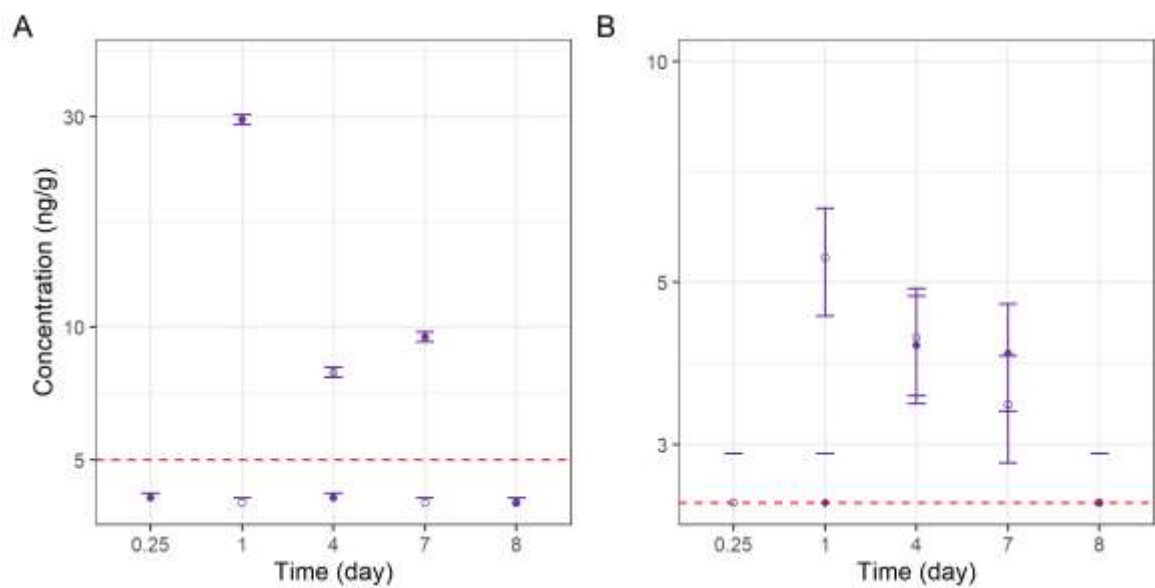
**Figure S2.** Blood concentrations of BPA measured by LC-MC/MS in stickleback.

Organ concentrations for the nominal dose of 10 µg/L and 100 µg/L of BPA are represented on panel A and B, respectively. Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification of 10 ng/g is represented by the red dashed line (points below LOQ were represented for the sake of comprehension). The precision of the measure for each organ was estimated to draw the error for each point ( $n = 1$ ).



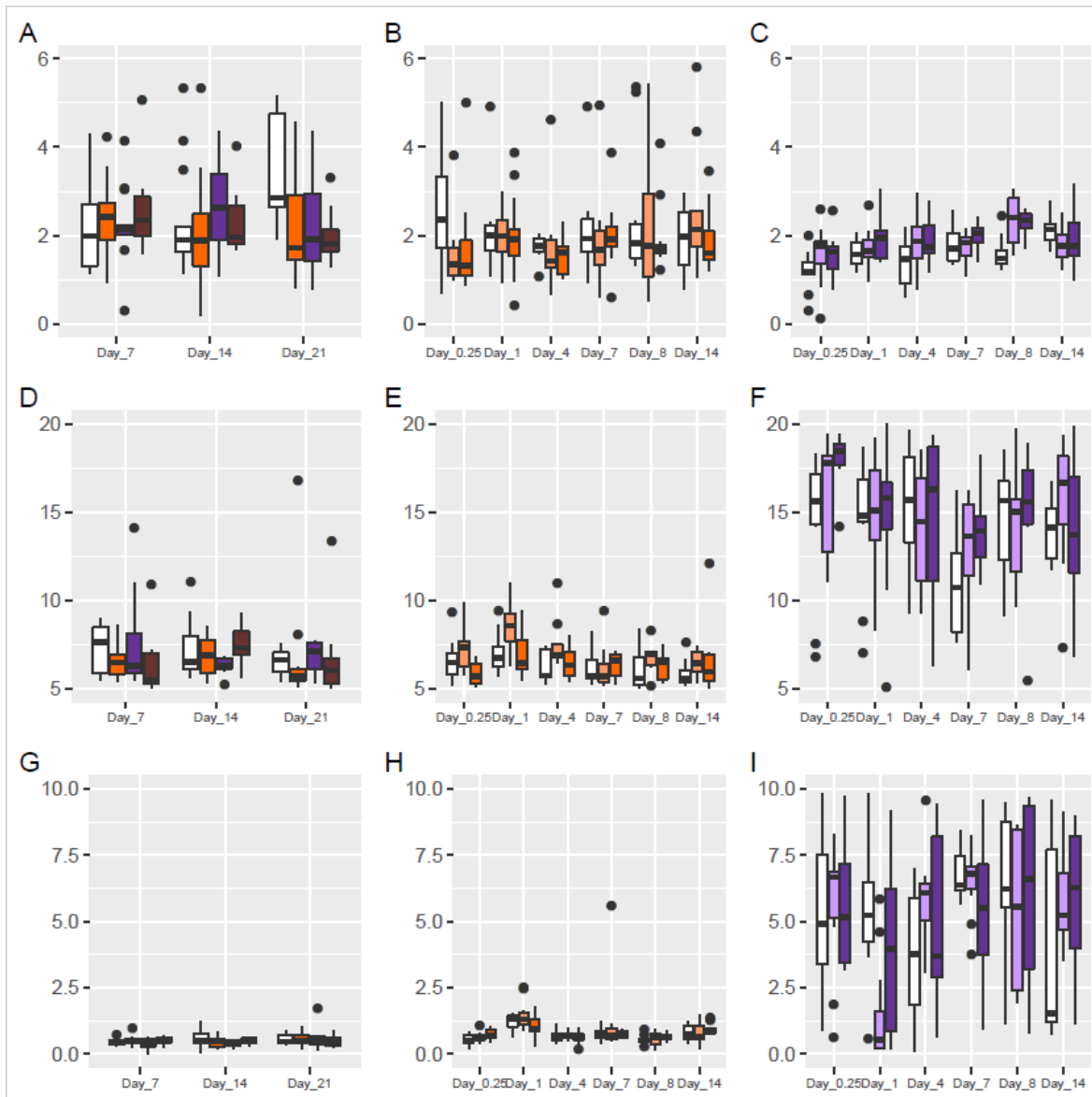
**Figure S3.** Carcass concentrations of BPA measured by LC-MC/MS in stickleback.

Organ concentrations for the nominal dose of 10 µg/L and 100 µg/L of BPA are represented on panel A and B, respectively. Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limit of quantification of 20 ng/g is represented by the red dashed line. The precision of the measure for each organ was estimated to draw the error for each point (n = 1).



**Figure S4.** Liver (panel A) and carcass (panel B) concentrations of BPS measured by LC-MC/MS in stickleback for the nominal dose of 100 µg/L of BPS.

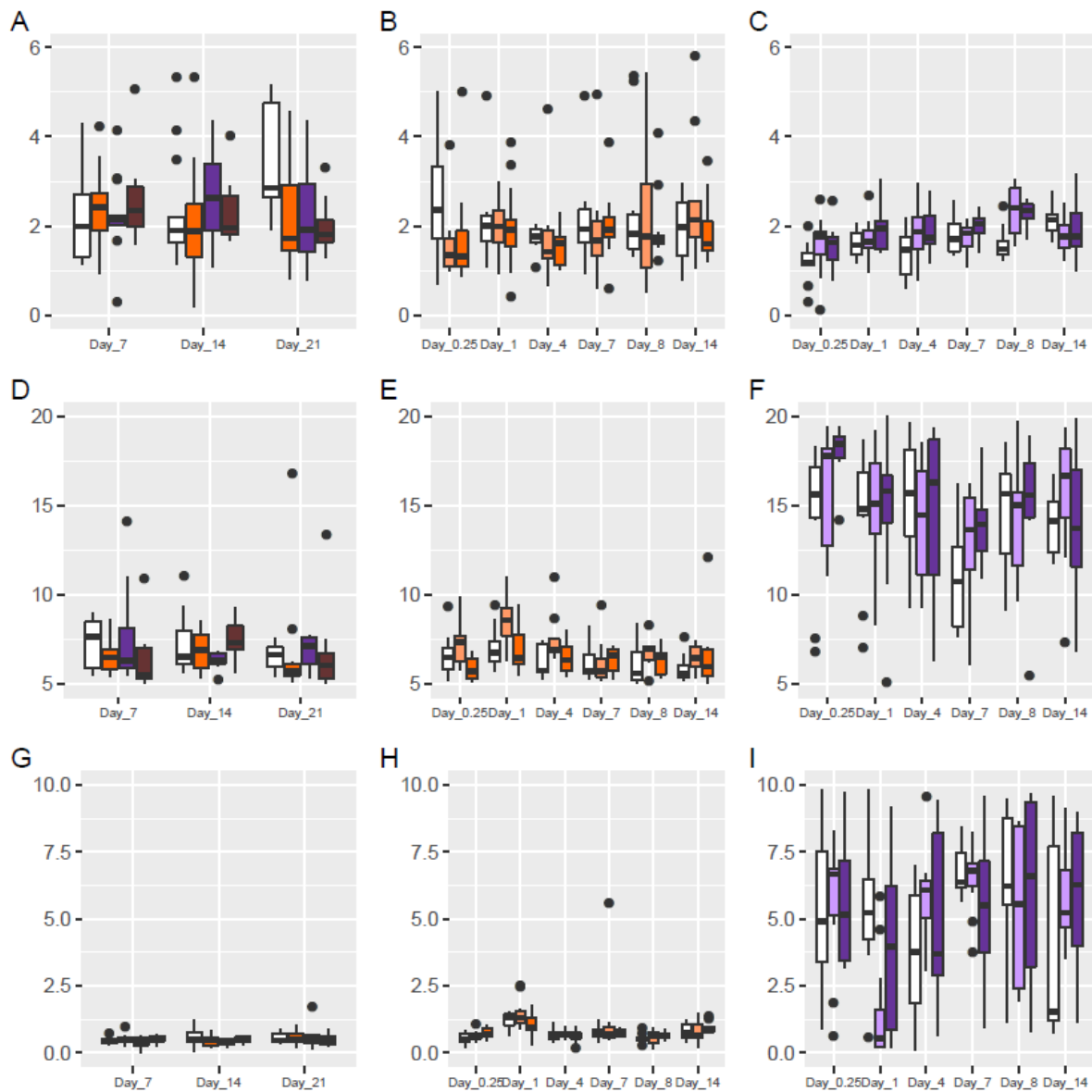
Concentrations in female and male tanks are symbolized with empty and full dots, respectively. The limits of quantification of 5 ng/g (liver) and 2.5 ng/g (carcass) are represented by the red dashed line. The precision of the measure for each organ was estimated to draw the error for each point ( $n = 1$ ).



**Figure S5.** Biological indices for the long-term exposure to BPA (in orange), BPS (in purple) and BPF (in brown) and the short-term exposures to BPA (nominal dose, 10  $\mu\text{g/L}$  in light orange and 100  $\mu\text{g/L}$  in orange) and BPS (nominal dose, 10  $\mu\text{g/L}$  in light purple and 100  $\mu\text{g/L}$  in purple).

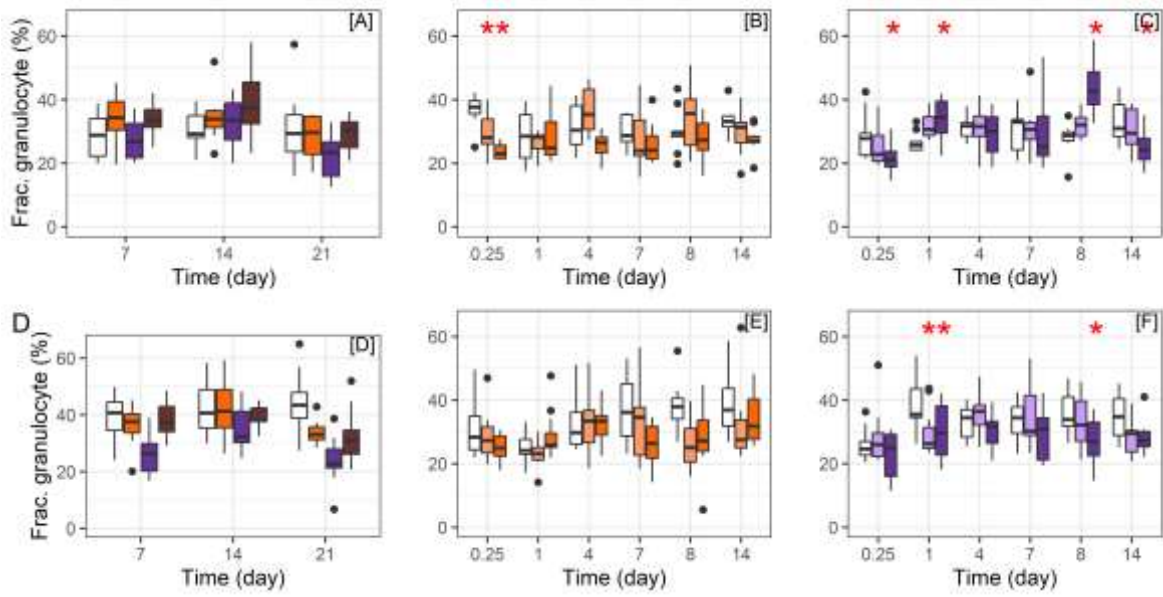
GSI (no unit) is represented on panel A, B and C. KSI (no unit) is represented on panel D, E and F. HSI (no unit) is represented on panel G, H and I. For the long-term (panel A, D and G) and short-term (panel B, C, E, F, H and I) exposures only female responses are represented ( $n = 10$  per boxplot).





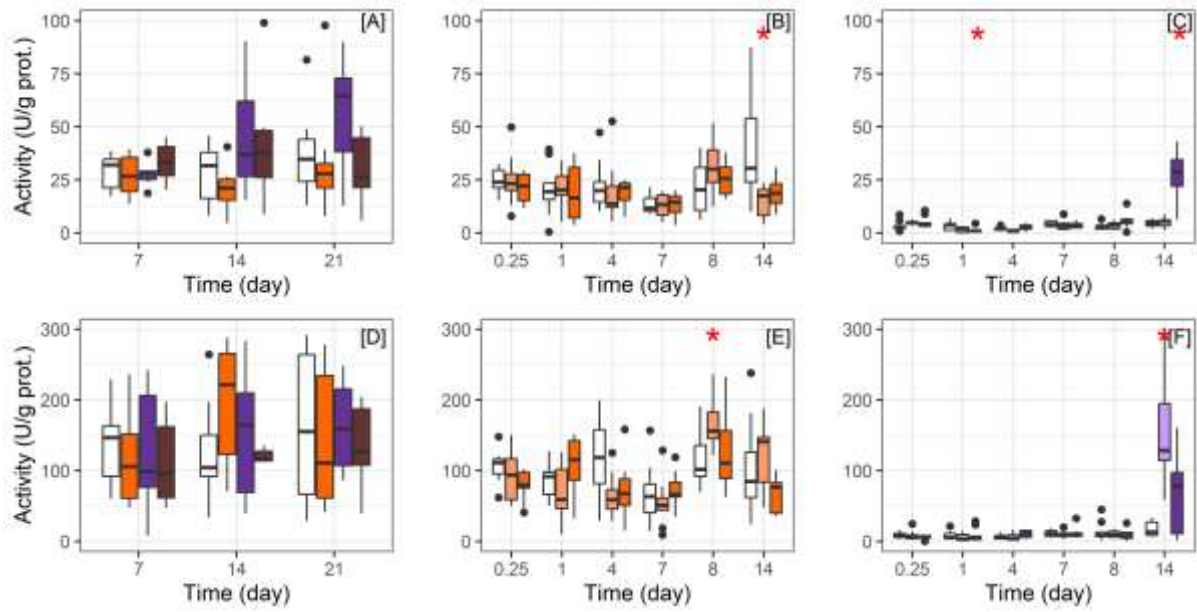
**Figure S6.** Biological indices for the long-term exposure to BPA (in orange), BPS (in purple) and BPF (in brown) and the short-term exposures to BPA (nominal dose, 10  $\mu\text{g/L}$  in light orange and 100  $\mu\text{g/L}$  in orange) and BPS (nominal dose, 10  $\mu\text{g/L}$  in light purple and 100  $\mu\text{g/L}$  in purple).

GSI (no unit) is represented on panel A, B and C. KSI (no unit) is represented on panel D, E and F. HSI (no unit) is represented on panel G, H and I. For the long-term (panel A, D and G) and short-term (panel B, C, E, F, H and I) exposures only male responses are represented ( $n = 10$  per boxplot).



**Figure S7.** Leucocyte distribution for the long-term exposure to BPA (in orange), BPS (in purple) and BPF (in brown) and the short-term exposures to BPA (nominal dose, 10  $\mu\text{g/L}$  in light orange and 100  $\mu\text{g/L}$  in orange) and BPS (nominal dose, 10  $\mu\text{g/L}$  in light purple and 100  $\mu\text{g/L}$  in purple).

Male responses are represented on panel A, B and C ( $n = 10$  per boxplot). Female responses are represented on panel D, E and F ( $n=10$  per boxplot). Asterisks correspond to a statistical difference ( $p$ -value  $< 0.05$ ) between control (blank) and condition.



**Figure S8.** GPx activity for the long-term exposure to BPA (in orange), BPS (in purple) and BPF (in brown) and the short-term exposures to BPA (nominal dose, 10 µg/L in light orange and 100 µg/L in orange) and BPS (nominal dose, 10 µg/L in light purple and 100 µg/L in purple).

GPx activity (U/gprot) is represented on panel A, B and C for male fish and on panel D, E and F for female fish ( $n = 10$  per boxplot). Asterisks correspond to a statistical difference ( $p$ -value  $< 0.05$ ) between control (blank) and condition.