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Analysis of Bisphenol-A contamination in fish liver and muscle tissue:

A method development and first assessment of BPA contamination in invasive round goby *Neogobius melanostomus* in the swiss Rhine River (Basel, Switzerland)

Master's thesis

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Keywords

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Abstract

Endocrine disrupting chemicals (EDCs) are of growing concern in aquatic ecosystems due to the potential of modulating the endocrine system in aquatic species by interfering with reproduction and developmental processes. Bisphenol-A (BPA) belongs to the EDCs and has been documented to induce estrogenic effects in fish. This study aimed to develop a reliable method based on molecularly imprinted polymers solid phase extraction (SPE MIPs) and LC-MS/MS for the analysis of BPA in liver and muscle samples in invasive round goby *Neogobius melanostomus* sampled in the river Rhine in Basel, Switzerland. Additionally, all fish samples were analysed for the liver protein vitellogenin to provide an unspecific indication of EDC induced endocrine effects. The study also aimed to investigate any sex- and size-specific differences in BPA contamination and the overall condition of the target species, by documenting condition indicators including total length, total weight, liver weight and hepatosomatic index (HSI). BPA contamination of the sampled round goby was below limit of quantification. A further improvement of the method is needed to detect low contaminations especially in liver samples, where high matrix interferences occurred. Vitellogenin measurements indicated the presence of EDCs.

Directory

1 Introduction

Thousands of chemicals of anthropogenic origin are released into the environment, including many chemicals with endocrine disrupting properties. Endocrine disrupting chemicals (EDCs) are substances that exhibit a certain hormonal activity in organisms, through interfering with hormonal regulation and the endocrine system, thus causing adverse effects on animal and human health (Lv et al., 2019). Of these, chemicals with oestrogen-like effects are of particular concern: the so-called xenoestrogens (Lahr et al., 2006). A well-known xenoestrogen is Bisphenol A (BPA), which is detectable almost everywhere in the environment. It has already been detected in environmental samples in air, dust, soil and water (Vandenberg et al., 2007). BPA has been produced for industrial use since around the 1960s, and its production is now estimated to exceed 8 million tonnes per year, and approximately 100 tons may be released into the atmosphere within one year (Vandenberg et al., 2010). It is mainly used in the production of epoxy resins, as well as polycarbonates for the manufacture of products such as CDs, certain water bottles, and a variety of other products (Kang et al., 2007).

BPA has an acute toxic effect on aquatic organisms: for example, it has been shown that it can lead to reduced fertility in fish (Lahnsteiner et al., 2005). Sperm quality is reduced if BPA is present above certain concentrations in the body, gonadal development can be inhibited, and gonadal intersexuality or complete sex reversal can occur in fish that are naturally male (Mandich et al., 2007). Oestrogen-like effects in male fish can be measured as vitellogenin content in the body. Vitellogenin is an egg yolk protein whose production in female fish is triggered by the hormone oestrogen. The gene responsible for vitellogenin production is also present in males. If vitellogenin can be detected in males, it can be concluded that an oestrogen-like substance must be present in the environment (water, sediment or food sources) that triggers vitellogenin production in the males' bodies (Rankouhi et al., 2002). The presence of BPA can lead to vitellogenin production in male fish (Kang et al., 2007). However, vitellogenin screening in river ecosystems indicated the presence of numerous xenoestrogenic compounds almost worldwide, as there are numerous compounds with similar modes of action present in river ecosystems.

BPA is biodegradable under aerobic conditions with a half-life of 4.5-4.7 days (Crain et al., 2007). However, BPA can constantly leach from plastics (polycarbonates) and epoxy resins (Biles et al., 1997; Howdeshell et al., 2003; Takao et al., 2002) or enter aquatic ecosystems via wastewater plants effluents (Fromme et al., 2002) posing a risk of chronic contamination input. Furthermore, water bodies act as sinks and can potentially accumulate BPA (Crain et al., 2007). The biodegradability of BPA is drastically limited under low-oxygen conditions, as shown for example by Kawahata et al.

(2004) and Voordeckers et al. (2002). Consequently, accumulation of BPA in sediments has been reported in a variety of studies due to low oxygen content.

Bioconcentration factor (*BCF*), bioaccumulation factor (*BAF*) and biomagnification are important parameters in understanding the potential impact of chemical pollutants on the environment (Wassenaar et al., 2020). *BCF* is a measure of the ability of accumulation capacity of chemicals via aquatic exposure only without considering dietary means, while *BAF* takes complex uptake processes into account and is therefore often used to assess chemical enrichment in the environment (Grisoni et al., 2015). Biomagnification is the process by which toxins become more concentrated as they move up the food chain (Wang et al., 2017). The *BCF* of BPA was often classified as low in the laboratory, but comparisons with field sites show that *BAF* was increased up to tenfold in some cases (J. Yang et al., 2014). According to Corrales et al. (2015) and Yang et al. (2014), possible explanations for this are the different conditions under which the measured values were collected. For example, most of the used fish species in laboratory BPA exposure experiments were predominantly pelagic living species. In the field, however, the highest BAFs show up precisely in benthic fish species, which are exposed to sediment that potentially is high in BPA content. Species-specific differences in metabolism may also be an explanation for the differences. Macrozoobenthos as a food source present another potential source of biomagnification during ingestion (Corrales et al., 2015).

Numerous studies deal with the hormonal activity of BPA. In vivo, correlations between microplastic emission and BPA bioaccumulation have been demonstrated (Chen et al., 2017), as well as an impairment of reproductive capacity at environmentally relevant BPA concentrations (Mandich et al., 2007), whereby structural changes in the gonads of carp males and increased malformation of oocytes, as well as a reduction in the ratio of oestrogen to androgen in the blood, have already been demonstrated at relatively low BPA concentrations in the range of 1-10 µg/l. Also in vivo, Lahnsteiner et al. (2005) observed reduced sperm quality in brown trout at low BPA exposure (1.75 µg/l) and lack of ovulation at 5 µg/l.

Various studies describe the occurrence of BPA in environmental samples (**Table 1**). In river water, very different values were found, for example in the Elbe (DE) 9-776 ng/l (Heemken et al., 2001), in the Netherlands a maximum value of 21 µg/l and median of 0.14 µg/l (Belfroid et al., 2002b), and again in the Netherlands, however, in sediment: <1.1-43 µg/kg d.w., median 3.2 µg/kg (Vethaak et al., 2005). In biota samples from fish, very different BPA measurement values were recorded. Examples are again known from the Netherlands: in liver samples of various fish species (2-75 ng/g) and in muscle (1-11 ng/g) (Belfroid et al., 2002). In the Northeast Atlantic, the median was 16.2 ± 48.3 ng/g in liver samples and 12.7 ± 20 ng/g in muscle samples (Barboza et al., 2020). The presence of vitellogenin in male fish after BPA exposure has been confirmed several times in different fish

species (Correia et al., 2007; Larsen et al., 2006). Vitellogenin analysis can therefore be a good indicator to provide an overview to xenoestrogenic induced endocrine abnormalities in male fish (Lahr et al., 2006).

Table 1. Bisphenol A in the aquatic environment: water, sediment, and fish tissue samples. Overview of important literature.

BPA levels				
Surface water (µg/l)	Sediment (µg/kg)	Fish tissue (ng/g)	Location	Reference
0.009-0.776	—[a]	—	Elbe, Germany	Heemken et al. (2001)
21 (max) 0.14 (median)	—	2-75 (liver) 1-11 (muscle)	Netherlands	Belfroid et al. (2002)
0.009-1 0.045 (median)	<1.1-43 3.2 (median)	1.2-2.6 (muscle) 1.3 (median)	Netherlands	Vethaak et al. (2005)
—	—	16.2 ± 48.3 (liver) 12.7 ± 20 (muscle)	Northeast Atlantic	Barboza et al. (2020)
4.9 (max) No data for median	—	—	Feldbach ZH, Switzerland	Gälli et al. (2009)
0.03-0.16 (treated sewage water)	—	—	Canton of Zurich	Fölmi, I. & Spycher, S. (2021)

[a] *no data.*

In Switzerland, there is hardly any data available on the distribution of BPA in water bodies; so far, only individual analyses of wastewater treating plants effluents and the receiving waters are known (Fölmli & Spycher, 2021; Gälli et al., 2009; St. Gallen Baudepartement, 2012; Voutsa et al., 2006). Otherwise, the data situation in this area is still patchy and no data on BPA in aquatic organisms are known. Gaining knowledge about individual pollutants and their abundance in the environment is important and can prevent problems that could potentially arise in the future.

The primary objective of this master's thesis was to develop a reliable method for the analysis of Bisphenol A (BPA) in liver and muscle samples, using rainbow trout Oncorhynchus mykiss and round goby Neogobius melanostomus as model species. The main challenge was to ensure that the samples were not contaminated, as BPA is a ubiquitous chemical. The goal was to achieve a limit of quantification in tissue samples as low as < 2 ng/g of BPA. Additionally, the study aimed to provide an overview of BPA contamination in the round goby, as well as in water and sediment in a selected sampling location at the Rhine River in the city of Basel, Switzerland. The research also aimed to investigate any sex- and size-specific differences in BPA contamination and the overall condition of the target species, by documenting condition indicators including total length, total weight, liver weight and hepatosomatic index (HSI). Additionally, all fish samples were analysed for the liver protein vitellogenin to provide an unspecific indication of EDC induced endocrine effects. The study

was conducted in collaboration with the EAWAG Oekotoxzentrum and the Bern University of Applied Sciences.

1.1 Round goby, *Neogobius melanostomus*

The round goby *Neogobius melanostomus* (Pallas, 1814) was identified as ideal model species for this master thesis. Originally native to the Black and Caspian Sea regions of Eastern Europe, the round goby has been introduced to numerous other locations, both freshwater and marine ecosystems on both sides of the Atlantic Ocean (Corkum et al., 2004), including the River Rhine and some of its tributaries (Baer et al., 2017). Population densities of 10-100 individuals/m^2 have been reported for newly invaded habitats and the species can account for up to 90 % of the total fish community, as reported in the lower Rhine (Borcherding et al., 2011). Since its first occurrence in 2012 the River Rhine at Basel it has established a high population density in a short period of time and presents a threat to native species by predating on the eggs and larvae of native species, as well as competing for food resources (Holm et al., 2014). The species is adapted to a benthic habitat, it is lacking a swim bladder and therefore primarily feeds at the bottom of water courses. Thereby, it is continuously in contact with sediment, where it feeds on mussels or by picking other prey items (Bussmann & Burkhardt-Holm, 2020) and potentially ingests BPA containing sediment. At least, ingested sand particles were found in a recent study (Bosshart et al., 2020), what supports the choice for this species as study species. Round goby rarely exceeds 15 cm in size, standard length differs depending on the study site and age class. For example, a standard length of up to 42 mm (year 0), 77 mm (year 1) and 93 mm (year 2) was reported for the Danube river in Prahovo (Simonoviç et al., 2001), whereas in the Azov Sea values of 70 mm (year 0), 120 mm (year 1) and 140 mm (year 2) were reported (Berg, 1949). Brandner et al. (2018) suggests, that newly invaded areas comprise populations with a higher body size compared to those at longer inhabited areas, due to the high availability of food and the lack of competition. Besides total length, most studies include the measurement of total weight, and some also liver weight which allows to calculate the hepatosomatic inded (HSI) (Azour et al., 2015). The HSI provides a good indication of energy reserve and can therefore indicate the general condition of an individual (Chellappa et al., 1995).

2 Material

This chapter provides information about all material and samples used during this study.

2.1 Sampling sites

As sampling site for the main analysis, one location at the Rhine river in Basel was with coordinates 47°33′45.347″N 7°37′49.033″E (WGS 84) representing the centre, sampling was conducted in a transect along the coordinate centre ± 150 m. The site was located in the backwater area upstream of the river power plant Birsfelden. The ARA Rhein AG wastewater treatment plant was located 6 km above of the sampling site. It was responsible for the treatment of 15'858 m^3 of waste water in 2021, of which 10 % were fed by the chemical industry located at Schweizerhalle (ARA Rhein AG, 2022). The percentage of wastewater in the pre-flood at low water is indicated with 6.6 % (BAFU, 2022). A large number of round gobies *N. melanostomus* was present at the site.

In addition to the *N. melanostomus* samples from the Rhine river, a small sample of *N. fluviatilis* was used as a reference group. The sample was collected by the Institute for Fisheries (Weilheimer Str. 8, 82319 Starnberg, Germany) from the Aisch river in Willersdorf, Bavaria (Germany) at a site which was assumed to be only marginally polluted by human activities.

2.2 Sample collection

2.2.1 Rainbow trout *Oncorhynchus mykiss* liver for preliminary analysis

As will be explained in section 2, the method establishment comprised a number of test series for optimizing the sample extraction and BPA analysis. For method development in test series 1 and 3, liver samples from *O. mykiss* were purchased from Biofischzucht Nadler AG in Rohr (canton of Aargau, Switzerland). The freshly collected liver was provided in plastic bags in a quantity of 100 g in total. The sample was picked up by the author immediately after dissection of the fish and transported within 2 hours to the laboratory, where the sample was transferred to BPA-free glass jars and frozen at – 20 °C until analysis.

2.2.2 Round goby, *N. melanostomus*

Samples of round goby were collected on various days between August and September 2022 along the previously described transect. For catching of the fish, both fishing rods and minnow traps (2 halves, Gee's Minnow Trap, Tackle Factory, length 44 cm, height 23 cm, mesh size 0.6 cm), were used. Cheese, Cervelat (local sausage) and corn kernels were used alternately as bait for the fishing rods, the minnow traps were stocked with Frolic dog food rings (Frolic Complete with beef, MARS

GmbH). All specimens were sacrificed instantly after catch and stored in a BPA-free glass jar on ice and then stored at the laboratory at – 20 °C until dissection.

2.2.3 Monkey goby, *N. fluviatilis*

A small sample of monkey goby (*N. fluviatilis*) was kindly provided by the Institute for Fisheries (Weilheimer Str. 8, 82319 Starnberg, Germany) as a reference group representing a small river ecosystem with lower anthropogenic activity. The sample individuals were collected in October 2022 and sent frozen to Switzerland, where they were continuously stored at – 20 °C until dissection.

2.2.4 Water

Water samples were taken on one day (October 18th 2022) at the Rhine transect previously described. The samples were taken by filling three BPA-free and pre-washed (twice with acetonitrile and methanol) glass jars by immersing the jar to approximately 30 cm water depth, which was the middle between the river ground and surface, open and fully filling it, then closing it under water and storing the water samples on ice until arrival to the laboratory, where they were stored at – 20 °C until analysis.

2.2.5 Sediment

Sediment samples were taken on one day (October 18th 2022) at the Rhine transect previously described. The samples were taken by filling three BPA-free and pre-washed (twice with acetonitrile and methanol) glass jars by immersing the opened jar into the sediment layer, closing it under water and storing the sediment samples on ice until arrival to the laboratory, where remaining water was removed, then stored at – 20 °C until analysis.

2.3 Laboratory

This section lists all the equipment, materials and chemicals used during the method development and the vitellogenin screening.

2.3.1 Equipment

Homogenisation of the samples was conducted using a mixer mill MM 400 supplied by Retsch GmbH (Haan, Germany) with cryogenic grinding compatible grinding jars (stainless steel, 50 mL volume), a disperging device (T25 easy clean ULTRA TURRAX® obtained from IKA®, Staufen im Breisgau, Germany) (only for test series 1) and an ultrasonic bath (SONOREX SUPER RK 106, acquired from BANDELIN electronic GmbH & Co. KG, Berlin, Germany).

Precise weighting was ensured by precision scales ML-4002T/00 (± 0.01 g) for total fish weight and XSR 204 (± 0.0001 g / ± 0.1 mg) for liver and muscle weight, as well as for the calibration of Pasteur pipettes. Both scales were obtained from Mettler Toledo Inc. (Greifensee, Switzerland). The samples were centrifuged by using an eppendorf® 5910R centrifuge provided by Eppendorf SE (Hamburg, Germany). To accelerate evaporation of the eluate, a magnetic stirrer with heating and contact thermometer (C-MAG HS 7, acquired from IKA®, Staufen im Breisgau, Germany) was used, which allowed to evaporate under a constant temperature of 40°C. The LC-MS/MS system used was an Agilent G6495A Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Inc., Santa Clara, United States) which comprised an Agilent G7120A high pressure binary pump, a ACQUITY BEH Shield RP18 Column (Waters Corporation, Milford MA, United States) and an ACQUITY UPLC BEH Shield RP18 VanGuard pre-column, and Agilent MassHunter Workstation Quantitative Analysis for QQQ version 10.1.733.0 analytical software.

ELISA and BCA were performed by using a compatible plate reader (infinite 200Pro, Tecan Group Ltd, Männedorf, Switzerland).

2.3.2 Material

Avoiding any possible contamination with BPA originating from plastic materials, glassware and chemicals was a prerequisite for the analyses. All plastic material that could be used to facilitate the BPA extraction process were previously analysed for BPA contamination and only used if no contamination was found. During the sample clean up basic laboratory material was used to store solutions and samples: glass bottles (50 mL, 150 mL, 250 mL, 500 mL, 1000 mL) and beakers (50 mL, 100 mL) were procured from Simax (KAVALIERGLASS a.s., Sázava, Czech Republic) and SCHOTT Schweiz AG (St. Gallen, Switzerland), test tubes ROTILABO® (borosilicate) were obtained from ROTH AG (Arlesheim, Switzerland) and 60 mL clear borosilicate collection vials were purchased from Fisher Scientific AG (Reinach, Switzerland). Aluminium foil was used as a cover for beakers to protect the frozen liver and muscle samples from BPA contamination during the processing whenever possible. Pasteur pipettes (Pipet-Lite™ XLS; L-200, L-100) were supplied by Mettler Toledo Inc. (Greifensee, Switzerland) and borosilicate Pasteur pipettes obtained from DWK Life Sciences Limited (Staffordshire, United Kingdom) were used for volume measurements and transfer of solvents and samples. For centrifugation borosilicate centrifuge tubes obtained from Faust Laborbedarf AG (Schaffhausen, Switzerland) (used in test series 1 and 2) and Corning® PP centrifuge tubes (15 mL) obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) (for test series 2-5) were used. For test series 1 additionally glass funnels purchased from SCHOTT Schweiz AG (St. Gallen, Switzerland) and separatory funnels acquired from Lenz Laborglas GmbH & Co. KG (Wertheim am Main, Germany) were used for phase separation. Furthermore, for test series 1

ROTILABO® PTFE filters (0.2 µm pore size) acquired from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) were used. The molecularly imprinted polymers solid-phase extraction columns (AFFINIMIP®SPE Bisphenols, 6 ml with 100 mg of sorbent) were supplied by AFFINISEP (Petit Couronne, France). An SPE Vacuum Manifold Visiprep 12 was supplied by Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). For evaporation of the elution, a selfmade construction (see photo in Appendix 9.1.3) imitating the principle of a MiniVap system by using a PP hose connected to the nitrogen source was built, which ended in connected Sterican® Hypodermic needles acquired from B.Braun SE (Melsungen, Germany) to allow the nitrogen to reach the solvent surface in a fine bundled stream coupled with the magnetic stirrer with heating previously mentioned (see 2.3.1). The readily prepared samples were sent to the Oekotoxzentrum in Agilent compatible (HP) screw vials with 250 µL and 2 mL volume with non-pigmented ms-Pure PTFE/silicone/PTFE septum both ordered from infochroma ag (Goldau, Switzerland).

A TECO®Perch (*Perciformes*) Vitellogenin ELISA kit TE1035 was obtained from TECOmedical AG (Sissach, Switzerland) for VTG analysis, which in addition to the supplied material required a multichannel pipette and tips (50 – 100 µl), vortex mixer, and an ELISA plate shaker (500 rpm, orbital shaker).

2.3.3 Chemicals

Reference standards

A certified reference standard of BPA was obtained as a power of 99.0 % analytical purity from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). The stock standard solution of 0.8 mg/ml was prepared in acetonitrile (MeCN) and kept frozen (at – 20 °C). The working standard solution contained 80 ng/ml and was used only for test series 1 and kept frozen in between usage (at – 20 °C).

All test series were based on the use of an internal standard of ^{13}C labelled BPA (hereafter abbreviated as *IS*) at a concentration of 400 ng/ml in acetonitrile, prepared and kindly provided by Daniel Olbrich (Oekotoxzentrum) from RING-13C12 (99 atom %, 100 µg/ml in MeCN), ordered from LGC Ltd (United Kingdom). The *IS* was kept frozen at – 20 °C.

All standard vial caps were securely resealed after use and stored in amber coloured glass vials.

Reagents and consumables

The MeCN (≥99,9%, HiPerSolv CHROMANORM®) and the MeOH (≥99,9% HiPerSolv CHROMANORM®) used were HPLC gradient-grade purity standard and purchased from VWR International GmbH (Dietikon, Switzerland). Glacial acetic acid (AcOH) (ROTIPURAN® 100 %, p.a.)

was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). HPLC Plus water was acquired from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Nitrogen 4.5 and liquid nitrogen were supplied by PanGas AG (Dagmersellen, Germany).

Vitellogenin ELISA

A TECO®Perch (*Perciformes*) Vitellogenin ELISA kit TE1035 was obtained from TECOmedical AG (Sissach, Switzerland) for VTG analysis, supplying all necessary reagents for the assay except destilled water (provided by Bern University of Applied Sciences BFH).

3 Methods

3.1 Workspace preparation

All work surfaces and materials were rinsed twice with both acetonitrile (MeCN) and methanol (MeOH) prior to usage to avoid possible BPA contamination during the sample extraction process.

3.2 Sample preparation (*N. melanostomus*)

The gathered data for all sample individuals consisted of their sex, total length (cm), total weight (g), liver weight (g) and the amount of muscle (g) dissected for analysis. Two size categories were defined to detect any differences in BPA concentration depending on the age of the fish, therefore all individuals < 11 cm were assigned to the size category "small" whereas individuals ≥ 11 cm were assigned to the size category "tall". Sex categories were defined as "male" and "female". Thus, four categories were recorded for analysis: "small females", "small males", "tall females", "tall males". Furthermore, the catch dates were treated as separate categories to detect possible differences among different days.

The collected samples were first thawed at room temperature. All samples were then separated according to their sex (male / female) determined based on the visual sex characteristics. If an individual could not be assigned to a sex with certainty, it was recorded as a female (some individuals showed signs of intersex). This was decided to ensure that all recorded males were allocated correctly for subsequent vitellogenin screening. Thereafter, all individuals were consecutively measured in length (± 1 mm) and weighed for total weight with a precision scale (± 0.01 g), then dissected to take the liver and muscle samples. To remove the liver, the fish were opened longitudinally starting from the anus up to the beginning of the gills with a knife or scissors (which were consistently cleaned after each individual dissection). All livers were measured by a precision scale (± 0.0001 g / ± 0.1 mg) and pooled by sex, size category, and catch date category until a minimum of 2.2 g was collected to compensate for the loss during homogenisation and ensure a minimum of 2 g sample size per test if possible. The hepatosomatic index (HSI), which serves as a measure of energy reserves in fish was calculated as liver weight/body weight x 100. Muscle samples were taken from the left side, weighted, and pooled similarly. The samples were then stored in beakers covered with closely fitted aluminium foil at – 20 °C until analysis.

The reference samples of *N. fluviatilis* were processed by an identical procedure.

3.3 Method development

Through an extensive literature review, two methods were selected to serve as the basis for the present method development for BPA analysis suitable for fish tissue (liver and muscle) samples (Cerkvenik-Flajs & Šturm, 2021; Di Marco Pisciottano et al., 2020). For further refinement, the procedures of both methods were combined in their characteristics and simultaneously optimised through adjustments and adapted to the available laboratory infrastructure and equipment. The method development was based on repeated verification and included verification of intermediate steps with the aim of avoiding potential contamination factors, decreasing the limit of detection (LOD) and the limit of quantification (LOQ) of BPA as far as possible and besides optimising effort and accuracy in the process. In the end, this method development comprised four test series resulting in the final method used in the main experiment (see Figure 1).

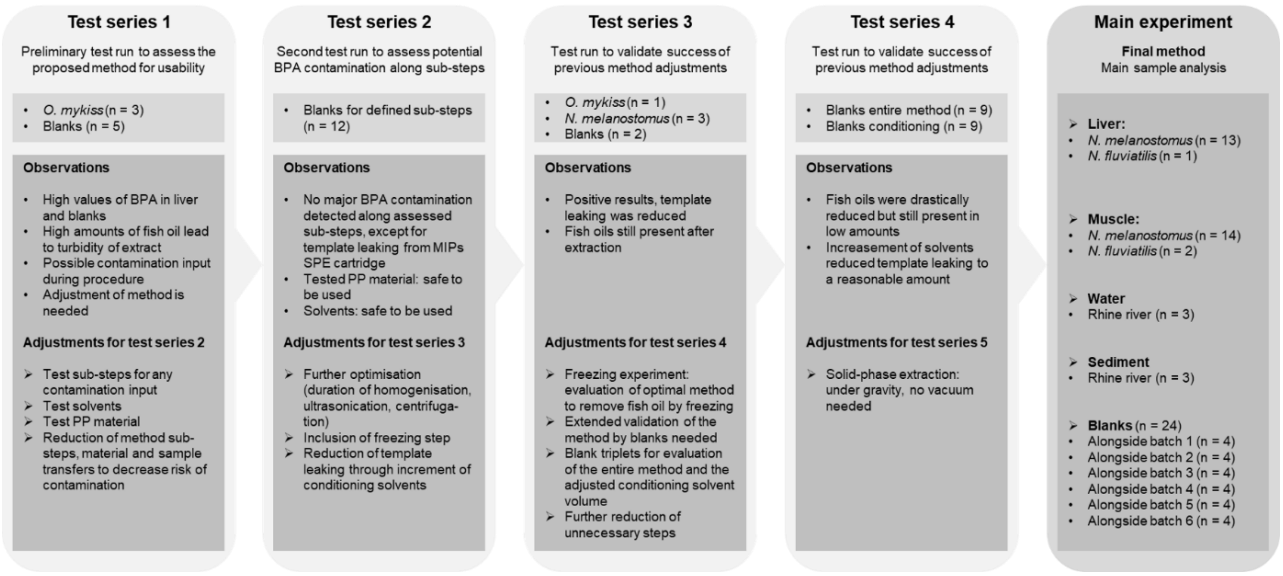


Figure 1. Schematic structure of the method development among the four test series. The developed method was applied in the main experiment to analyse the collected round goby, monkey goby, water, and sediment samples for BPA contamination.

Readily extracted samples were sent to Daniel Olbrich (Oekotoxzentrum) for measurement by LC-MS/MS (see 0).

3.4 Test series 1

In a first preliminary test, the method was qualitatively assessed for its usability with fish tissue samples. The sample size was kept low in order to reduce expenses, as changes of the method were expected depending on the results of this first test run. Liver tissue was considered more difficult to analyse due to its complex matrix containing high amounts of fish oils. In order to assess possible difficulties caused by matrix interferences, only liver tissue was used for test series 1 – 4, while the less complex muscle tissue was only examined in the main experiment. The liver samples

for test series 1 belonged to rainbow trout (*Oncorhynchus mykiss*). In test series 1, cryogenic homogenisation of the sample was added to the original methods due to the availability of the material in the laboratory and the reliability in breaking cell walls effectively. The detailed sampling plan for test series 1 is presented in chapter 3.4.1.

Homogenisation

The liver samples were thawed at room temperature and cut into small pieces with a razor blade, then frozen in a porcelain mortar by adding liquid nitrogen to prevent the sample and grinding ball to stick together while freezing. Subsequently, the frozen sample material was placed into grinding jars and again deep-frozen to – 196 °C in a Styrofoam container filled with liquid nitrogen. The sample was then grinded using a mixer mill (Retsch MM400) at 30.0 1/s for 90 s.

Sample extraction

For each sample, 2.00 g ± 0.01 g of homogenised tissue was weighed into beakers (50 ml) followed by addition of 50 µl of IS standard solution at 400 ng/ml. For the purpose of validation, the samples in test series 1 were spiked with the reference standard BPA at different levels (see Table 2). Thereafter, 7.0 ml of HPLC Plus water/MeCN 1/1 (v/v) solution was added to the sample and then homogenised again for 2 min by ultra-turrax disperging and transferred into a glass centrifuge vial. The sample then got sonicated in an ultrasonic bath for 15 min at room temperature, then centrifuged at 1`700 *g* and 4 °C for 15 min. The supernatant was then transferred into a glass tube, diluted with 7.0 ml of HPLC Plus water and mixed by vortex for 30 s.

To the sample residue, 5.0 ml of MeCN was added, the mixture was sonicated for 25 min at room temperature and centrifuged at 1`700 *g* and 4 °C for 15 min. The supernatant was collected, 15 ml of HPLC Plus water added, mixed by vortex for 30 s, and then gathered with the first extract.

Solid-phase extraction (SPE MIPs)

The sample extract was loaded onto an AFFINIMIP® Bisphenol SPE 6 ml PP cartridge, previously conditioned with 5.0 ml of MeOH/AcOH (98/2, v/v), 5.0 ml of MeCN and 5.0 ml of HPLC Plus water. The cartridge then got washed with 10.0 ml of HPLC Plus water and 6.0 ml of HPLC Plus water/MeCN 60/40 (v/v). The elution then was carried out with 3.0 ml of methanol, evaporated to approx. 1 ml at room temperature under a gentle nitrogen stream, then transferred into an Agilent compatible (HP) screw vial and evaporated just to dryness. The remains were combined with 100 µl of MeOH and 100 µl of HPLC Plus water for subsequent LC-MS/MS analysis at the Oekotoxzentrum by Daniel Olbrich. Due to the turbidity of the extract upon the addition of water by fish oils, the eluate had to be additionally filtered with a PTFE filter (20 µm diameter) as an unscheduled measure before analysis.

3.4.1 Sampling plan

The sampling plan for test series 1 is presented in Table 2. Liver samples were treated exactly as described above. For blank samples, 2 ml ± 0.01 ml of HPLC Plus water was used instead of liver homogenate. The blank samples were not cryomilled and directly pipetted into a glass centrifuge tube. The homogenisation step by ultra-turrax was skipped. Blank no. *3* was used to check whether the HPLC Plus water itself could contain BPA contamination. For this purpose, the water used in no. *3* was purified by a conditioned cartridge, with the aim of removing any BPA present in the water through the MIPs, which should bind the BPA.

Table 2. Sampling plan for test series 1. *IS* = ^{13}C labelled BPA in a concentration of 400 ng/ml in MeCN, Spike = certified reference standard BPA, working solution in MeCN (80 ng/ml).

No.	Quantity	*IS* (50 µl)	Spike	Tissue type	Category
1	1x	*none*	*none*	*none*	Doubleblank
2.1	2x	20.0 ng	*none*	*none*	Blank
2.2					
3	1x	20.0 ng	*none*	*none*	Blank, *H_2O "filtered"*
4	1x	20.0 ng	2.00 ng (25 µl)	*none*	Blank
5	1x	20.0 ng	2.00 ng (25 µl)	Liver (2 g) *O. mykiss*	Tissue sample
6	1x	20.0 ng	10.00 ng (125 µl)	Liver (2 g) *O. mykiss*	Tissue sample
7	1x	20.0 ng	50.00 ng (625 µl)	Liver (2 g) *O. mykiss*	Tissue sample

3.5 Test series 2

The results of test series 1 indicated that BPA contamination may occur during extraction (see chapter 4.2.1). These initial results served as the basis for the further development of the method in test series 2. In order to trace any BPA contamination back to a specific sub-step, all sub-steps were examined individually in test series 2 by using blank samples. All potentialsolvents and PP materials were also tested. In addition, it was considered whether PP centrifuge tubes could be used instead of glass centrifuge tubes, which would reduce the number of intermediate steps and transfers between the sample containers. The homogenisation step using ultra-turrax was dispensed altogether, as there may be BPA residues in the thread that cannot be sufficiently removed by cleaning, and one homogenisation step (cryomilling) was found to be sufficient. The inclusion of the filtration step in test series 2 was deliberately omitted, as the liver samples were subsequently to be freed from remaining fats by means of an intermediate step consisting of freezing overnight. The second centrifugation of the sample residue was also omitted in the further course.

3.5.1 Sampling plan

The sampling plan for test series 2 was defined as presented in Table 3.

Table 3. Sampling plan for test series 2. The intermediate steps and solvents were analysed and validated separately to detect and localise any possible BPA contamination input to the extraction process. The sampling solvent were added with *IS* (mentioned as "BPA IS") and in some cases additionally spiked with certified standard BPA (mentioned as "BPA cs").

Category	No.	Sampling solvent	BPA *IS* / BPA cs
Solvents (transferred by using glass pipettes)	*1.1*	MeCN	20 ng BPA IS
	1.2	MeCN	20 ng BPA IS
			20 ng BPA cs
	2.1	MeOH/AcOH (98/2)	20 ng BPA IS
	2.2	MeOH/AcOH (98/2)	20 ng BPA IS
			20 ng BPA cs
	3	MeOH/MeCN (50/50) glassware washout	20 ng BPA IS
	4	MeOH	20 ng BPA IS
			20 ng BPA cs
Solvents (transferred by using PP pipette tips)	*5.1*	MeOH	20 ng BPA IS
	5.2	MeOH	20 ng BPA IS
			20 ng BPA cs
Cartridge (AFFINIMIP® Bisphenol SPE)	*6*	5 ml MeOH/AcOH (98/2) and 5 ml MeCN	20 ng BPA IS
	7	Elution with MeOH	20 ng BPA IS
PP centrifuge tubes	*8*	MeCN/MeOH (50/50)	20 ng BPA IS
Cryomilling	*9*	MeOH	20 ng BPA IS

The method used for each sample is specified in Table 3. The hereby assessed solvents were from the same bottles used for all following test series.

Table 4. Detailed description of the individual validation steps for test series 2.

General method used for preparation of test series 2 variants: The solvent samples were prepared as specified below and evaporated to approx. 1 ml under a gentle nitrogen stream and in a 40 °C water bath, then transferred into an Agilent compatible (HP) screw vial and evaporated just to dryness. The remains were added with 100 µl of methanol and 100 µl of HPLC Plus water for LC-MS/MS analysis.	
No.	**Solvents** (transferred by using ***glass pipettes*** previously rinsed twice with MeOH and MeCN to rule out contamination input by any plastic items):
1.1 *1.2*	**MeCN:** 20 ng BPA *IS* was added to a 10 ml MeCN sample (sample *1.2* was additionally added with 20 ng BPA cs).
	MeOH/AcOH (98/2):
2.1 *2.2*	20 ng BPA *IS* was added to a 10 ml MeOH/AcOH (98/2) sample (sample *2.2* was additionally added with 20 ng BPA cs).
	MeOH/MeCN (50/50) (glassware washout):
3	The MeOH and MeCN used for rinsing the glass material was also analysed for BPA to assess the extent of BPA contamination of the unrinsed material. 20 ng BPA *IS* was added to a 10 ml MeOH/MeCN (50/50) sample.
4	**MeOH (spiked):** 20 ng BPA *IS* and 20 ng BPA cs were added to a 10 ml MeOH sample.
	Solvents (transferred by using ***PP pipette tips*** previously rinsed twice with MeOH and MeCN to rule out contamination input by any plastic items):
5.1 *5.2*	This step was planned to check whether PP pipette tips lead to BPA input. Only MeOH was used here due to the higher solubility of BPA in MeOH compared to MeCN according to Sun et al., (2020). 10 ml MeOH was pipetted into a test tube using a 1 ml PP pipette tip, then 20 ng BPA *IS* was added (sample *5.2* was additionally added with 20 ng BPA cs).
	Cartridge (AFFINIMIP® Bisphenol SPE)
6	The cartridge may contain residues of BPA, whereupon it was checked for template leaking by conditioning with 5.0 ml of MeOH/AcOH (98/2, v/v), previously added with 20 ng of BPA *IS* and 5 ml of MeCN according to the recommendation by AFFINIMIP®. The solvents were collected and processed as indicated above.
7	The third conditioning step was also carried out (5 ml HPLC Plus water), after which the cartridge was loaded with a further 10 ml HPLC Plus water to which 20 ng BPA *IS* had been added to check whether the HPLC Plus water contained BPA or whether BPA was still leaching from the cartridge. The cartridge was eluted with 3 ml MeOH and prepared for analysis as indicated above.
	PP centrifuge tubes
8	PP centrifuge tubes were tested for BPA leaking. The use of PP centrifuge tubes would simplify the method. For this purpose, 10 ml MeCN/MeOH (50/50) added with 20 ng BPA *IS* were pipetted into a PP centrifuge tube, mixed by vortex for 30 s and sonicated for 25 min.
	Cryomilling
9	During cryomilling, condensation of BPA from air caused by the low temperatures could be a source of contamination. Therefore, 20 ng BPA *IS* and 10 ml MeOH were added to a porcelain mortar bowl, poured with liquid nitrogen, and transferred to the cryogenic grinding jar which was again frozen to liquid nitrogen temperature and then milled at 30.0 1/s for 90 seconds in a mixer mill. After thawing to room temperature, the sample was prepared for analysis as indicated above after removing a thin metal abrasion dust by magnets.

3.6 Test series 3

Test series 3 consisted of blanks and liver samples whereupon *N. melanostomus* liver samples were included for the first time. The method was optimised in some steps using the previously obtained results and experience from test series 1 and 2. For example, the duration of homogenisation, ultrasonication and centrifugation was extended, solvent quantity was adjusted for some steps and most importantly a freezing step was included aiming to remove any fish oils from the sample and therefore no filtration of the extract would be needed. Other steps were also excluded such as the homogenisation by ultra-turrax and the second centrifugation step. Two blanks were planned to assess the template leaking from the MIPs SPE cartridges previously observed while increasing solvent volume used during conditioning. The new method was hence defined as follows:

Homogenisation

The liver samples were thawed at room temperature and cut into small pieces with a razor blade, then frozen in a porcelain mortar by adding of liquid nitrogen to prevent the sample and grinding ball to stick together while freezing. Subsequently, the frozen sample material was placed into grinding jars and again deep-frozen to – 196 °C in a Styrofoam container filled with liquid nitrogen. The sample was then grinded using a mixer mill (Retsch MM400) at 30.0 1/s for 2.5 min.

Sample extraction

For each sample, 2.00 g ± 0.01 g of homogenised tissue was weighed into a PP centrifuge tube (15 ml) followed by addition of 50 µl of IS standard solution at 400 ng/ml. Then 3.5 ml of HPLC Plus water was added and mixed by vortex for 30 s, then 3.5 ml of MeCN were added and mixed again by vortex for 30 s. The sample then was sonicated in an ultrasonic bath for 30 min at room temperature, then centrifuged at 1`700 *g* and 4 °C for 20 min. The supernatant was transferred into a PP centrifuge tube (50 ml), diluted with 12 ml of HPLC Plus water and mixed by vortex for 30 s before transferring into the assigned separating container* for freezing over night at – 20 °C to remove fish oils.

Solid-phase extraction (SPE MIPs)

The clear supernatant was collected the next day and loaded onto an AFFINIMIP® Bisphenol SPE 6 ml PP cartridge (under vacuum at 1 drop/s), previously conditioned with 5.0 ml of MeOH/AcOH (98/2, v/v), 5.0 ml of MeCN and 5.0 ml of HPLC Plus water (under vacuum at 2 drops/s). The cartridge then got washed with 10.0 ml of HPLC Plus water and 6.0 ml of HPLC Plus water/MeCN 60/40 (v/v) at 1 drop/s. The elution then was carried out with 3.0 ml of methanol at 1 drop/s, evaporated to approx. 1 ml at room temperature under a gentle nitrogen stream, then transferred

into an Agilent compatible (HP) screw vial and evaporated just to dryness. The remains were combined with 100 µl of MeOH and 100 µl of HPLC Plus water for LC-MS/MS analysis.

*The freezing step was tested with either separating funnels or graduated cylinders, see Table 6 for details.

3.7 Sampling plan

The sampling plan for test series 3 was defined as presented in Table 5.

Table 5. Sampling plan for test series 3. IS = ^{13}C labelled BPA in a concentration of 400 ng/ml in MeCN.

No.	*IS* (50 µl)	Category	Tissue type	Focus
1.2	20 ng	Blank	*none*	SPE template leaking test
1.3	20 ng	Blank	*none*	SPE template leaking test
2.1	20 ng	Tissue	Trout liver	Whole method, freezing step effectivity
2.2	20 ng	Tissue	Round goby liver	Whole method, freezing step effectivity
2.3	20 ng	Tissue	Round goby liver (1 g)	Whole method, freezing step effectivity
2.4	20 ng	Tissue	Round goby liver (1 g)	Whole method, freezing step effectivity

The exact method used for each sample was specified in Table 6.

Table 6. Detailed description of the samples analysed in test series 3.

No.	Detailed description
1.2	BPA template leaking from the cartridges was detected in test series 2, whereupon it was checked if increased solvent volume during conditioning would reduce BPA contamination. The cartridge was therefore conditioned with 10 ml of MeOH/AcOH (98/2, v/v), 10 ml of MeCN and 5 ml of HPLC Pure water. Then 10 ml of HPLC Pure water previously added with 20 ng of BPA *IS* was applied to the cartridge, washed with 6 ml of HPLC Pure water/MeCN (50/50, v/v) and eluated with 3 ml MeOH, then prepared for LC-MS/MS analysis following the usual method.
1.3	BPA template leaking from the cartridges was detected in test series 2, whereupon it was checked if increased solvent volume during conditioning would reduce BPA contamination. The cartridge was therefore conditioned with 15 ml of MeOH/AcOH (98/2, v/v), 15 ml of MeCN and 5 ml of HPLC Pure water. Then 10 ml of HPLC Pure water previously added with 20 ng of BPA *IS* was applied to the cartridge, washed with 6 ml of HPLC Pure water/MeCN (50/50, v/v) and eluated with 3 ml MeOH, then prepared for LC-MS/MS analysis following the usual method.
2.1	*O. mykiss* liver samples were used following the method described in chapter 3.6. The freezing step was carried out in a glass separating funnel.
2.2	*N. melanostomus* liver samples were used following the method described in chapter 3.6. The freezing step was carried out in a glass separating funnel.
2.3	*N. melanostomus* liver samples were used following the method described in chapter 3.6. The freezing step was carried out in a glass graduated cylinder. Sample size was 1 g ±0.01 g instead of 2 g.
2.4	*N. melanostomus* liver samples were used following the method described in chapter 3.6. The freezing step was carried out in a glass separating funnel. Sample size was 1 g ±0.01 g instead of 2 g.

3.8 Test series 4

Due to the promising results from test series 3 concerning the reduction of template leaching during solid-phase extraction using an increased solvent volume for the conditioning of the cartridges, a last method validation by extended blanks was planned for test series 4 to present a reliable final method in main experiment. Therefore, triplets of blanks for three solvent volumes (5 ml; 10 ml; 15 ml of both conditioning solvents MeOH/AcOH (98/2, v/v) and MeCN) were defined, which were run in two variants: one focusing on the conditioning step and the other including the whole method process. The method was adjusted again by further reduction of unnecessary steps and reduction of potential contamination inputs: the pre-freezing of the sample in the porcelain mortar was dispensed by inserting them directly into the opened grinding jars, previously filled with liquid nitrogen, what still prevented the samples from sticking to the jars wall and the grinding ball. Besides, before the start of test series 4 the freezing step had to be adjusted hence no satisfactory result was achieved during test series 3 by using separating funnels nor graduated cylinders, see chapter 3.9.1. As a result, the method was further reduced in complexity by avoiding the need of the sample to be transferred into a separating funnel or graduated cylinder, instead conducting the freezing inside a PP centrifuge tube.

The method developed for test series 4 was hence defined as follows.

Homogenisation

The samples were thawed at room temperature and placed into the open grinding jars previously filled with liquid nitrogen to prevent the samples through immediate freezing to stick to the walls and the grinding ball. The jars were then closed and again deep-frozen to – 196 °C in a Styrofoam container filled with liquid nitrogen. The samples were then grinded using a mixer mill (Retsch MM400) at 30.0 1/s for 2.5 min.

Sample extraction

For each sample, 2.00 g ± 0.01 g of homogenised tissue was weighed into a PP centrifuge tube (15 ml) followed by addition of 50 µl of IS standard solution at 400 ng/ml. Then 3.5 ml of HPLC Plus water was added and mixed by vortex for 30 s, then 3.5 ml of MeCN was added to the sample and mixed again by vortex for 30 s. The sample then was sonicated in an ultrasonic bath for 30 min at room temperature, then centrifuged at 1`700 *g* and 4 °C for 20 min. The supernatant was transferred into a PP centrifuge tube (15 ml) and stored over night at – 20 °C. The clear supernatant phase was then immediately transferred into another PP centrifuge tube by carefully pipetting (approx. 2 ml) and added with 8 ml HPLC Plus water to ensure that the water/MeCN ratio was ≤ 80/20, then mixed by vortex for 30 s.

Solid-phase extraction (SPE MIPs)

The clear supernatant was collected the next day and loaded onto an AFFINIMIP® Bisphenol SPE 6 ml PP cartridge (under vacuum at 1 drop/s), previously conditioned with 5.0 ml* of MeOH/AcOH (98/2, v/v), 5.0 ml* of acetonitrile and 5.0 ml of HPLC Plus water (under vacuum at 2 drops/s). The cartridge then got washed with 10.0 ml of HPLC Plus water** and 6.0 ml of HPLC Plus water/MeCN 60/40 (v/v) at 1 drop/s. The elution then was carried out with 3.0 ml of methanol at 1 drop/s, evaporated to approx. 1 ml at room temperature under a gentle nitrogen stream, then transferred into an Agilent compatible (HP) screw vial and evaporated just to dryness. The remains were added with 100 µl of methanol and 100 µl of HPLC Plus water for LC-MS/MS analysis.

*Depending on the blank number, a different amount of solvent volume was used (see Table 7).

**In case of B1 samples, the 10 ml HPLC Plus water were added with 20 ng of *IS*.

3.9 Sampling plan

The sampling plan for test series 4 was defined as presented in Table 7. All B1 only included the solid-phase extraction (SPE MIPs) step. All B2 were prepared according to the full test series 4 method. Instead of tissue sample, HPLC Plus water was used. The water was first "homogenised" by cryomilling to simulate the full method. 2 ml ± 0.01 ml was then pipetted into a PP centrifuge tube and 20 ng of *IS* added. After freezing, the supernatant was collected (approx. 2 ml) just as the tissue samples would be treated.

For B1 20 ng of *IS* was added to the 10 ml HPLC Plus water used in the washing step.

Table 7. Detailed sampling plan for test series 4. All samples were blanks and arranged into triplets to validate the method and elicit the optimal solvent volume for conditioning of the SPE MIPs cartridges. B1 blanks consisted of the solid-phase extraction step only, B2 blanks involved the entire method procedure.

No.	*IS* (50 µl)	Details and solvent volume for conditioning
B1.1a	20 ng	Original volume
B1.1b	20 ng	5 ml of MeOH/AcOH (98/2, v/v), 5 ml of MeCN and 5 ml of HPLC Pure water
B1.1c	20 ng	
B1.2a	20 ng	Adjusted volume
B1.2b	20 ng	10 ml of MeOH/AcOH (98/2, v/v), 10 ml of MeCN and 5 ml of HPLC Pure water
B1.2c	20 ng	
B1.3a	20 ng	Adjusted volume
B1.3b	20 ng	15 ml of MeOH/AcOH (98/2, v/v), 15 ml of MeCN and 5 ml of HPLC Pure water
B1.3c	20 ng	
B2.1a	20 ng	Original volume
B2.1b	20 ng	5 ml of MeOH/AcOH (98/2, v/v), 5 ml of MeCN and 5 ml of HPLC Pure water
B2.1c	20 ng	
B2.2a	20 ng	Adjusted volume
B2.2b	20 ng	10 ml of MeOH/AcOH (98/2, v/v), 10 ml of MeCN and 5 ml of HPLC Pure water
B2.2c	20 ng	
B2.3a	20 ng	Adjusted volume
B2.3b	20 ng	15 ml of MeOH/AcOH (98/2, v/v), 15 ml of MeCN and 5 ml of HPLC Pure water
B2.3c	20 ng	

3.9.1 Freezing experiment

By the freezing step previously used in test series 3 the fish oils could not be removed successfully. Therefore, a freezing experiment was planned to improve the effectivity through changing of the solvent/water ratio and switching to PP centrifuge tubes instead of separating funnels and graduated cylinder, thus reducing contamination risk by elimination of another sample transfer.

O. mykiss and *N. melanostomus* liver samples were homogenised and processed according to the test series 3 method until centrifugation, adjusting the water/MeCN ratio to 50/50; 60/40; 70/30; 80/20 (v/v) at the beginning of the sample extraction step. The supernatant was collected and filled into a PP centrifuge tube, then stored overnight at – 20 °C. The next day, the clear supernatant was collected, added with HPLC Plus water at a 80/20 ratio to assure that the analyte was not flushed out during solid-phase extraction and the best resulting variant was determined and implemented to the method for test series 4 and the final method (main experiment).

3.10 Main experiment

By discussing and validating all results and experiences of the test series 1-4, following final method was proposed and used for the main sample analysis in main experiment. The solid-phase extraction was conducted under gravity without vacuum expect after the washing step, hence difficulties

occurred while processing a large sample quantity at the same time, whereas drying of the sample during conditioning had to be prevented. The centrifugation was adjusted to maximum *g* as supported by the PP centrifuge tubes.

Final method

Homogenisation

The samples were thawed at room temperature and placed into the open grinding jars previously filled with liquid nitrogen to prevent the samples through immediate freezing to stick to the walls and the grinding ball. The jars were then closed and again deep-frozen to – 196 °C in a Styrofoam container filled with liquid nitrogen. The samples were then grinded using a mixer mill (Retsch MM400) at 30.0 1/s for 2.5 min.

Sample extraction

For each sample, 2.00 g ± 0.01 g of homogenised tissue was weighed into a PP centrifuge tube (15 ml) followed by addition of 50 µl of IS standard solution at 400 ng/ml. Then 3.5 ml of HPLC Plus water was added and mixed by vortex for 30 s, then 3.5 ml of MeCN was added to the sample and mixed again by vortex for 30 s. The sample was sonicated in an ultrasonic bath for 30 min at room temperature, then centrifuged at 4347 *g* and 4°C for 20 min. The supernatant was carefully transferred into a PP centrifuge tube (15 ml) and stored over night at – 20 °C. The clear supernatant phase was then transferred into another PP centrifuge tube by pipetting (approx. 2 ml). The transfer was done quickly to prevent the precipitated matter from re-dissolving. The clear extract was then added with 8 ml HPLC Plus water to ensure water/MeCN ratio was ≤ 80/20, then mixed by vortex for 30 s.

Solid-phase extraction (SPE MIPs)

The clear supernatant was collected the next day and loaded onto an AFFINIMIP® Bisphenol SPE 6 ml PP cartridge, previously conditioned under gravity with 10.0 ml of MeOH/AcOH (98/2, v/v), 10.0 ml of MeCN and 5.0 ml of HPLC Plus water. The cartridge then got washed with 10.0 ml of HPLC Plus water and 6.0 ml of HPLC Plus water/MeCN 60/40 (v/v) and dried by applying a vacuum. The elution then was carried out under gravity with 3.0 ml of MeOH, evaporated to approx. 1 ml at room temperature under a gentle nitrogen stream, then transferred into an Agilent compatible (HP) screw vial and evaporated just to dryness. The remains were combined with 100 µl of MeOH and 100 µl of HPLC Plus water for LC-MS/MS analysis.

3.10.1 Sampling plan

A total of 36 pooled samples, including three sediment and three river water samples, as well as three *N. fluviatilis* pooled samples were analysed in main experiment. The capacity limit for samples simultaneously processed was 12, limited by the capacity of the vacuum manifold used for solid-phase extraction. The sample analysis was therefore divided into six batches, extracting either three, six or seven samples simultaneously. Each batch was accompanied by four blank samples along the complete extraction process for verification, resulting in a total of 24 blank samples. HPLC Plus water was used as replacement for the tissue in blank samples.

Tissue samples

The samples for this main analysis were pooled according to the categories and process described in chapter 3.2. Since not all liver samples obtained the 2 g sample size, a lower quantity was used for some samples as indicated in

Table 8 and Table 9. Due to fish oils still present albeit in much lower concentration, all liver samples were dissolved in 50 µl of MeCN after evaporating, without addition of water instead of 100 µl of methanol and 100 µl of HPLC Plus water, then transferred into a narrowed Agilent compatible (HP) screw vial (see chapter discussion 5.2.5). The same procedure was used for the corresponding blank samples. Muscle samples followed the method without changes.

Table 8. Sampling plan for *N. melanostomus* liver and muscle samples for main analysis in main experiment.

Batch	Sample No.	Date of catch	Tissue	Size category (t=tall; t=small)	Sex (m=male; f=female)	Fitted sample weight if not 2 g (g)
1	1	08.08.2022	Liver	t	m	0.7
1	2	02.09.2022	Liver	t	m	
1	3	02.09.2022	Liver	t	m	
1	4	02.09.2022	Liver	t	m	
1	5	02.09.2022	Liver	t	m	
1	6	02.09.2022	Liver	t	m	1.8
2	7	02.09.2022	Liver	t	f	
2	8	10.09.2022	Liver	s	m	1.3
2	9	10.09.2022	Liver	s	f	1.6
2	10	10.09.2022	Liver	t	m	
2	11	02.09.2022	Liver	s	m	
2	12	02.09.2022	Liver	s	f	
2	13	08.08.2022	Liver	s	f (mf)	0.7
3	14	08.08.2022	Muscle	t	m	
3	15	02.09.2022	Muscle	t	m	
3	16	02.09.2022	Muscle	t	m	
3	17	02.09.2022	Muscle	t	m	
3	18	02.09.2022	Muscle	t	m	
3	19	02.09.2022	Muscle	t	m	
3	20	02.09.2022	Muscle	t	f	
4	21	10.09.2022	Muscle	s	m	
4	22	10.09.2022	Muscle	s	f	
4	23	10.09.2022	Muscle	t	m	
4	24	02.09.2022	Muscle	s	m	
4	25	02.09.2022	Muscle	s	f	
4	26	08.08.2022	Muscle	s	m	
4	27	08.08.2022	Muscle	s	f	

The *N. fluviatilis* sample consisted of a small sample size, therefore liver samples of both male and female had to be combined, see Table 9.

Table 9. Sampling plan for *N. fluviatilis* liver and muscle samples for main analysis in main experiment.

Batch	Sample No.	Tissue	Size category (t=tall; s=small)	Sex (m=male; f=female)	Fitted sample weight for extraction if not 2 g (g)
5	1	Liver	s	f (mf)	0.1
5	2	Muscle	s	m	1.5
5	3	Muscle	s	m	

River Rhine water and sediment samples

The water and sediment samples were processed according to the developed method, but the homogenisation step via cryomilling was skipped, since no pulping of cells was needed. Instead, the sonication time was doubled (60 min).

For the sediment samples 2 g ±0.01 g wet weight was used, any visible organic matter such as leaves was excluded. Three samples for each type were extracted for analysis, see Table 10.

Table 10. Sampling plan for Rhine river water and sediment samples for main analysis in main experiment.

Batch	Sample No.	Sample Type	Sample weight (g)
6	*1*	Water	2
6	*2*	Water	2
6	*3*	Water	2
6	*4*	Sediment	2
6	*5*	Sediment	2
6	*6*	Sediment	2

3.11 Measurements by LC-MS/MS

Chemical analyses were performed using electrospray ionization (ESI) in negative mode on an Agilent G6495A Triple Quadrupole (QQQ) mass spectrometer (for parameter settings see Appendix 10.4, Table A). Chromatographic parameters see Appendix 10.4, Table B. Briefly, a Waters 100 x 2.1 mm ACQUITY BEH C18 column with 1.7 µm particle size was used within a column compartment maintained at 40 °C and with an upstream security guard cartridge and an eluent flow rate of 0.5 ml/min. Eluent A comprised of 2.4 mM ammonium hydroxide in water (from a 25 % solution 0.38 ml was added to 1 l of water). Eluent B comprised of 100 % LCMS grade methanol. 0.6 mM ammonium fluoride was added post-column with a T-connector at a flow rate of 0.05 ml/min. A sample run lasted 17 min and started with 90 % A and 10 % B and this condition was maintained for 0.19 min and then quickly raised to 30 % B at 0.2 min. For the next 8.8 min Eluent A was reduced to 74 % then for the next 2.3 min reduced to 98 % and this condition was maintained for a further 3.7 min. At 14 min into the run, the initial condition (90 % A and 10 % B) was reintroduced and maintained until minute 17 min, subsequently, the next run starts.

The monitored mass transitions and compound specific tuning parameters of target analytes and their isotope-labeled analogs in ESI-ionization mode are shown in Appendix 10.4 (Table C). The source of the analytes and matching internal standards are listed in Appendix 10.4 (Table D).

3.12 Method validation and quality assurance

In each batch (main experiment) control samples consisting of HPLC Plus water added with 20 ng of *IS* were included and were treated with the same procedure as the tissue, water and sediment samples. In test series 1 – 4 control samples were also included as specified in the corresponding chapters. The limit of detection (LOD) and limit of quantification were determined separately

depending on the test series and the sample blanks / sample type and were indicated in each test series in chapter results.

3.13 Vitellogenin

For measurement of the VTG with ELISA, the remains of the homogenised samples were pre-diluted in the dilution buffer provided by the manufacturer, vortexed, and prepared for assay in duplicates. The ELISA was carried out according to the manufacturer's instructions (TECO®Perch (Perciformes) Vitellogenin ELISA) and the results based on a 4-parameter calibration curve were back-calculated by multiplying the value read off the calibration curve by the dilution factor of the samples.

To complement the ELISA and correct any influencing factors, a parallel BCA protein assay was used to quantify the total amount of protein and correct the vitellogenin results by the protein concentration.

3.14 Data analysis

Data visualisation and statistical analysis was performed using R statistical software (v3.6.3; R Core Team, 2020). The size and sex categories data was tested for normal distribution by using Shapiro-Wilk test if $n < 30$, otherwise checked visually by EDC (boxplots) (Stone, 2010). Parametric unpaired two-samples Welch's t-test was used if normality was assumed, non-parametric unpaired two-samples Wilcoxon test was used if the data was not normally distributed. Differences were regarded as significant at $p < 0.05$. The visualisation was performed using ggplot2 package (Wickham, 2016).

4 Results

4.1 Sample composition

This section refers to the samples used in the main experiment.

Round goby

In total, n = 173 specimen of round goby and one bighead goby *Ponticola kessleri* (Günther, 1861), which was excluded from the analysis, were caught at the established sampling location in Basel. The overall sex ratio of females to males was 1:1.34 (females n = 74; males n = 99). Total length (L_T) ranged from 4.6 to 13.1 cm in females and from 5.5 to 14.5 cm in males. Only one specimen (4.6 cm) was < 5 cm, which according to Kornis et al. (2012) is the limit for juveniles, and cannot be determined to a sex with certainty. It was therefore listed as small female to prevent wrongly assigned males despite showing rather male morphology features. The mean L_T of all collected samples was 9.77 cm (SD = 2.33 cm), mean $L_{T\,females}$ was 8.92 cm (SD = 1.91 cm) and mean $L_{T\,males}$ was 10.4 cm (SD = 2.41 cm). Tall specimen (≥ 11 cm) count was n = 13 for females and n = 45 for males, whereas small specimen (< 11 cm) count was n = 61 for females and n = 54 for males. Overall, males tended to appear more frequent in taller sizes than females, while females were more abundant in the category "small". Mean $L_{T\,tall\,females}$ was 12.02 cm (SD = 0.73 cm) and mean $L_{T\,tall\,males}$ was 12.66 cm (SD = 1.02 cm), mean $L_{T\,small\,females}$ was 8.26 cm (SD = 1.35 cm) and mean $L_{T\,small\,males}$ was 8.52 cm (SD = 1.41 cm). While males had a higher mean length in all categories, significance was only found for L_T (Wilcoxon test, $p < 0.001$), differences in mean of $L_{T\,tall}$ and $L_{T\,small}$ were not significant (Wilcoxon test, $p > 0.05$; resp. Welch's t-test, $p > 0.05$), see also Table 11. Figure 2 shows the frequency in length across all sizes in counts per 0.5 cm for both male and female.

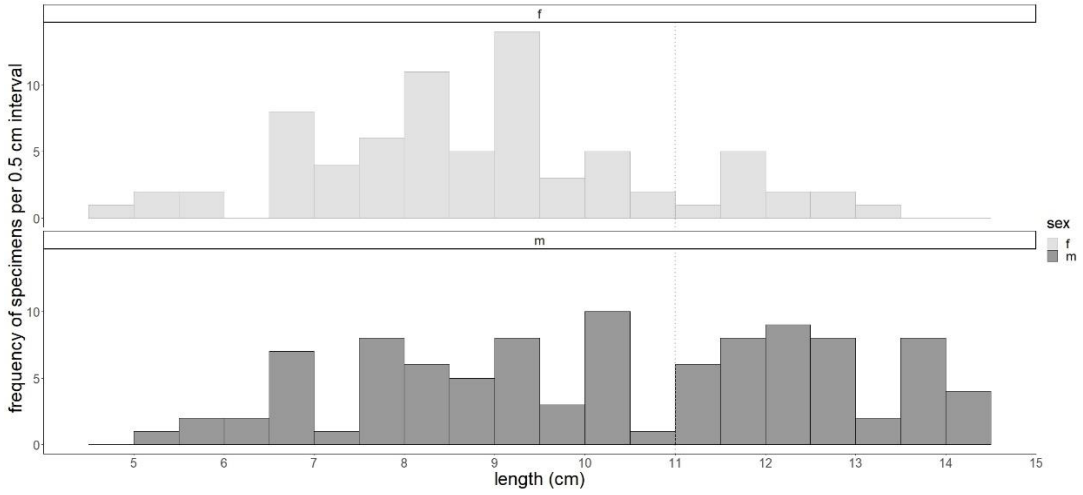


Figure 2. Length frequency of all sampled *N. melanostomus* specimen per 0.5 cm interval displayed for females (f) and males (m). The dotted line (cm 11) marks the parting line between size category small (< 11 cm) and tall (> 11 cm).

Total weight (W_T) varied from 1.6 to 32.6 g in females and 1.8 to 48.0 g in males (see Figure 3). The mean W_T of all collected specimen was 14.08 g (SD = 10.29 g), mean $W_{T\ females}$ was 10.08 g (SD = 6.82 g) and $W_{T\ males}$ was 17.08 g (SD = 11.4 g). Concerning the size categories, mean $W_{T\ tall\ females}$ was 22.45 g (SD = 5.25 g), respectively 27.62 g (SD = 7.82 g) in $W_{T\ tall\ males}$. Mean $W_{T\ small\ females}$ was 7.44 g (SD = 3.28 g), and mean $W_{T\ small\ males}$ was 8.29 g (SD = 4.01 g). Overall, males had a higher total weight than females in all categories, and for W_T the difference was highly significant (Wilcoxon test, $p < 0.001$) and significant in $W_{T\ tall}$ (Welch's t-test, $p < 0.01$) while for $W_{T\ small}$ no significant difference was found (Welch's t-test, $p > 0.05$).

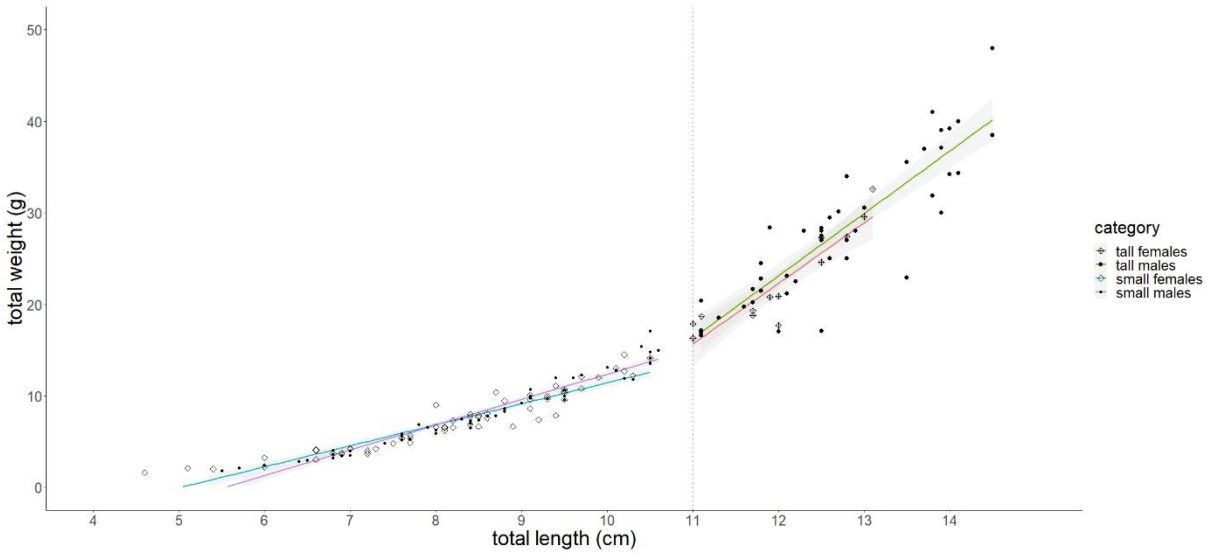


Figure 3. Round goby specimen plotted by total length (cm) and total weight (g) with linear regression lines displaying all round goby specimen categorised into sex and size.

Liver weight (W_L) varied from 0.002 to 0.75 g in females and 0.001 to 1.3 g in males (see Figure 4). The mean W_L of all collected samples was 0.26 g (SD = 0.28 g), mean $W_{L\ females}$ was 0.16 g (SD = 0.14 g), and $W_{L\ males}$ was 0.32 g (SD = 0.33 g). Concerning the size categories, mean $W_{L\ tall\ females}$ was 0.32 g (SD = 0.18 g), and $W_{L\ tall\ males}$ was 0.53 g (SD = 0.35 g). Mean $W_{L\ small\ females}$ was 0.12 g (SD = 0.07 g), and $W_{L\ small\ males}$ was 0.11 g (SD = 0.07 g). Overall, males had a higher liver weight than females in the categories except for $W_{L\ small}$, and although mean $W_{L\ tall}$ was higher for males, significant sex specific differences were only found for W_L (Wilcoxon test, $p < 0.05$).

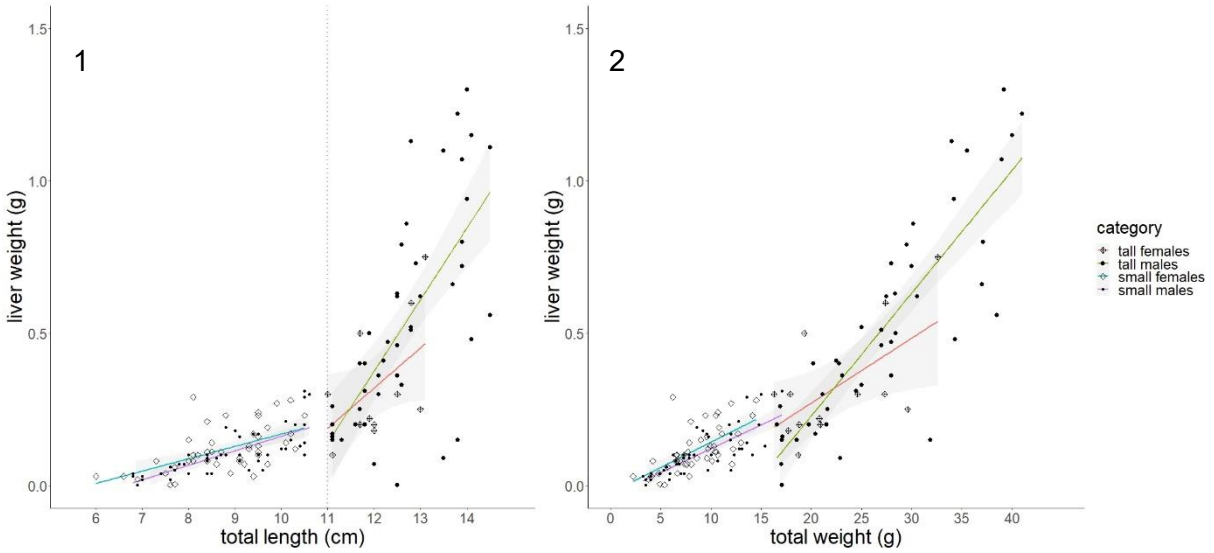


Figure 4. Round goby specimen plotted by total length (cm) [1] and total weight (g) [2] in relation to the liver weight (g) with linear regression lines displaying all specimen categorised into sex and size categories.

Hepatosomatic index (*HSI*) varied from < 0.3 to 4.7 in females and < 0.3 to 3.3 g in males (see Figure in Appendix 10.1.3Figure 4). The mean *HSI* of all collected samples was 1.4 (SD = 0.74), mean $HSI_{T\ females}$ was 1.36 (SD = 0.73) and $HSI_{T\ males}$ was 1.43 (SD = 0.76). Concerning the size categories, mean $HSI_{T\ tall\ females}$ was 1.41 (SD = 0.64) and $HSI_{T\ tall\ males}$ was 1.76 (SD = 0.83). Mean $HSI_{T\ small\ \ females}$ was 1.34 (SD = 0.75) and $HSI_{T\ small\ \ males}$ was 1.10 (SD = 0.50). No significant difference in condition was found between male and female categories, see Table 11 and Figure 5. The condition of tall males however was significantly higher than in small males (Welch's t-test, p < 0.001 ***), see figure in Appendix 10.1.4.

All mean values for the indicators were summarised in Table 11, statistical significances between the means of all indicators were enclosed, see also Figure 5.

Table 11. Key indicators total length (L_T), total weight (W_T), liver weight (W_L) and hepatosomatic index (*HSI*) of all collected *N. melanostomus* specimen compared by sex and size categories. Significant differences were indicated if present (* denotes p ≤ 0.05; ** denotes p < 0.01; * denotes p < 0.001; ns denotes p > 0.05). Wilcoxon test[1] and Welch's t-test[2] were used to detect significances.**

indicators	*p*	females			males		
		n	mean	SD	n	mean	SD
L_T [cm]	***[1]	74	8.92	1.91	99	10.40	2.41
$L_{T\ tall}$ [cm]	ns[1]	13	12.02	0.73	45	12.66	1.02
$L_{T\ small}$ [cm]	ns[2]	61	8.26	1.35	54	8.52	1.41
W_T [g]	***[1]	74	10.08	6.82	99	17.08	11.40
$W_{T\ tall}$ [g]	**[2]	13	22.45	5.25	45	27.62	7.82
$W_{T\ small}$ [g]	ns[2]	61	7.44	3.28	54	8.29	4.01
W_L [g]	*[1]	74	0.16	0.14	99	0.32	0.33
$W_{L\ tall}$ [g]	ns[1]	13	0.32	0.18	45	0.53	0.35
$W_{L\ small}$ [g]	ns[1]	61	0.12	0.07	54	0.11	0.07
HSI_T	ns[1]	74	1.36	0.73	99	1.43	0.76
$HSI_{T\ tall}$	ns[2]	13	1.41	0.64	45	1.76	0.83
$HSI_{T\ small}$	ns[1]	61	1.34	0.75	54	1.10	0.50

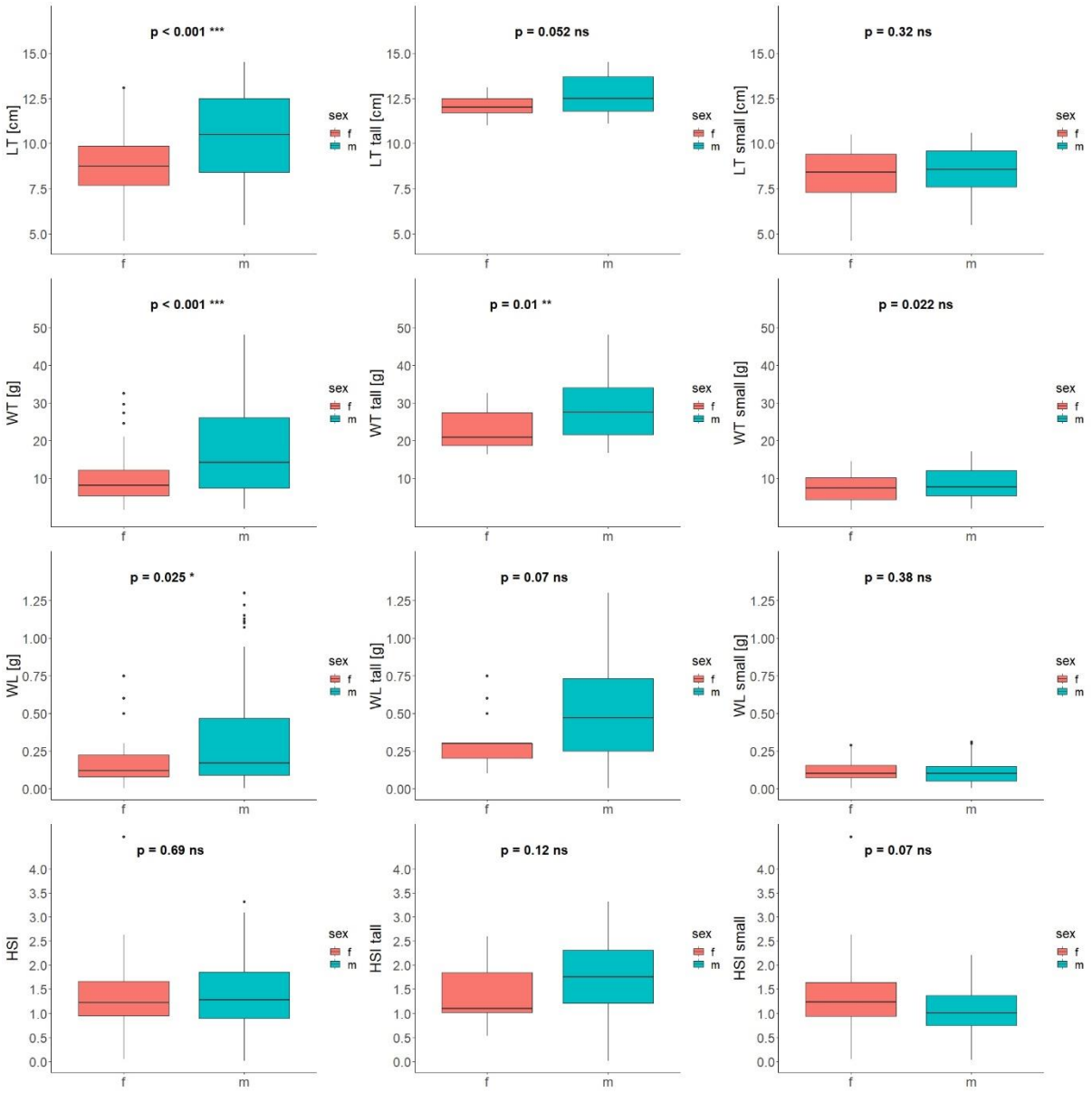


Figure 5. All compared morphological indicators total length (LT), total weight (WT), total liver weight (WL) and hepatosomatic index (HSI) plotted and marked with the corresponding p value.

The sample size of monkey goby received from Germany was small with a total of n = 14 specimen. The sex ratio of females to males was 1:0.75 (females n = 8; males n = 6). Total length (L_T) varied from 5.3 to 8.8 cm in females and from 5.2 to 7.1 cm in males. Total weight (W_T) varied from 0.99 to 6.05 g in females and from 1.0 to 3.42 g in males. Liver weight of all samples ranged from 0.001 g to 0.025 g in n = 8 specimen of both sexes, the rest of the specimen were too small to be dissected. Liver samples were collected in one pool without categorising by sex. Muscle tissue was analysed separately for both sexes. Due to the small sample size, no further comparison of indicators was conducted.

4.2 Method development BPA analysis in fish tissue samples

A total of 5 test series were conducted, of which the fifth test series represented the main experiment, where the round goby liver and muscle tissue was sampled for BPA contamination. The following sub-chapters list all results for each test series. Due to the trial-and-error approach of this method development, each test series method was changed based on the results obtained in the previous test series. The reasons for all adaptations and changes in methodology were indicated in chapter 5.2discussion.

The LOQ was defined as a signal-to-noise ratio of >10/1 for blank samples, while LOQ for fish tissue and water/sediment samples was >10/1 of the mean blank samples LOQ for each test series and patch. The LOD for fish tissue and water/sediment samples was calculated according to the equation LOD = LOQ/3.3. If not otherwise indicated, the LOQ of the BPA concentration in the samples was reached.

4.2.1 Test series 1

The first test series included blank samples and liver samples of *O. mykiss*, which were spiked at different levels (see Table 2). The *IS* was sufficiently visible in all samples for measurement, albeit low in liver samples. In all samples, BPA was present and could be quantified at different levels. The BPA concentration in 2 ml was 0.4 and 0.6 ng in the blank samples no. *2.1* and *2.2*, respectively 1.2 ng in blank sample no. *3*. In blank sample no. *4*, which was spiked with 2 ng, 2.1 ng BPA was measured (recovery [%]: 105). In the spiked 2 g liver samples, BPA concentration was exceptionally high in no. *5* and *6* (27.6 and 62.6 ng, recovery [%]: 1380 and 626), while in no. *7* it was lower (46.3 ng, recovery [%]: 92.6).

Table 12. BPA in test series 1. *IS* = ^{13}C labelled BPA, Spike = certified reference standard BPA, absolute amount added [ng].

No.	Quantity	*IS*	Spike	Tissue type	Category	BPA [ng]	BPA [ng/g]
2.1	2x	20.0 ng	*none*	*none*	Blank	***0.4***	
2.2						***0.6***	
3	1x	20.0 ng	*none*	*none*	Blank, *H_2O "filtered"*	***1.2***	
4	1x	20.0 ng	2.00 ng	*none*	Blank	***2.1***	
5	1x	20.0 ng	2.00 ng	Liver (2 g)	Tissue sample	***27.6***	***13.8***
6	1x	20.0 ng	10.00 ng	Liver (2 g)	Tissue sample	***62.6***	***31.3***
7	1x	20.0 ng	50.00 ng	Liver (2 g)	Tissue sample	***46.3***	***23.1***

4.2.2 Test series 2

All tested solvents (no. *1.1 – 5.2*) contained low amounts of BPA and risk of sample contamination by BPA containing solvents was assessed as low. The results for BPA are indicated as absolute amount [ng] measured in 10 ml solvent for each sample number (see Table 3). MeCN sample no. *1.1* contained the lowest BPA amount (0.02 ng), whereas MeOH contained 0.18 ng. The solvent sample no. *3* obtained from glassware washout contained 0.12 ng and for no. *2.1* (AcOH (2%) in MeOH) 0.18 ng were measured. The use of PP pipette tips (no. *5.1*) did not increase the BPA amount measured in MeOH compared to the use of glass pipettes, the solvents stored in PP centrifuge tubes (no. *8*) neither (0.09 ng). Elevated amounts of BPA were found in no. *6* and *7* with 3.51 and 1.27 ng respectively. No. *7* indicated template-leaking deriving from the SPE MIPs cartridge. Sample no. *9* contained 0.8 ng BPA, indicating contamination since compared to the other MeOH samples, an additional 0.62 ng BPA was measured. The spiked samples were measured around the spiked 20 ng BPA amount (± 0.7).

Table 13. BPA [ng] per 10 ml sample in test series 2. *IS* = ^{13}C labelled BPA, spike = certified reference standard BPA, added amounts indicated as absolute [ng].

Category	No.	Sampling solvent	*IS*	Spike	BPA [ng]
Solvents (transferred by using glass pipettes)	1.1	MeCN	20 ng		***0.02***
	1.2	MeCN	20 ng	20 ng	***19.1***
	2.1	MeOH/AcOH (98/2)	20 ng		***0.18***
	2.2	MeOH/ AcOH (98/2)	20 ng	20 ng	***20.4***
	3	MeOH/MeCN (50/50) glassware washout	20 ng		***0.12***
	4	MeOH	20 ng	20 ng	***19.7***
Solvents (transferred by using PP pipette tips)	5.1	MeOH	20 ng		***0.18***
	5.2	MeOH	20 ng	20 ng	***20.7***
Cartridge (AFFINIMIP® Bisphenol SPE)	6	5 ml MeOH/AcOH (98/2) and 5 ml MeCN	20 ng		***3.51***
	7	Elution with MeOH	20 ng		***1.27***
PP centrifuge tubes	8	MeCN/MeOH (50/50)	20 ng		***0.09***
Cryomilling	9	MeOH	20 ng		***0.8***

4.2.3 Test series 3

In test series 3, 0.7 and 0.56 ng BPA were measured in two blank samples with increased solvent volume for SPE MIPs conditioning step (no. *1.2*: 10 ml and no. *1.3*: 15 ml of MeOH/AcOH (98/2) and water/MeCN (60/40) respectively). The liver samples ranged from 5.9 – 16.1 ng BPA absolute amount or 4.8 – 8.4 ng/g as indicated in Table 5. Trout liver contained 155 % the amount of BPA compared to the round goby liver mean value.

Table 14. BPA concentration in test series 3. *IS* = ^{13}C labelled BPA.

No.	*IS*	Category	Tissue type	Focus	BPA [ng]	BPA [ng/g]
1.2	20 ng	Blank	*none*	SPE template leaking test	***0.7***	
1.3	20 ng	Blank	*none*	SPE template leaking test	***0.56***	
2.1	20 ng	Tissue	Trout liver	Whole method, freezing step effectivity	***16.1***	***8.0***
2.2	20 ng	Tissue	Round goby liver	Whole method, freezing step effectivity	***9.6***	***4.8***
2.3	20 ng	Tissue *1 g*	Round goby liver	Whole method, freezing step effectivity	***5.9***	***5.9***
2.4	20 ng	Tissue *1 g*	Round goby liver	Whole method, freezing step effectivity	***8.4***	***8.4***

The freezing steps for removing dissolved solids and dissolved fish oils performed visually best in separation funnels. In the measuring cylinder, the scooped supernatant was still slightly turbid. Approximately half of the sample volume was discarded for each sample. However, all samples became turbid when the eluate was partly evaporated.

4.2.4 Test series 4

The suspected template-leaking (see 4.2.2 test series 2) was confirmed in both blank sample types B1 and B2. By applying the original solvent volume for the conditioning of the SPE MIPs cartridges, a mean BPA amount of 1.25 (± *0.12*) and 3.0 (± *1.12*) ng was measured for B1 blanks (solid-phase extraction step only) and B2 blanks (entire method procedure) respectively. Applying an adjusted amount of solvent volume led to a clear reduction of BPA: mean values of 0.77 (± *0.18*) and 0.69 (± *0.14*) ng were measured at a 10 ml adjusted solvent volume in B1 and B2 respectively, 0.87 (± *0.41*) and 1.06 (± *0.62*) ng for 15 ml adjusted solvent volume. The mean values and standard deviation were calculated from triplets as indicated in Table 7 and plotted in Figure 6.

Table 15. BPA concentration in test series 3. *IS* = 13C labelled BPA, spike = certified reference standard BPA. All blank samples were arranged into triplets to validate the method and elicit the optimal solvent volume for conditioning of the SPE MIPs cartridges. B1 blanks consisted of the solid-phase extraction step only, B2 blanks involved the entire method procedure.

No.	*IS*	Conditioning solvent volume*	BPA [ng]	Mean BPA [ng]	SD
B1.1a	20 ng	Original volume[1]	*1.36*	***1.25***	*± 0.12*
B1.1b	20 ng		*1.12*		
B1.1c	20 ng		*1.26*		
B1.2a	20 ng	Adjusted volume[2]	*0.63*	***0.77***	*± 0.18*
B1.2b	20 ng		*0.97*		
B1.2c	20 ng		*0.72*		
B1.3a	20 ng	Adjusted volume[3]	*0.47*	***0.87***	*± 0.41*
B1.3b	20 ng		*0.84*		
B1.3c	20 ng		*1.29*		
B2.1a	20 ng	Original volume[1]	*1.71*	***3.0***	*± 1.12*
B2.1b	20 ng		*3.70*		
B2.1c	20 ng		*3.59*		
B2.2a	20 ng	Adjusted volume[2]	*0.85*	***0.69***	*± 0.14*
B2.2b	20 ng		*0.57*		
B2.2c	20 ng		*0.65*		
B2.3a	20 ng	Adjusted volume[3]	*0.56*	***1.06***	*± 0.62*
B2.3b	20 ng		*1.75*		
B2.3c	20 ng		*0.87*		

**Solvent volume used for conditioning:*
[1] 5 ml of MeOH/AcOH (98/2, v/v), 5 ml of MeCN and 5 ml of HPLC Pure water
[2] 10 ml of MeOH/AcOH (98/2, v/v), 10 ml of MeCN and 5 ml of HPLC Pure water
[3] 15 ml of MeOH/AcOH (98/2, v/v), 15 ml of MeCN and 5 ml of HPLC Pure water

The results were visualised as boxplots in Figure 6.

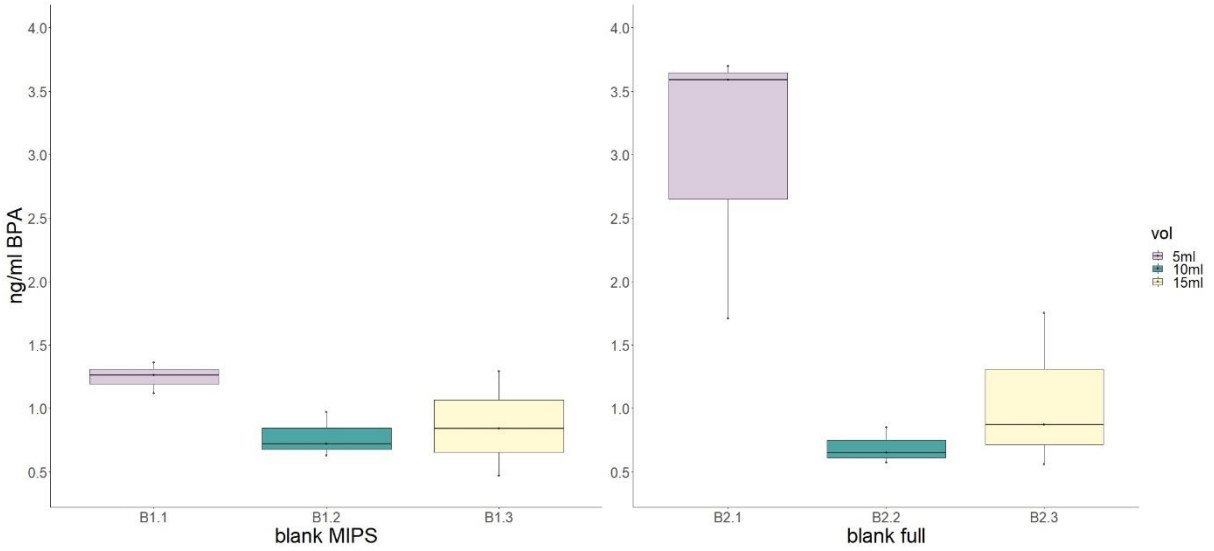
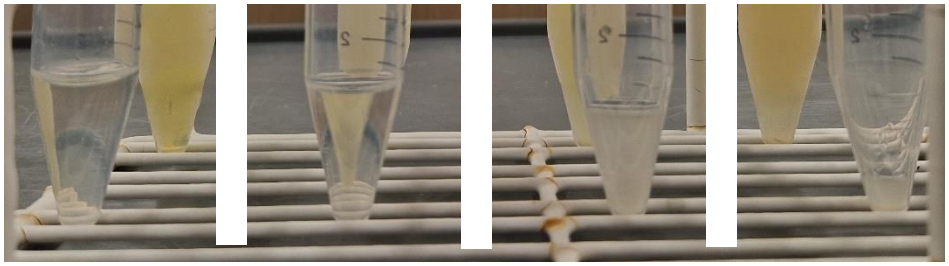


Figure 6. BPA concentration in all test series 4 blank samples. B1.2 and B2.2 performed best (both with adjusted solvent volume 10 ml). Template leaking was generally reduced by applying higher solvent volumes compared to the original method (5 ml) during conditioning of the SPE MIPs.

Freezing experiment

Four tissue samples were processed according to the method steps *homogenisation* and *sample extraction* applied in the previous test series 3, but not further extracted by SPE MIPs nor analysed for BPA. During the freezing experiment the samples were visually evaluated for clarity and volume by quickly transferring the clear supernatant extract into another PP centrifuge vial (see Table 16). The samples performed best at a water/MeCN ratio 50/50 (v/v), which was therefore implemented to the final method. The samples appeared to be clear, and no fish oil was visually detectable. The samples with water/MeCN ratio of 70/30 and 80/20 (v/v) were turbid and only a low volume could be transferred because they were almost completely frozen.

Table 16. Freezing test to find optimal water/MeCN ratio to remove fish oils from sample.

H_2O/MeCN ratio	50/50	60/40	70/30	80/20
Supernatant				
Results	***best***	*good*	*not suitable*	*not suitable*

4.2.5 Main experiment

Blank samples

Each of the six sample batches was accompanied by four blanks which served as the base for separate LOQ determination in each batch and indicated if any contamination was induced across the extraction process. Based on all blank samples the mean BPA values [ng] was calculated. The lowest blank sample mean value was found in batch *6* (0.32 ± 0.06), the highest blank sample mean value in batch *3* 0.85 (± 0.36). The other means were measured at 0.69 ± 0.19 in batch *1*, 0.61 ± 0.22 in batch *2*, 0.43 ± 0.12 in batch 4 and 0.36 ± 0.04 in batch *5* (see

Table 17 and Figure 7). All samples were added with 20 ng *IS.*

Table 17. BPA [ng] measured for all blank samples and batches in the main experiment.

Batch	Blank No.	BPA [ng]	Mean BPA [ng]	SD
1	1.1	0.61	0.69	*± 0.19*
1	1.2	0.94		
1	1.3	0.73		
1	1.4	0.48		
2	2.1	0.74	0.61	*± 0.22*
2	2.2	0.37		
2	2.3	0.49		
2	2.4	0.85		
3	3.1	1.24	0.85	*± 0.36*
3	3.2	0.52		
3	3.3	1.08		
3	3.4	0.57		
4	4.1	0.58	0.43	*± 0.12*
4	4.2	0.38		
4	4.3	0.46		
4	4.4	0.30		
5	5.1	0.37	0.36	*± 0.04*
5	5.2	0.40		
5	5.3	0.31		
5	5.4	0.35		
6	6.1	0.32	0.32	*± 0.06*
6	6.2	0.28		
6	6.3	0.40		
6	6.4	0.27		

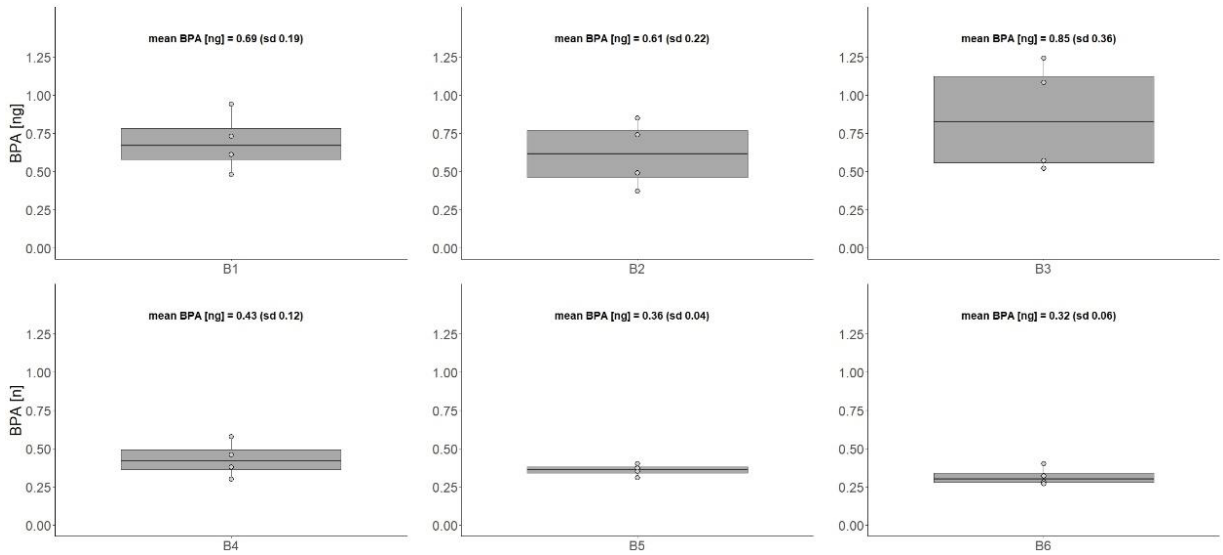


Figure 7. BPA [ng] measured in blank samples for batch 1 to batch 6 in the main experiment.

Main samples

For all batches, the LOQ was separately determined as the tenfold mean value of the corresponding blank samples and LOD as the mean blank value + standard deviation. All samples were below LOQ (see Figure 8).

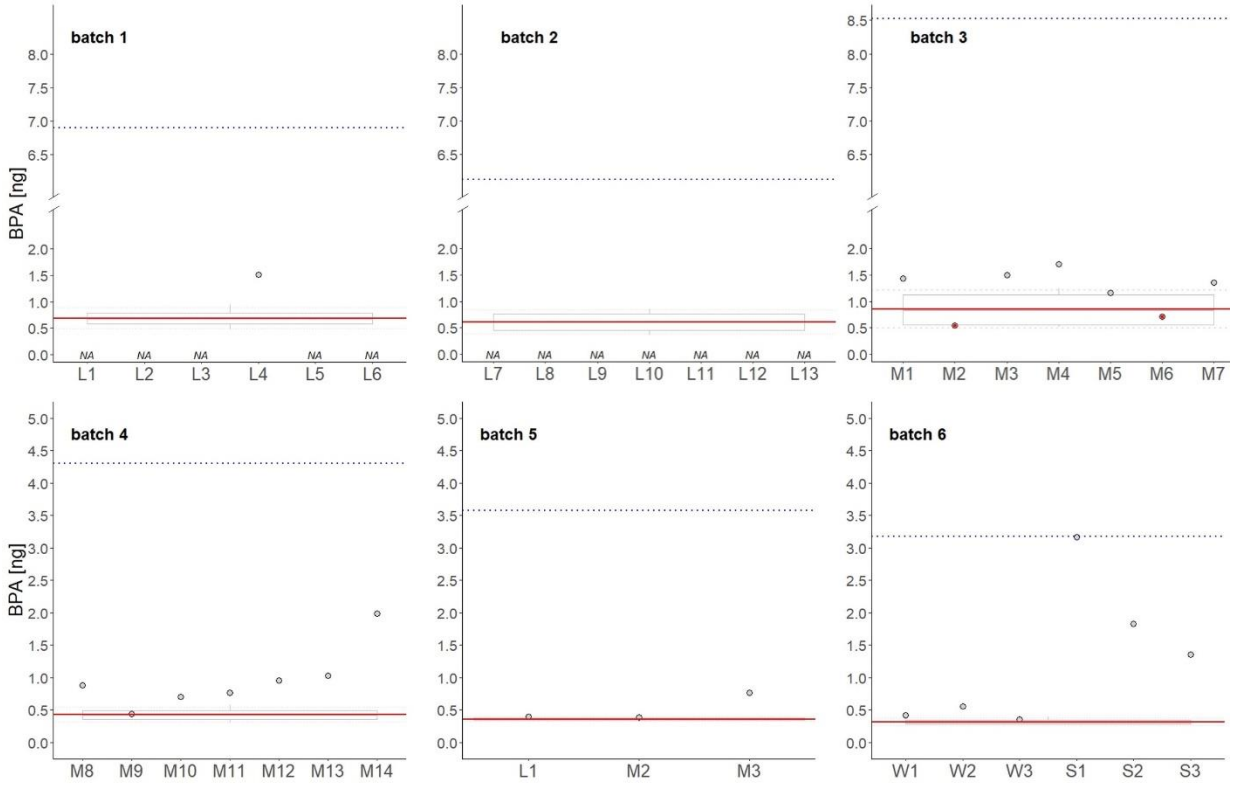


Figure 8. Total BPA [ng] measured in pooled samples for batch 1 – 6. The red line marks the mean blank value according to each batch (with standard deviation, fine dotted line). The blue dotted line marks the LOQ limit (tenfold mean blank value). Each value was indicated as point. Batch 1 – 4 were round goby liver (L) and muscle (M) samples, batch 5 monkey goby samples and batch 6 water (W) and sediment (S) samples.

Round goby tissue samples

The round goby samples were analysed in pooled samples containing of various specimen per pool. The exact number of specimen was indicated in

Table 8. Five samples were too low to include the aim of 2 g homogenised sample weight to the extraction, the exact sample weight was then specified in

Table 8.

Table 18. Round goby specimen amount per pool, tissue type and configuration of batches. If the sample was below 2 g, it was specified in row "fitted sample weight". Abbreviations: tf = tall female; sf = small female; tm = tall male; sm = small male.

Batch	Pool No.[1]		Tissue	Category[1]	specimen per pool	Fitted sample weight if not 2 g (g)
1	*L1*	1	Liver	tm	n = 5	0.7
1	*L2*	2	Liver	tm	n = 8	
1	*L3*	3	Liver	tm	n = 7	
1	*L4*	4	Liver	tm	n = 5	
1	*L5*	5	Liver	tm	n = 6	
1	*L6*	6	Liver	tm	n = 6	1.8
2	*L7*	7	Liver	tf	n = 12	
2	*L8*	8	Liver	sm	n = 18	1.3
2	*L9*	9	Liver	sf	n = 17	1.6
2	*L10*	10	Liver	tm	n = 8	
2	*L11*	11	Liver	sm	n = 25	
2	*L12*	12	Liver	sf	n = 25	
2	*L13*	13	Liver	sf[2]	n = 25	0.7
3	*M1*	14	Muscle	tm	n = 5	
3	*M2*	15	Muscle	tm	n = 8	
3	*M3*	16	Muscle	tm	n = 7	
3	*M4*	17	Muscle	tm	n = 5	
3	*M5*	18	Muscle	tm	n = 6	
3	*M6*	19	Muscle	tm	n = 6	
3	*M7*	20	Muscle	tf	n = 12	
4	*M8*	21	Muscle	sm	n = 18	
4	*M9*	22	Muscle	sf	n = 17	
4	*M10*	23	Muscle	tm	n = 8	
4	*M11*	24	Muscle	sm	n = 25	
4	*M12*	25	Muscle	sf	n = 25	
4	*M13*	26	Muscle	sm[2]	n = 11	
4	*M14*	27	Muscle	sf[2]	n = 14	

[1] The specimen used for pool no. 1-13 were the same used in pool no. 14-27, where instead of liver, the muscle tissue was analysed. Also indicated in grey (L1 – M14).

[2] Due to low liver weight, males and females were pooled in pool no. 13 (L13). Muscle tissue was then analysed as usual separated by sex (pool no. 26 and 27 or M13 and M24).

In none of the tissue samples BPA could be quantified, all measured samples ranged below LOQ indicating a low BPA contamination of round goby at the sampled location. BPA could not be quantified nor detected in liver samples because the *IS* was unexpectedly not visible in 12 out of 13 samples due to strong matrix effects. Only in pool no. *4*, 1.51 ng BPA was measured (< LOQ; recovery [%]: 13). The liver samples still contained fish oils after the adjusted freeze-step, although at a lower concentration, and were therefore prepared for LC-MS/MS analysis in a narrowed microvial dissolved in 50 µg MeOH. In muscle samples, the *IS* recovery rate varied greatly and ranged between 10 and 83 % but was sufficient for measurement. BPA could therefore be detected

in every pooled muscle sample, although the value was far below LOQ, and for pool no. *15*, *19* and *21* below LOD. All results are shown in Table 19.

Table 19. Results of BPA analysis in round goby tissue samples.

	Pool No.	Tissue	Rec [%]	BPA [ng]	BPA [ng/g]	LOQ 10*mean blank	Final BPA c
L1	1	Liver	0	No *IS* visible	*NA*	6.9	*NA*
L2	2	Liver	0	No *IS* visible	*NA*	6.9	*NA*
L3	3	Liver	0	No *IS* visible	*NA*	6.9	*NA*
L4	4	Liver	13	1.51	0.76	6.9	< LOQ
L5	5	Liver	0	No *IS* visible	*NA*	6.9	*NA*
L6	6	Liver	0	No *IS* visible	*NA*	6.9	*NA*
L7	7	Liver	0	No *IS* visible	*NA*	6.1	*NA*
L8	8	Liver	0	No *IS* visible	*NA*	6.1	*NA*
L9	9	Liver	0	No *IS* visible	*NA*	6.1	*NA*
L10	10	Liver	1	S/N < 1/10	*NA*	6.1	*NA*
L11	11	Liver	0	No *IS* visible	*NA*	6.1	*NA*
L12	12	Liver	0	No *IS* visible	*NA*	6.1	*NA*
L13	13	Liver	0	No *IS* visible	*NA*	6.1	*NA*
M1	14	Muscle	15	1.43	0.71	8.5	< LOQ
M2	15	Muscle	10	0.54	0.27	8.5	< LOD
M3	16	Muscle	19	1.49	0.75	8.5	< LOQ
M4	17	Muscle	39	1.70	0.85	8.5	< LOQ
M5	18	Muscle	32	1.16	0.58	8.5	< LOQ
M6	19	Muscle	83	0.71	0.36	8.5	< LOD
M7	20	Muscle	40	1.35	0.67	8.5	< LOQ
M8	21	Muscle	18	0.88	0.44	4.3	< LOQ
M9	22	Muscle	49	0.44	0.22	4.3	< LOD
M10	23	Muscle	28	0.70	0.35	4.3	< LOQ
M11	24	Muscle	25	0.77	0.38	4.3	< LOQ
M12	25	Muscle	14	0.95	0.48	4.3	< LOQ
M13	26	Muscle	11	1.03	0.51	4.3	< LOQ
M14	27	Muscle	12	1.99[1]	0.99	4.3	< LOQ

[1] Highest BPA value measured in fish tissue, but below LOQ (1.99/LOQ = 0.46)

N. fluviatilis

The monkey goby samples were analysed in three pooled samples, one containing liver tissue and two muscle tissue. The exact specimen number was indicated in

Table 8. Two samples were too low to include the aimed 2 g homogenised sample weight for extraction and due to the small sample size, liver samples for both sexes were pooled in the same pool at a total weight of 0.01 g liver tissue and only 1.5 g male muscle tissue was analysed.

Table 20. Monkey goby specimen amount per pool and tissue type. All samples belonged to batch 5. Abbreviations: sfm = small female and male; sm = small male; sf = small female.

Batch	Pool No.[1]	Tissue	Category	specimen per pool	Fitted sample weight if not 2 g (g)
5	1	Liver	sfm[1]	n = 8	0.01
5	2	Muscle	sm	n = 6	1.5
5	3	Muscle	sf	n = 8	

[1] Due to the low liver weight, males and females were pooled in pool no. 1 (eight tallest fish)*. Muscle tissue was then analysed as usual separated by sex (pool no. 2 and 3).*

In none of the tissue samples BPA could be quantified, all measured samples ranged below LOQ indicating a low BPA contamination in monkey goby from the sampled location. In the liver sample, 0.4 ng BPA was measured, well below LOQ. In muscle, 0.39 ng were measured in the pooled male sample (< LOD) and 0.77 ng in the pooled female sample (< LOQ). The results were indicated in Table 21.

Table 21. Results of BPA analysis in monkey goby tissue samples.

Pool No.		Tissue	Rec [%]	BPA [ng]	BPA [ng/g]	LOQ 10*mean blank	Final BPA c
L1	1	Liver	43	0.40	4.32	3.6	< LOD
M1	2	Muscle	32	0.39	0.26	3.6	< LOD
M2	3	Muscle	23	0.77	0.38	3.6	< LOQ

Water

None of the water samples could be quantified, all measured samples ranged below LOQ (see Table 22) indicating a low BPA contamination at the sampled location. The *IS* recovery rate was generally higher compared to the tissue samples.

Table 22. Results of BPA analysis in River Rhine water samples.

Batch	Sample No.	Rec [%]	BPA [ng]	BPA [ng/ml]	LOQ 10*mean blank	Final BPA c
6	1	56	0.42	0.21	3.2	< LOQ
6	2	71	0.55	0.28	3.2	< LOQ
6	3	49	0.36	0.18	3.2	< LOQ

Sediments

None of the sediment samples could be quantified, all measured samples ranged below LOQ (see Table 23), indicating a low BPA contamination at the sampled location. However, the measured BPA values were higher than in most tissue samples, with one sample value at the brink of quantification (3.16 ng, *3.16/LOQ = 0.99*).

Table 23. Results of BPA analysis in sediment samples.

Batch	Sample No.	Rec [%]	BPA [ng]	BPA [ng/g]	LOQ 10*mean blank	Final BPA c
6	4	29	3.16	1.58	3.2	< LOQ
6	5	23	1.83	0.92	3.2	< LOQ
6	6	31	1.36	0.68	3.2	< LOQ

4.3 Vitellogenin

Vitellogenin was detected in 8 out of 27 pooled round goby samples and quantified in three. Four samples were between lower limit of detection LLD and lower limit of quantification LLOQ. LLOQ was < 1 ng/ml as indicated by the manufacturer and lower limit of detection LLD (defined as the corresponding concentration of the mean optical density value OD zero standard plus 3 SD) was 0.37 ng/ml. In muscle tissue, VTG concentration in all pooled samples was below LLD. VTG was quantified in liver tissue of tall males (2/7 pooled samples) at 1.3 ng/mg and 1.2 ng/mg, and in small males (1/2 pooled samples) at 1.5 ng/mg, but not in tall females (0.95 ng/mg, < LLOQ) and small females (< LLD), see Figure 9.

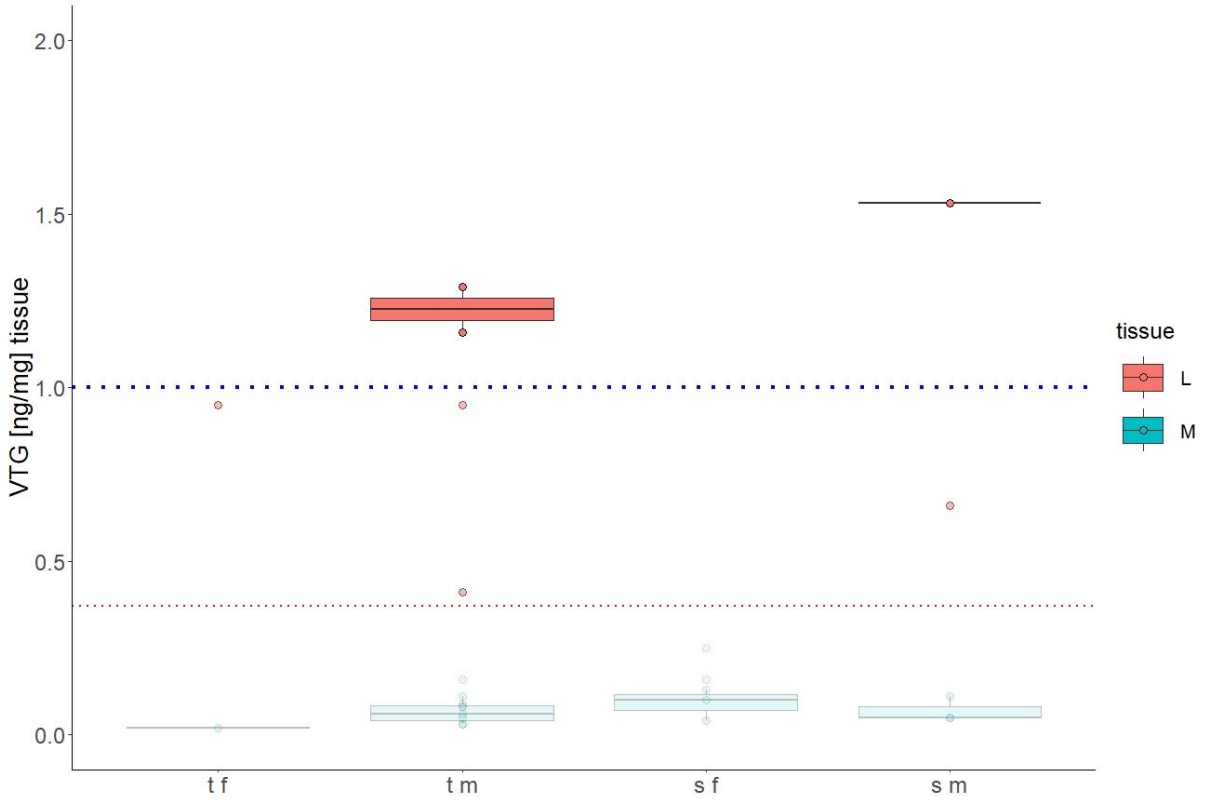


Figure 9. VTG concentration measured in fish tissue [ng/mg]. The red dotted line marks the LLD (0.37 ng/ml), LLOQ was 1 ng/mg. Samples below LLOQ and LLD were plotted transparent. Abbreviations: t f = tall females; t m = tall males; s f = small females; s m = small males; L = liver; M = muscle.

The normalised VTG amount corrected by the total protein amount remained largely unaltered when comparing the quantified samples of tall males and small males. VTG amounts in tall males corresponded to 0.17 and 0.2 % of the total protein content in the liver sample and in small males to 0.2 % in the quantified liver samples, see Figure 10. Tall female liver samples were slightly below LOQ and VTG in total protein concentration was around 0.14 %. Small female samples were below LLOD and VTG in total protein concentration <0.05 %.

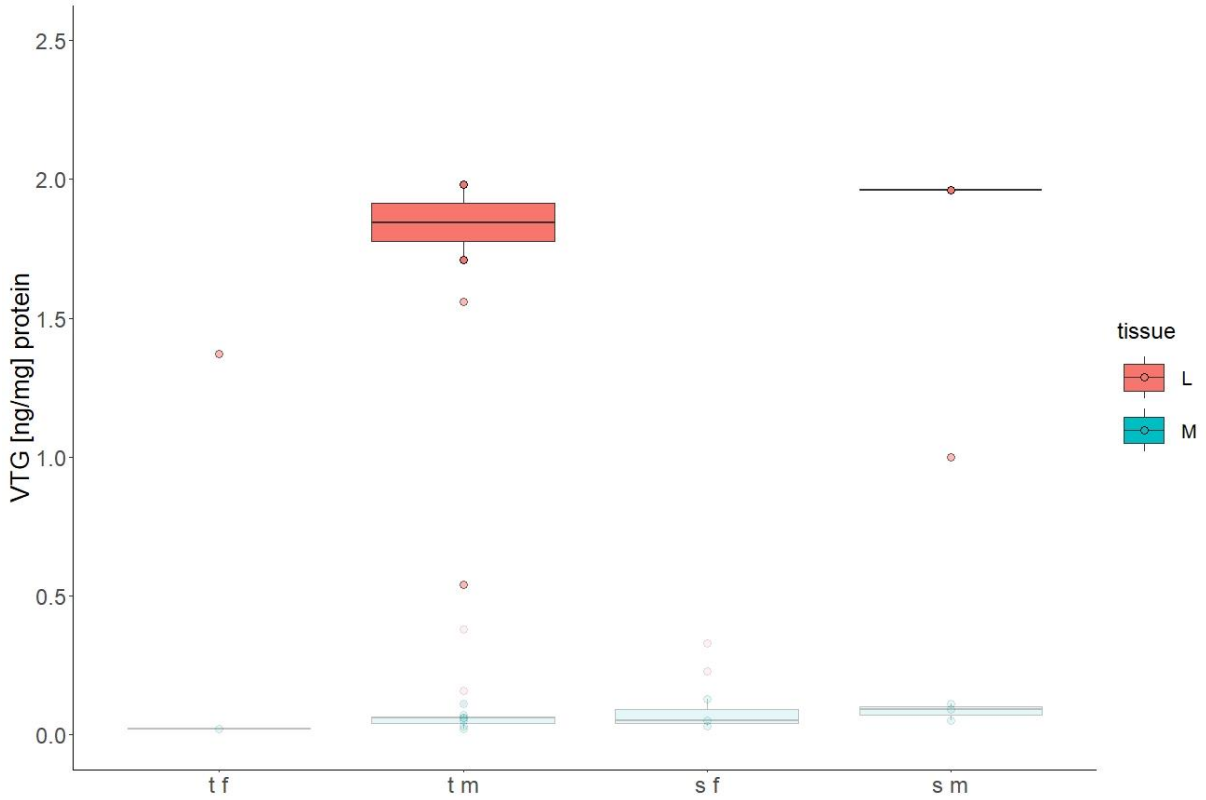


Figure 10. VTG concentration corrected by the protein concentration. Transparent data points and boxplots mark data points below LLOQ and LLD. Abbreviations: t f = tall females; t m = tall males; s f = small females; s m = small males; L = liver; M = muscle.

5 Discussion

5.1 Sample composition

Sex ratio in the sampled round goby specimens was male-biased (1:1.3 f:m), the same has been documented in other sources (Corkum et al., 2004; Lee F.G. Gutowsky & Fox, 2011; Young et al., 2010), but does not seem to apply to all populations, as female-biased sex ratios are also often reported (Brownscombe & Fox, 2012; L. F.G. Gutowsky & Fox, 2012). In the present study, it must be taken into account that the proportion of females may have been even slightly overestimated.in particular, it was not always possible to distinguish small individuals with certainty, as the distinguishing characteristics (morphology of the urogenital papilla) were sometimes small or not fully developed yet. Individuals that showed rather male traits but could not be classified as males with certainty were therefore classified as females to prevent false positive results in vitellogenin analysis. In general, however, most (> 95 %) individuals could be assigned to a gender with certainty based on well visible urogenital papilla morphology and the error rate is considered low (see photo in Appendix 10.1.1). One specimen was assigned as female that indicated intersex characteristics, as the external genital organs showed both male and female characteristics (see photo in Appendix 10.1.1). The occurrence of intersex in round gobies has been documented several times (L. Bowley, 2010; Guellard et al., 2015), and may be caused by the presence of EDCs, as confirmed at least by Bowley (2010).

Growth rates are highly variable and generally depend on the sample location. Most studies agree that males grow generally larger in total length than females (Kornis et al., 2012). The here documented mean total length (8.92 cm in females and 10.4 cm in males) was in line with the expected larger size in males but also exceeded most documented mean length observed in other studies, see Table 24. Brandner et al. (2018) and Cerwenka et al. (2018) compared long established (sub-)populations with newly colonised (sub-)populations and documented a general trend of higher abundance of taller specimens in the newly colonised populations, and a rather female biased population structure in these pioneering populations. As the specimens in this study were obtained from a newly colonised location (only 4 years of presence), the elevated total length mean value could be due to the same biological characteristics found by the cited studies, although the female bias could not be confirmed.

Table 24. Selection of round goby mean total length reported from different sampling locations.

L_T [cm] (mean ±SD)	Sex ratio (f:m)	n	Location	Reference
6.5 (f), 8.6 (m)	1:1.4	750	Lake Huron, US	Duan et al. (2016) [1]
7.5 (f), 9.9 (m)	1:0.9	2010	Lake Erie, US	Johnson et al. (2005) [1]
7.4 ± 0.2 (m, f)	1:2	4181	Hamilton, CA	Young et al. (2010) [1]
6.8 ± 0.7 (f). 7.7 ± 0.9 (m)	1:1.4	172	Trent River, CA	Gutowsky & Fox (2011)[1]
8.1 ± 0.2 (f). 9.1 ± 0.2 (m)	1:2.2	172	Trent River, CA	Gutowsky & Fox (2011)[2]
10.4 ± 2.3 (f), 10.2 ± 2.6 (m)	1:0.8	106	Danube River, DE	Brandner et al. (2013) [2]
8.9 ± 1.3 (f), 9.6 ± 2.0 (m)	1:0.6	375	Danube River, DE	Brandner et al. (2018) [2]
8.9 ± 1.9 (f), 10.4 ± 2.4 (m)	1:1.3	173	Rhine Rhiver	This study[2]

Seven datasets of round goby documenting total length in different regions of the world. The data was rounded to tone decimal place and re-calculated in case of not being explicitly presented in these studies to obtain comparability of data.

[1] locations with established round goby populations.

[2] locations where the habitat colonised newly (< 5 years).

The HSI documented in this study was lower than in most documented studies of recent invaded habitats (Azour et al., 2015; Brandner et al., 2013; Gutowsky & Fox, 2011), but higher in tall specimens (significantly in tall males) than in small categorised specimens and was highly variable across all categories. This could indicate the establishment of a dense population and increased intraspecific competition (especially in small specimens) for resources, therefore resulting in a lower condition and limited growth. The tall specimens with higher HSI were probably present in an early stage of the invasion and the therefore grew larger and outcompete smaller specimens. Similar patterns have been documented, also a decrease in gut content was found across the expansion phase between newly invaded sectors and already densely populated sites in the Trent-Severn Waterway (Ondario, Canada) (Raby et al., 2010).

5.2 Method development

5.2.1 Test series 1

Both the blank and liver samples contained BPA, with the liver samples containing the highest values, and two of the three showing values well above what was expected with the spike. It is likely that BPA entered the sample during the course of the experiment, which should be evaluated in a second pilot study by analysing sensitive sub-steps. Contaminations are possible at several steps, for example during homogenisation by ultra-turrax, filtration by PTFE-filter or the condensation of BPA from the air when the frozen sample powder was prepared for weighting.

The high values in the liver should be interpreted with caution: On the one hand, the liver was stored in a plastic bag by the fish farm where it was retrieved. On the other hand, several steps had to be added during the experiment. The liver samples were thawed and then cut into small pieces (with a razor blade), frozen in a ceramic bowl with the addition of liquid nitrogen, before being cooled again to – 196 °C in the grinding container, and only thereafter cryomilled. After eluting the extract, it became clear that there was still a high matrix content present after the SPE MIPs clean-up (fish oils), which had to be frozen out by further dilution with solvent and freezing (-20°C). The supernatant was eluted again through the cartridge and still showed matrix. This made a filtration step necessary (PTFE filter, 0.2 µm). The matrix could be greatly reduced but was still visibly present. It became clear that all potential sources of contamination should be checked, and the method adapted accordingly. On the one hand, the solvents and materials used should be analysed. On the other hand, the method should be reduced to the most essential steps. For example, dispensing the second homogenization step (by ultra turrax) as there may be residual contamination in the threads, and to plan only one centrifugation instead of two. After centrifugation, the freezing step should follow directly, in which the sample is stored at -20°C overnight and the clear supernatant is to be used the following day. This does not affect the results as the ratio of BPA *IS* to BPA remains unchanged, even if a majority of the sample got dispensed by only taking a small amount of the clear supernatant for the further process. The use of PP centrifuge tubes should also be checked, what would reduce the number of transfers of the sample. PP pipette tips also had to be checked to rule out contamination of the samples by BPA that dissolves from them.

Sample nr. *3* was included with the intent to check if the water used in the method contained BPA. The water was filtered through a SPE MIP cartridge assuming that the possibly present BPA would stick to the MIPs. The contrary was the case, BPA contamination was higher than in the blanks without filtered water, even though the water used was retrieved from the same bottle. This could be explained by leakage from the cartridges and the water instead of getting filtered, accumulated BPA.

5.2.2 Test series 2

The test results retrieved in test series 2 indicated that the use of the solvents was safe and should not cause contamination that would influence the analysis in a noteworthy extent. Upon examination of the spiked samples, the differences are likely due to small inaccuracies in pipetting, but the results are all close to the expected range of 20 ng. Glassware did not appear to be contaminated with BPA, the BPA amount in MeOH/MeCN 50/50 that was used to clean the glassware was exactly in the middle of the values of both solvents. The solvents used for conditioning of the SPE MIPs contained a far higher amount of BPA, which was interpreted as template-leaking from the cartridges. Even after conditioning, the blank eluate contained an elevated amount of BPA, which had to be reduced in the following test series to achieve the aim of a lower LOQ. However, an inquiry at the manufacturer clarified that unlike other scientific publications using BPA as template in the production of the MIP, another molecule was used for their product to avoid false positives and observing values of as low as 0.06 ng would be possible. Still the manufacturer confirmed that the use of a higher solvent volume could improve the reliability and reduce BPA leaking out of the cartridge. The BPA leaking from the cartridges could also be due to a defective patch or might have entered during transport or while storing in the laboratory.

In the cryomilling sub-step a possible contamination was detected, the BPA value was four times the detected amount of the other MeOH samples. This could be due to various factors. First, the MeOH was placed into a porcelain mortar bowl and deluged with liquid nitrogen. Then transferred into the grinding jar and milled. The retrieved solvent was slightly turbid due to steel abrasion from the grinding jar. The steel abrasion was removed by magnetically attracting the particles to one side of the test tube and then transferring the clear MeOH sample to another test tube (see pictures in Appendix 7.1.2). The whole process took longer than desired, and contamination could have entered along the prolonged process, or else by condensation in the porcelain mortar bowl. To reduce probability of contamination during the homogenisation, the samples in the following test series were no longer pre frozen in the porcelain bowl but directly in the grinding jars. Steel abrasion was not expected to occur if the grinding jars were filled with actual tissue samples.

5.2.3 Test series 3

In test series 3 round goby liver samples were included for the first time. Compared to the trout liver, the detected amount of BPA was lower and the high BPA value in the sampled trout liver also found in test series 1 was again confirmed. If the fish ingested BPA and therefore accumulated the compound or if it was due to the storage in a plastic bag remains unclear. The BPA content in the three round goby samples varied greatly and could be explained by a difference in the specimen composition or rather to the different freezing steps tested in this test series. Concerning the freezing

steps, no satisfactory result was obtained. All liver samples still contained fish oils and turned turbid during evaporation. Compared to test series 1 the samples were clearer at the beginning and turned turbid later, what indicated a certain reduction of fatty compounds, but the extract remained turbid (see pictures in Appendix 8.1.5).

A major difference was observed in the characteristics of the liver samples of the two species. While the trout liver separated well into one clear aqueous phase and one solid phase during centrifugation, the result with the round goby was different. Several phases formed: one phase of solid matter at the bottom, in the middle an aqueous phase with few suspended light matters and at the top a lid of light solids and a layer of oil (see photo in Appendix 8.1.2). As a result, the aqueous phase located in the middle had to be carefully scooped up with a glass pipette, preferably without contamination from the very fatty phase located at the top.

5.2.4 Test series 4

By analysing a triplet series of blanks treated with different solvent solutions, the method was validated, and last adjustments could be implemented to the main experiment method. As expected, the triplets confirmed the previously observed BPA leaking sourcing from the SPE MIPs cartridges. To our knowledge, no other publication using the same product observed this issue. It could be imaginable that the cartridges of only the lot used in this experiment was contaminated although this assumption was not further examined by using another lot.

The procedure for the main experiment was continued according to the best results in this test series with the 10 ml solvent volume for conditioning of the cartridges and was considered reliable enough, as further increasing of the solvent volume did not lead to better results. The remaining BPA background may have been due to a weak contamination of the water that accumulated at the MIPs during the clean-up. This would explain the relatively stable background contamination in B1.2 and B2.2 and why the manufacturer observed lower levels than the ones observed in this study. No higher contamination was found in the full process (B2) compared to the SPE MIPs process only (B1), which was suggestive of a well-optimized methodology. The occasionally higher values in B1.3 and B2.1 could be due to a brief drying out of two samples during conditioning, as the substrate may not have come into contact with the solvent uniformly due to enclosed air. It can be difficult to get the timing right to close the cartridge and prevent drying out when many samples are being run simultaneously. Based on this experience, it was decided to dispense the vacuum in the main experiment for cartridge conditioning, loading, washing and eluting, and instead process these steps under gravity. Only the actual intentional drying after cartridge-washing was still carried out under vacuum to get rid of the residual water before eluting with MeOH.

Freezing experiment

The experiment to optimize the freezing step was only visually evaluated and indicated a significantly improved result. Only in the main experiment did it become clear that fish oils were still present in the extract and although at reduced amounts they interfered with the analyte.

5.2.5 Main experiment

Liver and muscle analysis

During the preparation of the liver samples, it was observed that the samples behaved very heterogeneously. The round goby liver homogenates differed not only compared to trout liver but also differed from pool to pool, some containing a high amount of suspended light matter in the aqueous phase after centrifugation, others with an almost clear aqueous phase but a thick layer of a supernatant layer containing a high amount of fatty matter (see photo in Appendix 10.2.1 and 8.1.2). Although maximum 4347 *g* was applied for centrifugation, the samples remained inhomogeneous.

At first, the extraction procedure for the liver samples seemed to have improved significantly and showing no visual signs of any matrix contamination as observed in the previous test series. Only shortly before the eluate was fully evaporated, it still turned turbid and upon re-dissolving the first sample by MeOH and water it became entirely cloudy (see photos in Appendix 10.2.7). Fish oils thus still were present, although in a reduced amount, judging by the delayed occurrence of the turbidity compared to the previous test series samples. The decision to dissolve the liver samples in only MeOH and transfer the extract to a narrowed microvial came at short notice but seemed to work well. Concerning the LC-MS/MS analysis results, the equally processed blank samples were measured at an expected BPA value and no signs indicating an impairment due to the transfer was detected. The liver extracts however, although containing supposedly lower fish oil content and appearing as clear solution in MeOH, could unexpectedly not be assessed for their BPA content except for one sample. In all other round goby liver samples, the *IS* was not visible at all, presumably due to strong suppression caused by matrix interference. Two sub-steps were identified as the decisive factors: On one hand, the adjusted freezing step was still not effective to sufficiently eliminate all interfering fish oil matrix. The other factor originated from the SPE MIPs extraction. The washing of the cartridges by MeCN/water (40/60 v/v) did not sufficiently remove the nonselective bound matrix interference, which subsequently resulted to co-elute with the BPA analyte and causing the strong *IS* suppression observed in the LC-MS/MS analysis. These strong matrix effects were only observed in round goby liver. The applied method was suitable for muscle tissue analysis although recovery was rather low for some samples, all were in an acceptable range.

To mitigate the issue of the complex liver matrix, further improvements in the presented method are necessary. The use of smaller samples such as 1 g instead of 2 g liver tissue would already reduce

matrix effects but would also negatively impact the lower limit of BPA detectability if present in low concentrations. This was the case in the monkey goby sample nr. *5.1* where matrix interference was lower, but the BPA level was on the same level as the blanks. It may prove more effective to focus on the selectivity of the cartridges in addition to the freezing step, which already partly reduces the matrix compounds. Yang et al. (2014) suggested to add an adjusted washing step to the SPE MIPs if strong matrix interference was detected, which effectively improved the binding of the analyte to the molecularly imprinted polymers in canned food samples. By completely drying the cartridges after the usual washing step for 30 minutes, the interaction of the BPA with the polymer was improved. Then the authors washed the cartridge again with 2 ml of MeCN to induce the formation of specific interactions through hydrogen bonds between the analytes and the MIPs. By subsequently adding 2 ml of MeOH/MeCN (10/90 v/v), non-specifically bound apolar matrix interference was eluted. This did not affect the recoveries of BPA as long as MeOH was not applied in higher concentration than 10 % but greatly reduced the apolar matrix. By eluting the cartridge with MeOH, the selectively bound BPA was then released, and no matrix interference was detected.

While in most studies dealing with BPA extraction from fish or meat use MeCN for BPA solvent extraction (Thomson & Grounds, 2007), some also described the use of non-polar solvents such as *n*-hexane or *n*-heptane in combination with acetonitrile if the samples contain high amounts of fats (Sun et al., 2006). According to Thomson & Grounds (2007) the use of trimethylpentane resulted more effective fat removal than *n*-hexane. Another method reported the use of celite and activated natural alumina (Shao et al., 2007).

With regard to the BPA contamination of the muscle samples, no considerable contamination was be detected. The BPA level in all samples was below the LOQ and therefore absolute values were not meaningful to report (< 1 ng/g).

Conclusion fish tissue

The method needs to be further refined to be able to reliably quantify values in the single-digit ng range. The goals for liver analysis were not achieved due to strong matrix effects. However, it can be concluded that the samples are barely contaminated by BPA and no problematic values were detected in the sampled locations. However, it has to be noted that these data were based on pooled fish samples and give no insight in the individual variation and are limited to the sampling period. Neither can seasonal changes in BPA contamination of the sampled environments be ruled out nor do the data provide insights into BPA contamination in other fish species.

Water

No water contamination was found at higher values than observed in the blank samples. To improve the method for BPA analysis in water, a higher volume could be analysed. It is probable that only a

high BPA contamination would be detected by including only 2 ml of water in each sample. Lower traces could be found if the sample volume was increased e.g. ten- or fiftyfold or even up to one litre, as suggested by Belfroid et al. (2002). Due to the focus of this study on the method development for fish tissue samples, no separate method was implemented to optimise water analysis.

Sediments

In the present study, the highest trace contamination of BPA was detected in sediment samples, although the highest value remained narrowly below the LOQ. The three samples deviated considerably. The samples were collected at the same location and consisted of a sediment layer about 10 cm deep. Possibly BPA concentration depends on the depth of the sediment sample. To validate this assumption, more samples would be needed. The increased BPA content in sediment is in line with the studies of Kawahata et al. (2004) and Voordeckers et al. (2002), which found higher BPA values in sediment due to the reduced biodegradability in sediment caused by low oxygen conditions.

5.3 Vitellogenin

The quantified VTG concentration documented in this study was in a low range and only hardly above LOQ in only three out of 27 samples. 24 samples were below LOQ, and all muscle samples (n = 14) and six liver samples below LLD. Interestingly, three male liver samples (tall n = 2; small n = 1) indicated a higher VTG concentration than the tall female liver sample (n = 1). However, the tall female liver sample comprised of only one (pooled) sample and was not quantified with certainty, as the obtained value was slightly below LOQ, comparability was therefore limited. In follow-up studies we suggest using an increased tissue sample weight to provide more reliable values and quantify lower VTG concentrations. For liver, we suggest at least factor 3 (15 µg homogenate) and factor 10 for muscle samples (100 µg homogenate) in round goby samples obtained from the sampling location. Unfortunately, this experiment could not be repeated due to limited time. Another factor to consider is that vitellogenin, as a cleavable precursor of yolk proteins, is highly unstable. For this reason, the samples obtained must be immediately deep-frozen and analysed immediately upon thawing. It is possible that a temporary thawing while preparing of the samples resulted in a loss. The here observed slightly increased VTG values in male round gobies (exceeding the female VTG level) can, however, be considered as an indication of the presence of xenoestrogens in the sampled Rhine River area and should be analysed in more detail in follow-up studies. Round goby as an 'easy-to-catch' invasive species with high population densities could serve as an important model species in monitoring endocrine disruption in invaded ecosystems.

To the authors' knowledge, VTG has not been reportedly quantified in round goby via ELISA, however one study reports the development and use of qPCR assays to measure VTG gene

expression and upregulation caused by (estrogenic) EDCs in round goby (L. A. Bowley et al., 2010). ELISA was used for the VTG determination in the closely related Japanese common goby (*Acanthogobius flavimanus*) in several studies (Nagae et al., n.d.; Ohkubo et al., 2003; Song et al., 2018). The ELISA system applied in the present study supported *Neogobius sp.* according to the manufacturer through sufficient cross-reactivity, although standard stock solution and vitellogenin controls were based on perch vitellogenin.

5.4 Conclusion

While the final method was successful for muscle tissue analysis, it was not yet fully reliable for liver tissue analysis and requires further improvement. The limit of quantification was too high to accurately quantify low contaminated samples, and matrix interferences in liver samples require an extra step to remove fish oils before sample preparation. However, the study found that BPA contamination in round goby from the sampling location was low and of no concern. However, the findings are only applicable to the specific sampling location and fish species, as other habitats and species may have different characteristics. The sediment samples indicated higher levels of BPA, but the levels were below the limit of quantification and therefore could not be quantified. In addition, the study faced some limitations in the method development, namely the limited number of cartridges (100) and a balance between sample quantity, cost, and time had to be implemented. Therefore, test series 1 – 3 were planned without triplets to provide a rough overview and provide the lead to a fine-tuning of the method when main factors were identified. One drawback of this approach was that outliers were more difficult to detect. The main experiment was then accompanied by 4 blanks for each batch, but outlier in tissue samples were still possible and prevented by setting LOQ to tenfold blank mean level. A further reduction of LOQ could therefore be achieved by analysing tissue samples in triplets too in future approaches.

The size- and sex-specific conditions in the round goby population indicated that males were taller than females and that the population structure and HSI showed signs of intraspecific competition, indicating a high population density and reduced population- and individual specimen condition, compared to other studies. This could indicate a current transition from "newly invaded habitat characteristics" to "established habitat characteristics".

Finally, the presence of endocrine disrupting chemicals was indicated by detecting vitellogenin in males, although at low levels, but in higher concentrations than in females. Round goby could pose a valuable model species for the monitoring of EDCs activity in invaded ecosystems; however, more research is needed.

This study contributes a small piece to the overall understanding of BPA contamination in biota in Switzerland, where there is currently limited knowledge.

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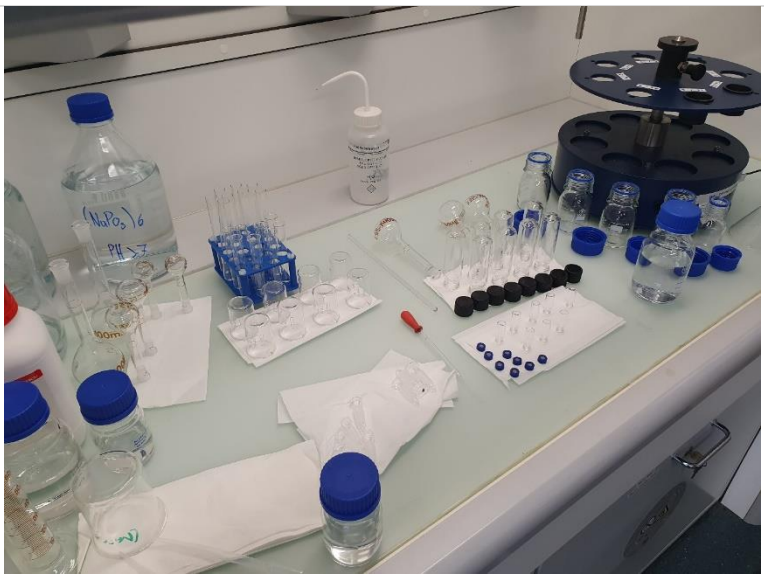
List of figures

List of tables

Appendix

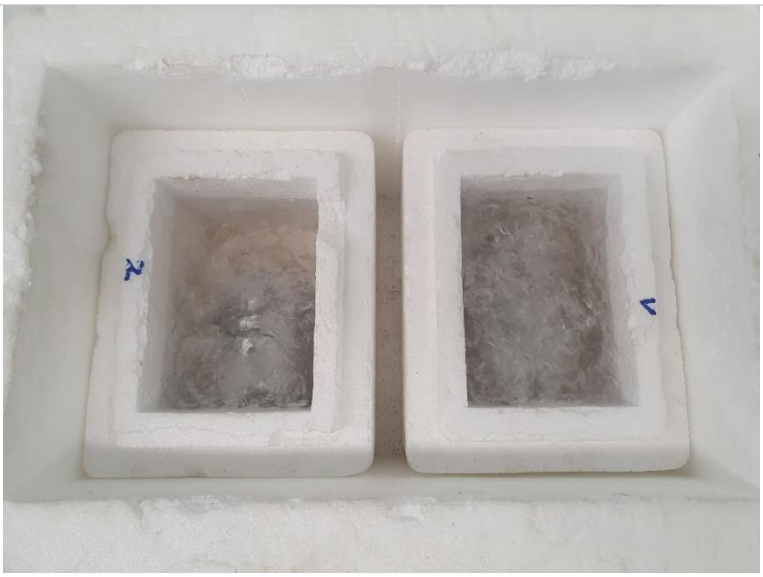
6 Appendix: Test series 1

6.1 Photos process



6.1.1 **All glassware was cleaned by MeCH and MeOH before using.**

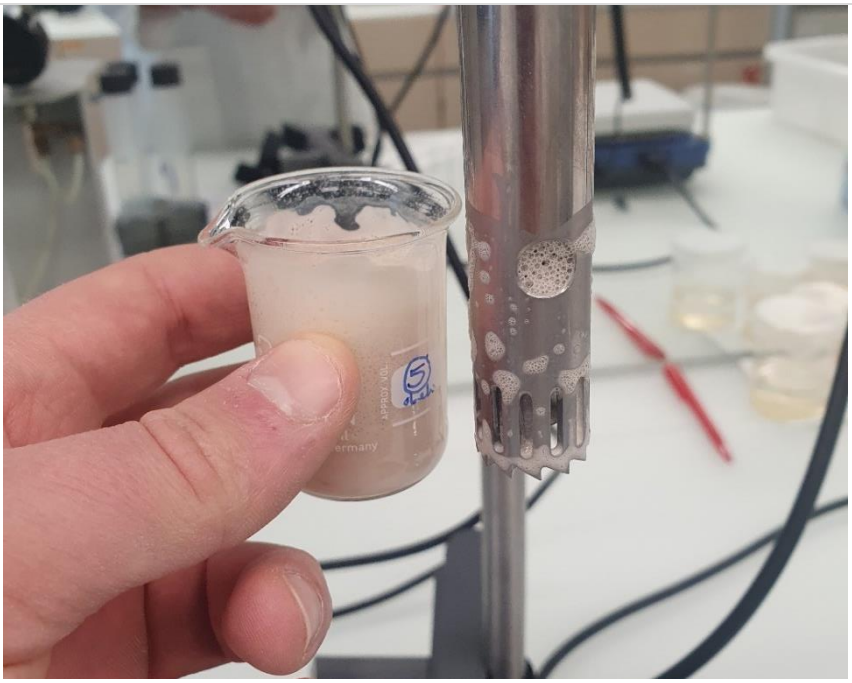
6.1.2 ***O. mykiss*** **liver sample had to be pre-frozen in a mortar bowl to prevent the grinding ball and the homogenate from sticking together.**



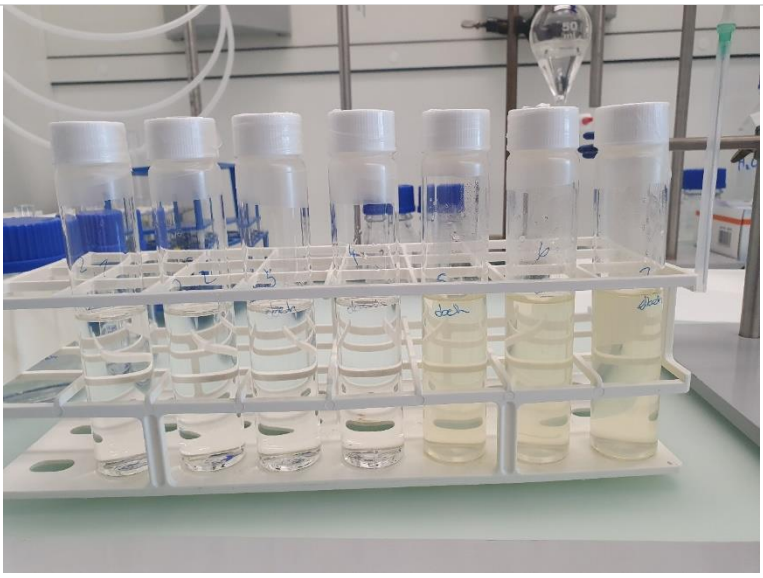
6.1.3 **The liver sample was placed into the grinding jars and frozen to – 196 °C in liquid nitrogen.**

6.1.4 **Program set for the mixer mill MM 400.**

6.1.5 **Frozen *O. mykiss* liver homogenate after cryomilling.**



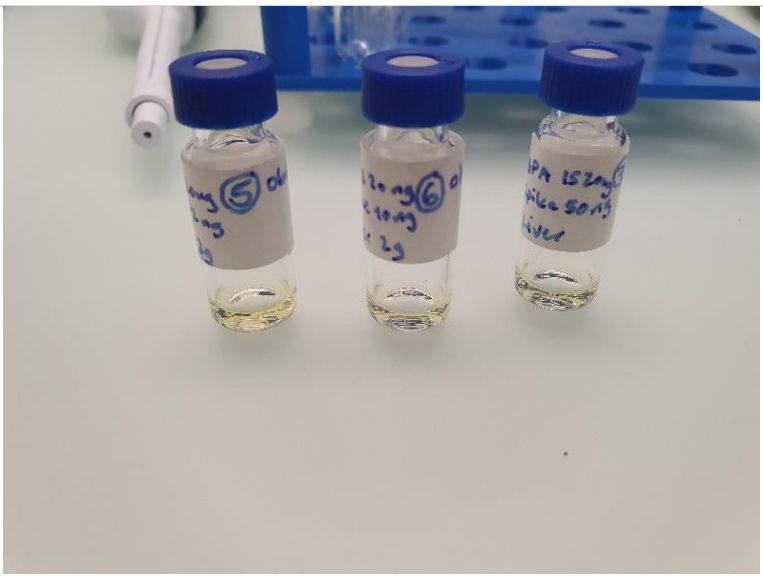
6.1.6 **Ultra-turrax homogenisation.**



6.1.7 Diluted sample extract after centrifugation.

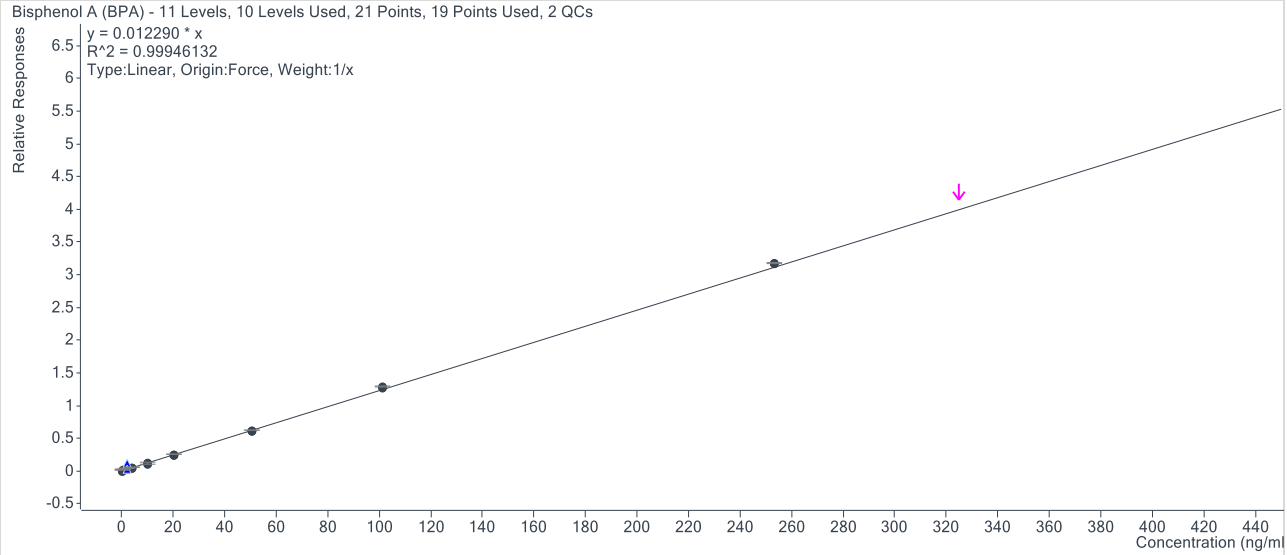
6.1.8 Evaporation with gentle nitrogen stream.

6.1.9 **Fish oil and matrix visible after evaporation to dryness.**

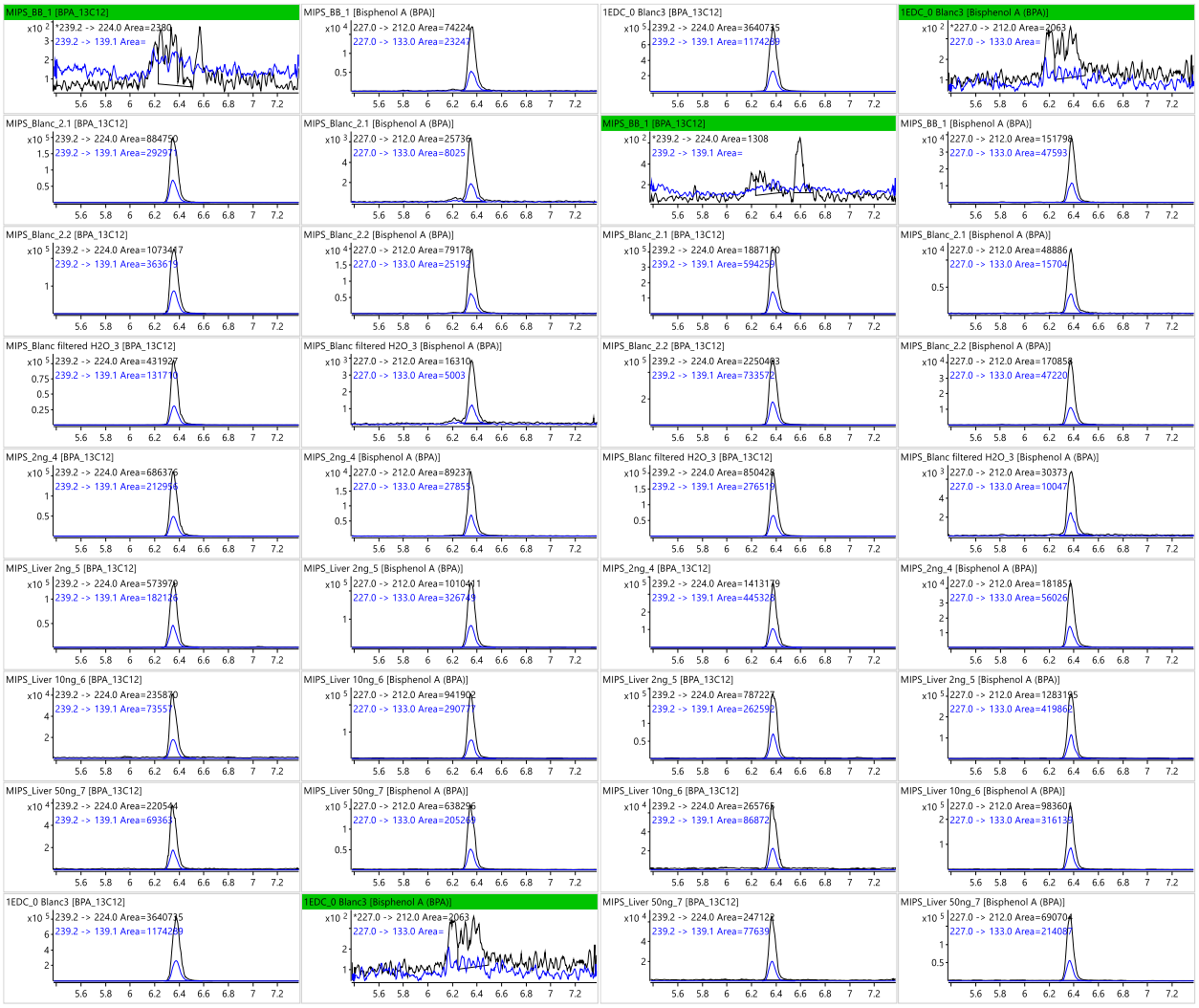


6.1.10 **Redissolved extract in 100 µl MeOH. The extract turned turbid afterwards when adding 100 µl H_2O.**

6.2 Complementary information LC-MS/MS



6.2.1 Calibration curve test series 1.



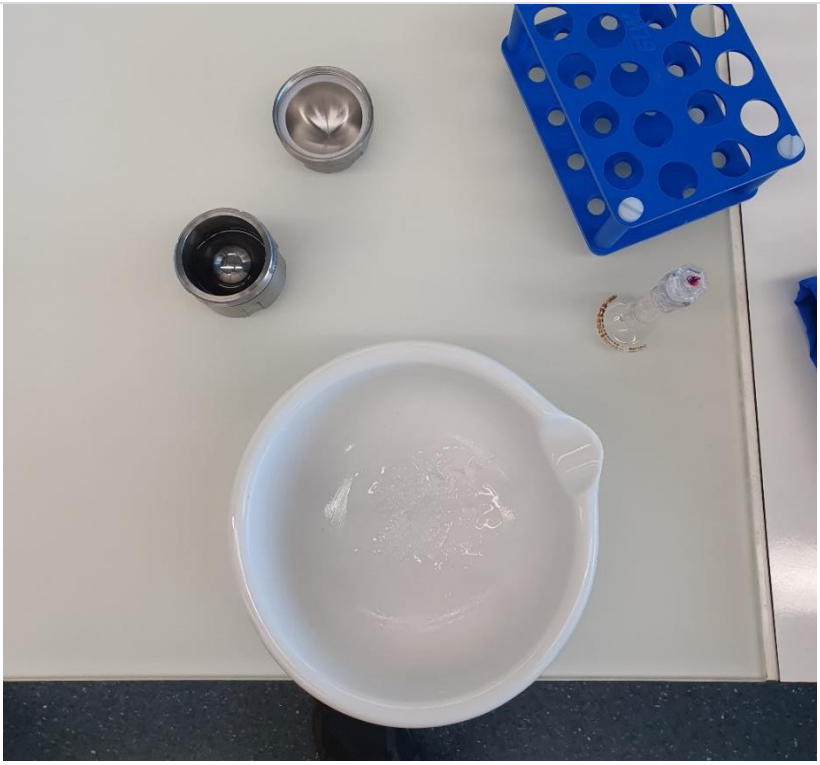
6.2.2 LC-MS/MS chromatograms obtained in test series 1.

6.2.3 **Signal-to-noise ratio and response in test series 1 samples for *IS* and BPA.**

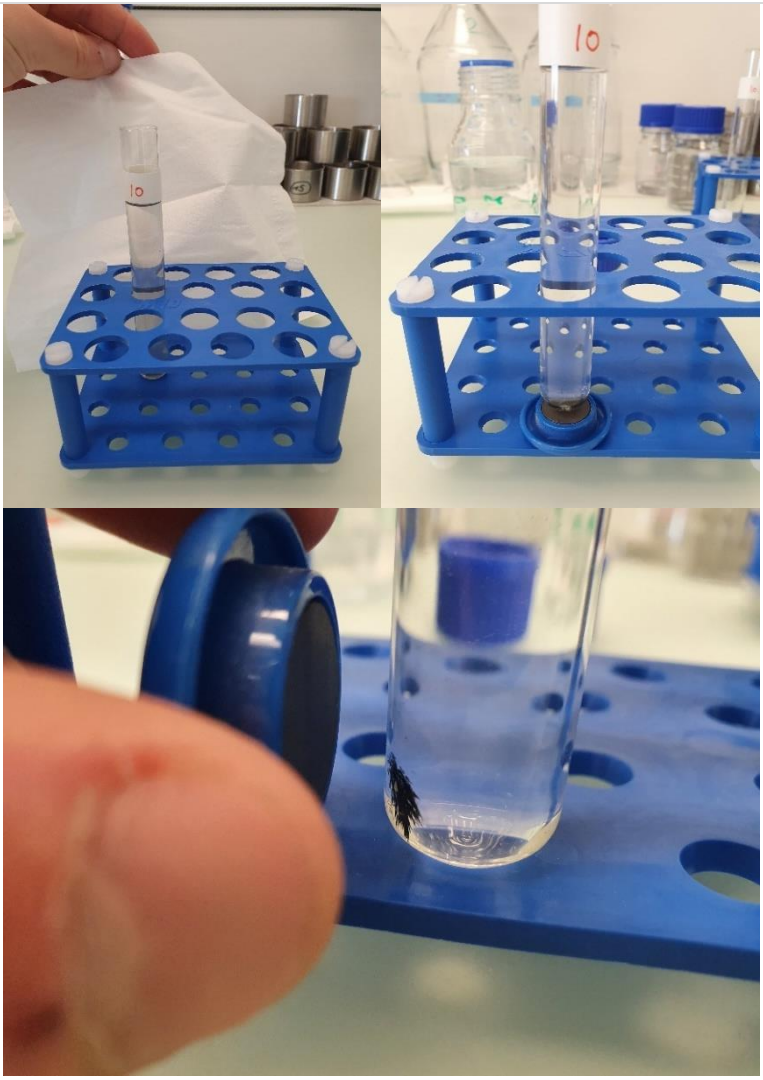
No.	S/N *IS*	S/N BPA	*IS* response	*BPA* response
2.1	*4397.0*	*81.7*	*1385930*	*37311*
2.2	*4393.0*	*129.0*	*1661910*	*125018*
3	*2083.0*	*40.5*	641178	23341
4	*4556.0*	*272.4*	1049778	135544
5	*249.0*	*404.9*	680603	1146803
6	*48.7*	*193.4*	250817	962752
7	*72.4*	*199.7*	233833	664500

7 Appendix: Test series 2

7.1 Photos process



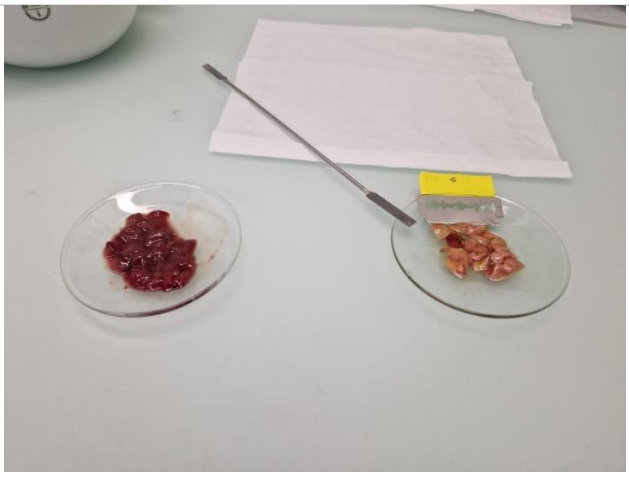
7.1.1 Frozen MeCN to simulate the cryomill step while evaluating potential contamination sources.



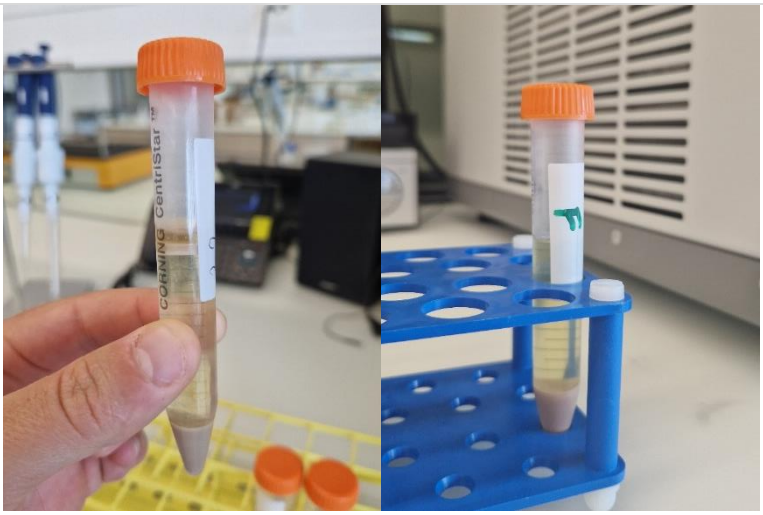
7.1.2 The MeOH blank sample contained steel abrasion sourcing from the grinding jars. The impurities were removed by magnets.

8 Appendix: Test series 3

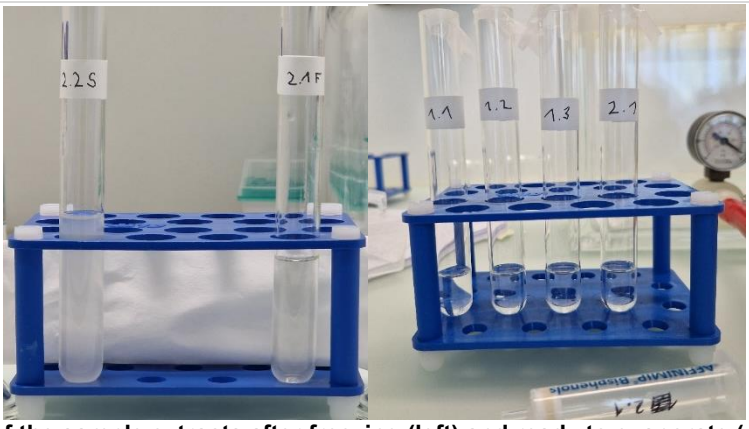
8.1 Photos process



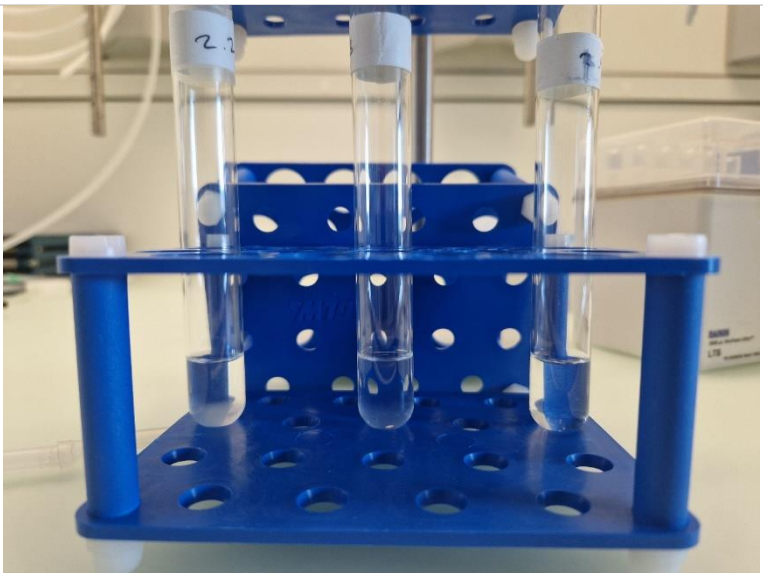
8.1.1 ***O. mykiss*** **(left) and** ***N.melanostomus*** **(right) liver.**



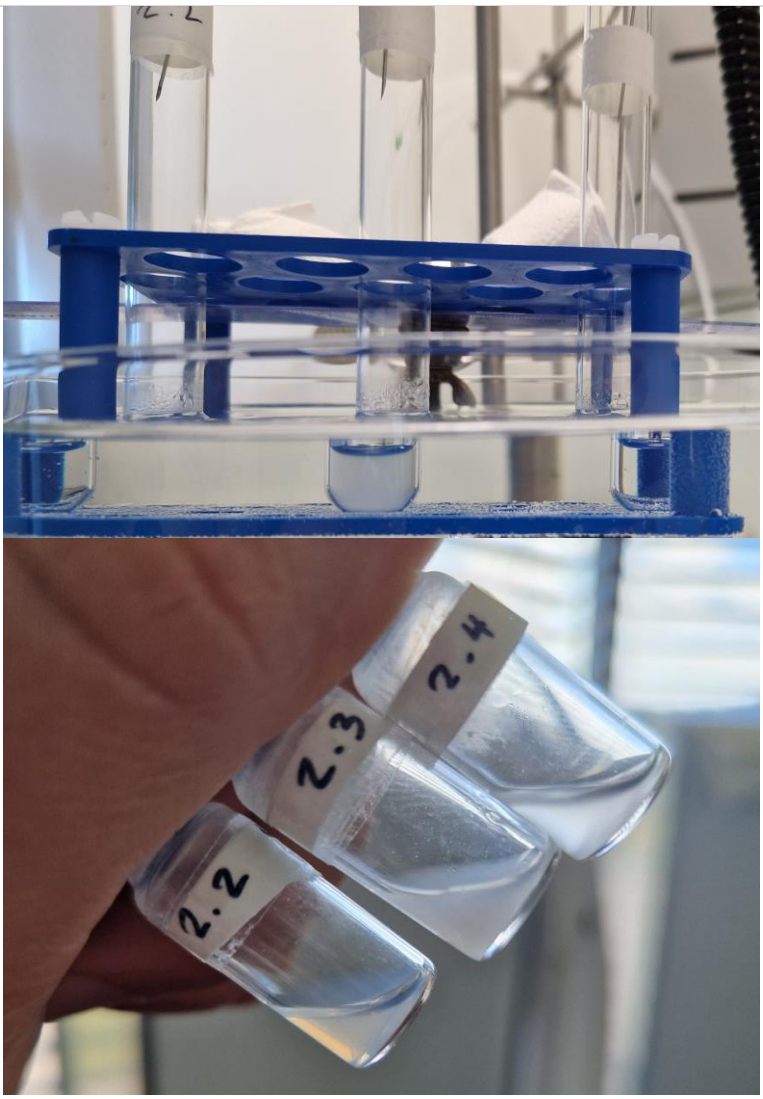
8.1.2 ***N.melanostomus*** **(left) and** ***O.mykiss*** **(right) liver tissue samples after centrifugation. The round goby sample separated into several phases while the trout sample separated well.**



8.1.3 **Part of the sample extracts after freezing (left) and ready to evaporate (right).**

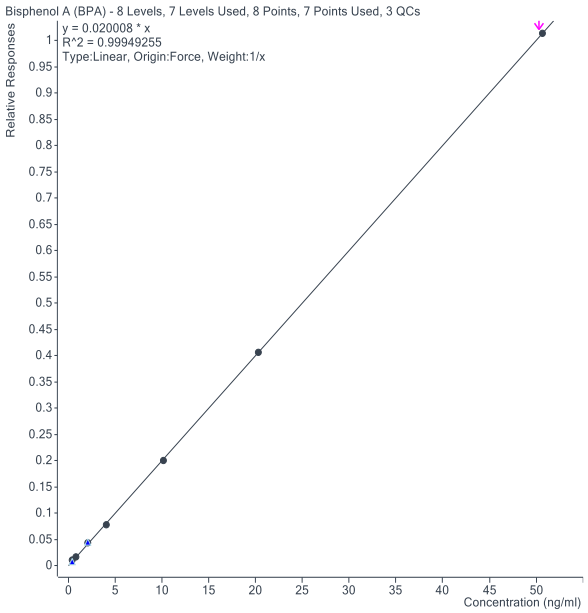


8.1.4 **Some liver sample extracts already clouded afther the elution from the cartridges, while others appeared clean.**

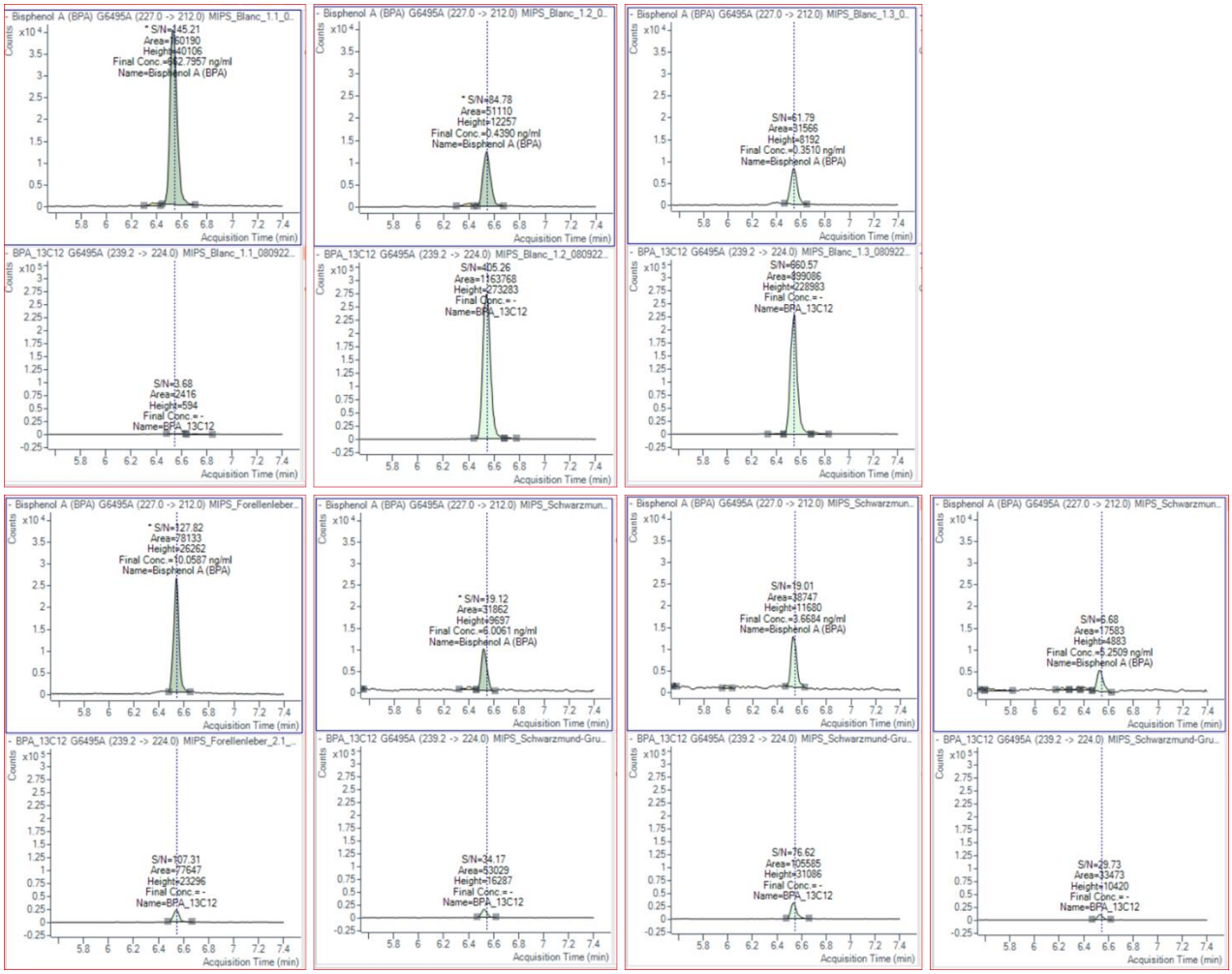


8.1.5 **During the evaporation, all liver samples became turbid at a certain volume.**

8.2 Complementary information LC-MS/MS



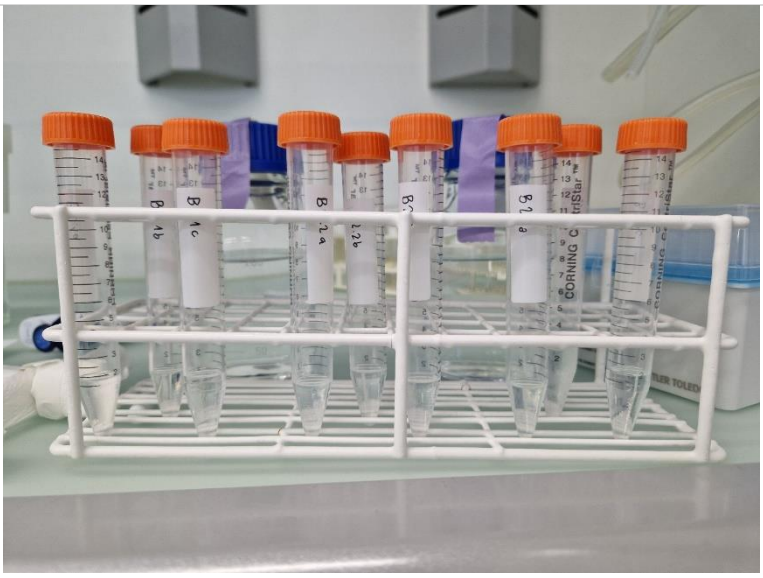
8.2.1 LC-MS/MS calibration curve test series 3.



8.2.2 LC-MS/MS chromatograms obtained for test series 3.

9 Appendix: Test series 4

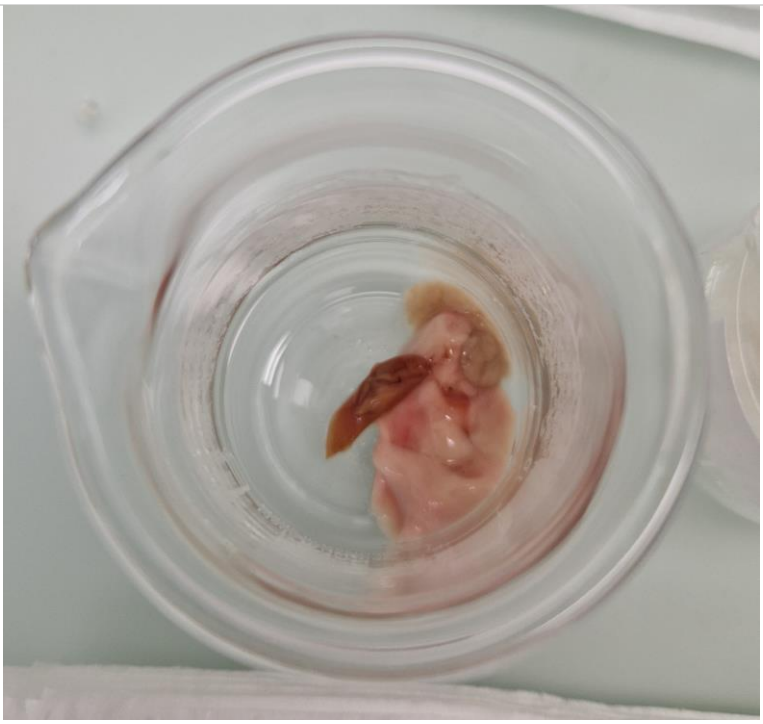
9.1 Photos process



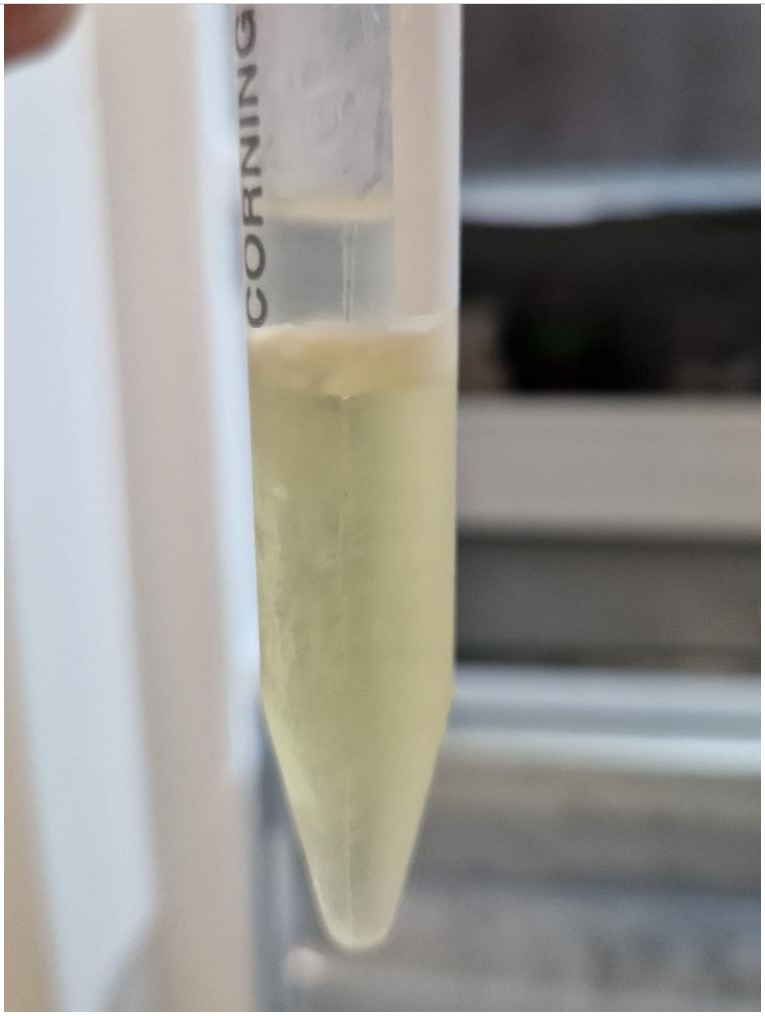
9.1.1 Sample volume retrieved after freezing step was similar in all blanks.

9.1.2 SPE MIPs setup.

9.1.3 Setup for the evaporation of the eluates.



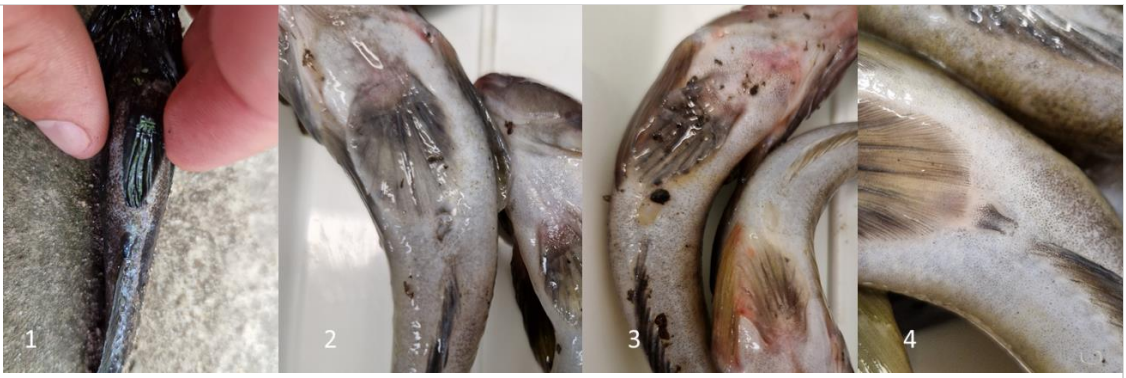
9.1.4 Round goby liver had a highly heterogenous appearance.



9.1.5 Nicely separated and clear supernatant phase after freezing overnight.

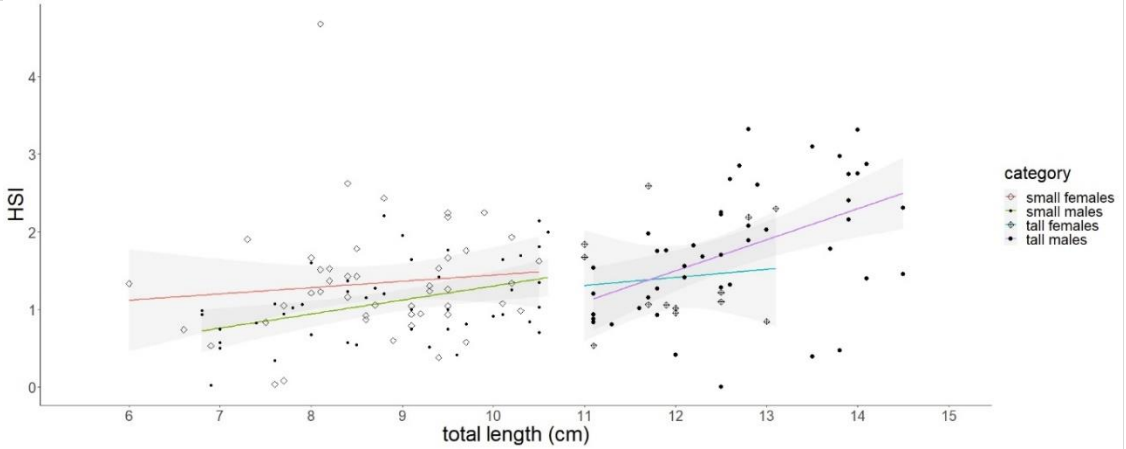
10 Appendix: Main experiment

10.1 Sample composition

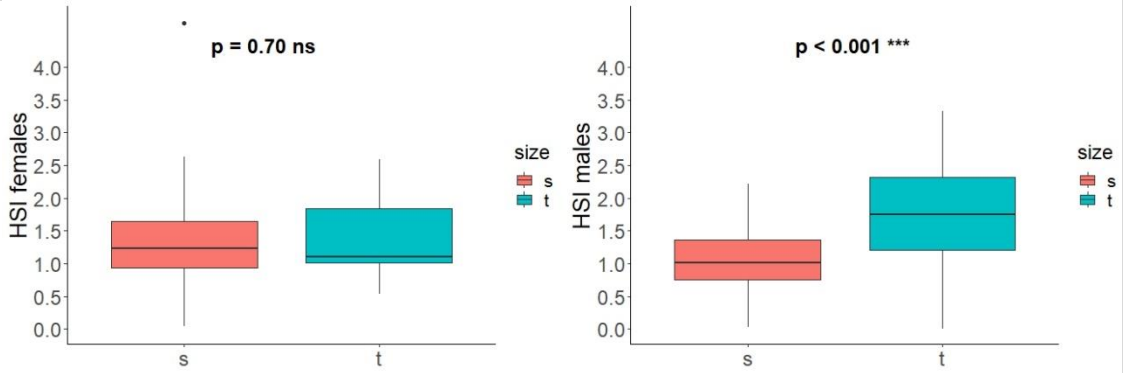


10.1.1 **Determination of sex was simple for males (1) if they were pigmented black ("guarder males" when breeding) and if the urogenital papillae was undoubtedly male shaped (2) or female shaped (3). A wider papilla with rather male characteristic but not explicitly pointed end (4) could indicate an intersex individual (see also figure 1 in Guellard et al., 2014).**

10.1.2 Monkey goby sample (males left, females right side).

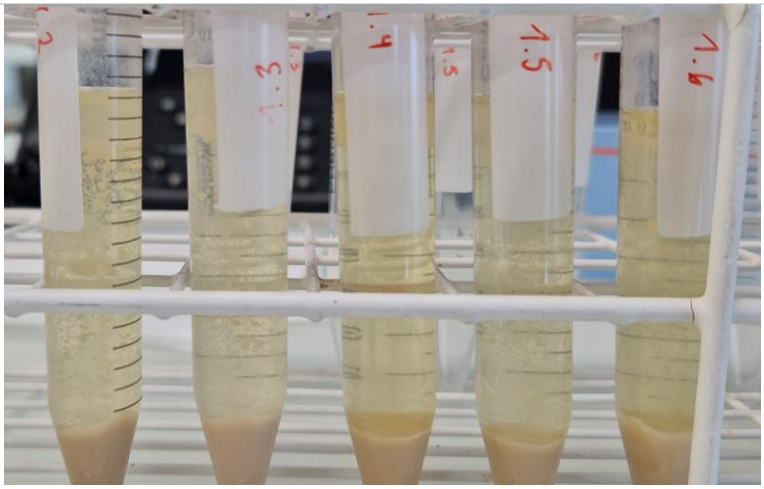


10.1.3 HSI for each round goby specimen and corresponding sex and size category.

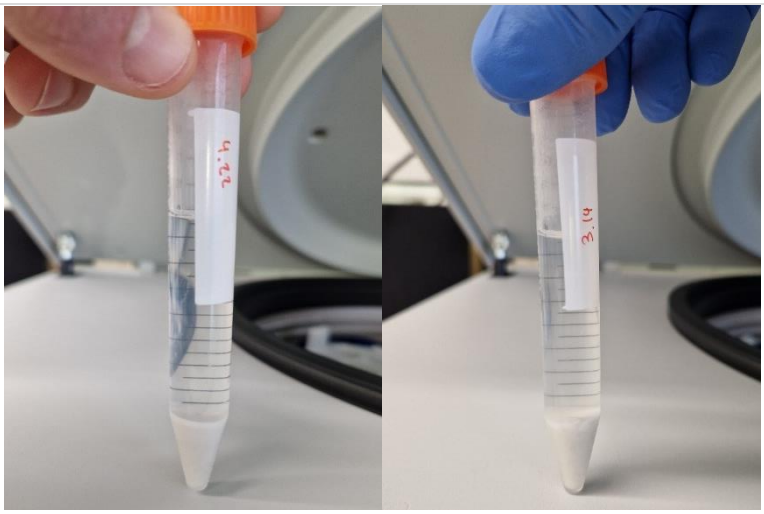


10.1.4 **HSI between sex categories of female (t = tall and s = small), and males (t = tall and s = small). Tall males HSI was significantly higher (Welchs' t-test, p < 0.001 ***).**

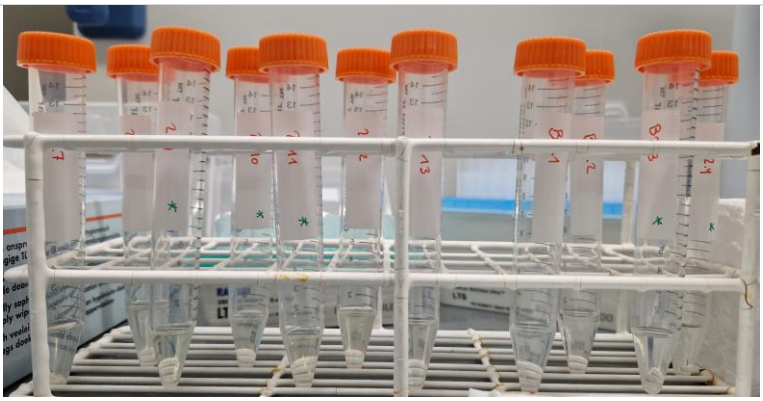
10.2 Photos process



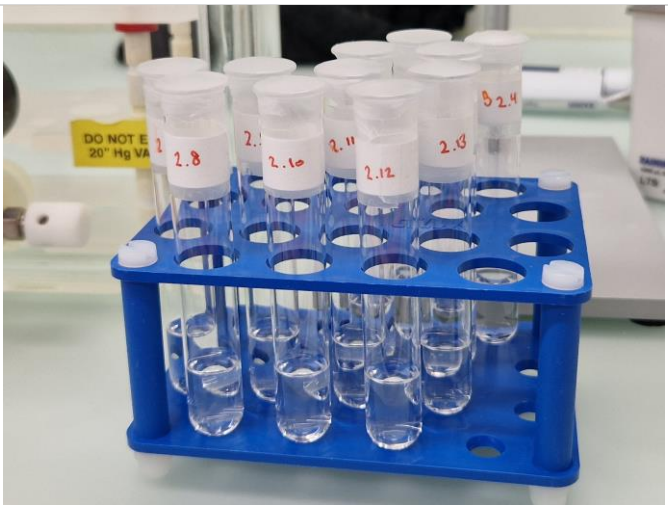
10.2.1 **Phase separation by centrifugation was difficult in round goby liver samples, while some separated relatively well (see also Appendix 8.1.2), others could not be separated into a clear aqueous phase.**



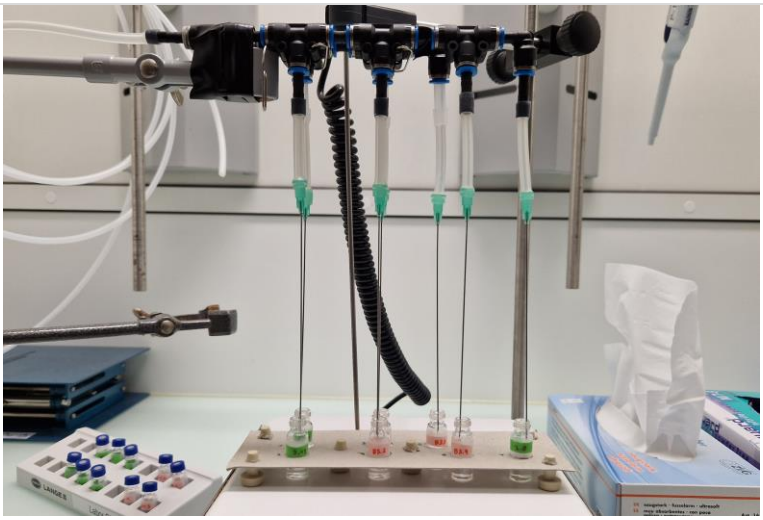
10.2.2 **Muscle tissue was clear and separated well.**



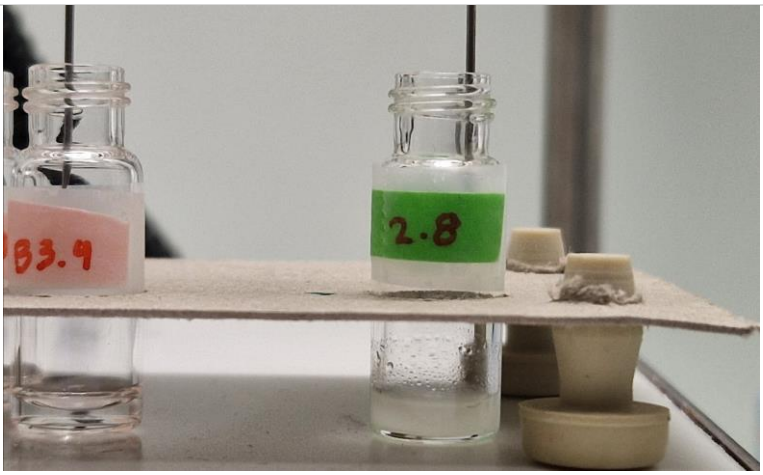
10.2.3 Sample volume retrieved after freezing step in round goby liver samples (patch 2).



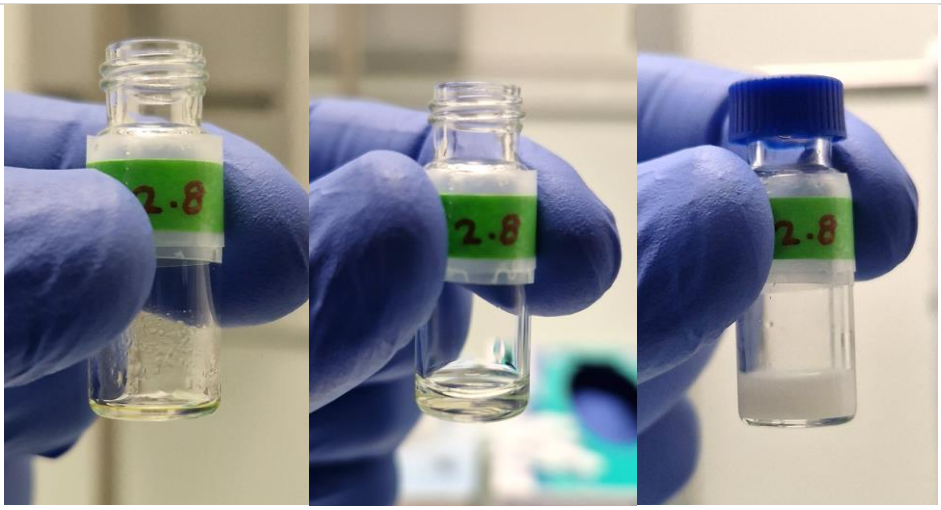
10.2.4 The eluate was colorless and clear (liver samples, patch 2), no indication of fish oils was visible.



10.2.5 Setup for sample evaporation to dryness.



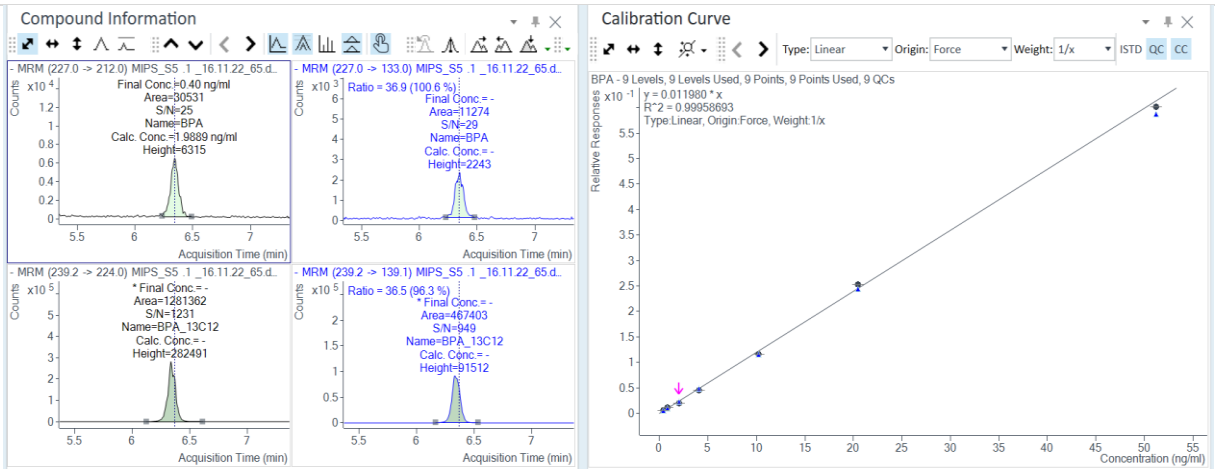
10.2.6 **Sudden turbidity in liver samples at low volume. The samples turned turbid much later than in test series 1 – 3, indicating an improved fish oil removal by the adjusted freezing step, but was still not sufficient.**



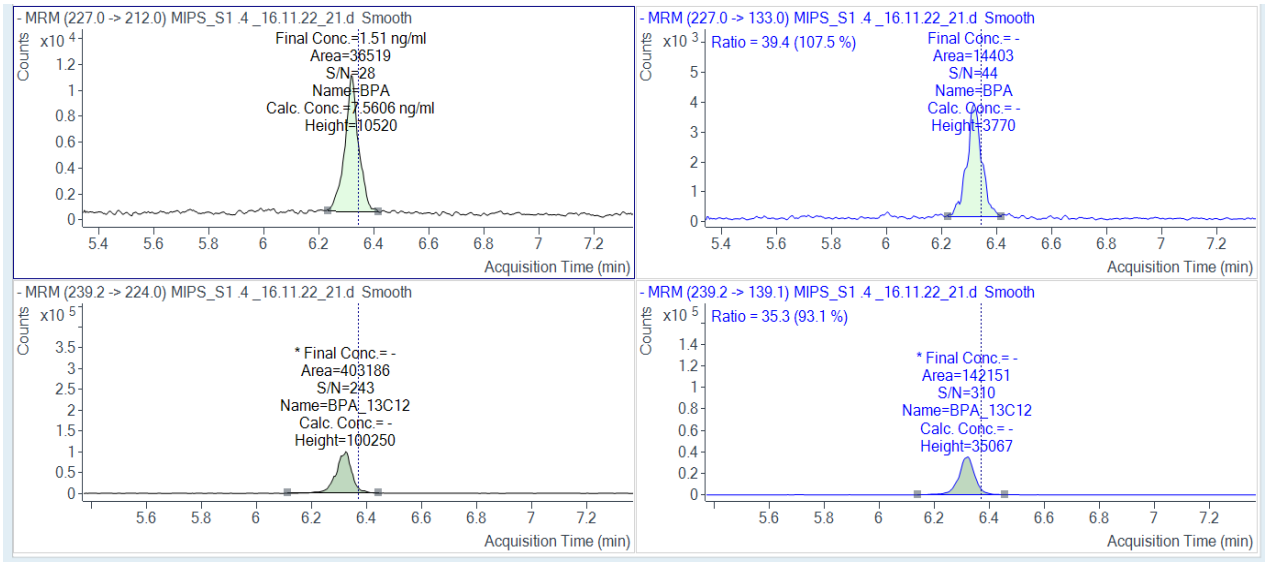
10.2.7 **After re-dissolving the first extract with MeCN and water, the decision was made to evaporate it again and re-dissolve all liver extracts in 50 µl MeOH only and transfer the final (clean) extract into a narrowed microvial.**

10.2.8 **Readily extracted samples for LC-MS/MS analysis. The narrowed microvials are located above.**

10.3 Complementary information LC-MS/MS



10.3.1 LC-MS/MS calibration curve for main experiment (right) and chromatograms obtained for monkey goby *N. fluviatilis* liver sample (left).



10.3.2 LC-MS/MS chromatogram obtained for round goby liver sample no. 4, the only quantified round goby liver sample. The *IS* was not visible in the other round goby liver samples.

10.4 Parameter settings LS-MS/MS

Table A. Parameter settings of the Agilent G6495A Triple Quadrupole (QQQ) mass spectrometer used in negative mode.

Parameter	Value
Gas Temp (°C)	200
Gas Flow (l/min)	17
Nebulizer (psi)	25
SheathGasHeater	350
SheathGasFlow	12
Capillary (V)	3300
VCharging	1800
Delta EMV (V)	400

Ion Funnel Parameters	Value
Neg High Pressure RF	150
Neg Low Pressure RF	100

Table B. Parameter settings of the Agilent G7120A high pressure binary pump

Time (min)	A (%)	B (%)
0.19 min	90.00%	10.00%
0.20 min	70.00 %	30.00 %
9.00 min	26.00 %	74.00 %
10.30 min	2.00 %	98.00 %
14.00 min	2.00 %	98.00 %
14.00 min	90.00%	10.00%
17.00 min	90.00%	10.00%

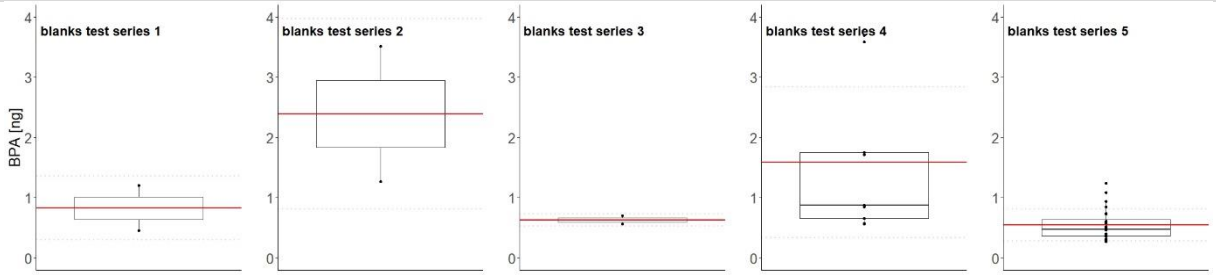
Table C. Retention time and mass transitions of analyzed compounds (unit resolution).

Compound [M-H]-	Retention Time (min)	precursor ion (m/z)	Quantifier MRM product ion (m/z)/ collision energie [V]	Qualifier MRM product ion (m/z)/ collision energie [V]
Bisphenol A (BPA)	3.25	227.0	212 / 17	133 / 29
BPA_13C12	3.26	239.2	224 / 18	139.1 / 28
Estron (E1)	3.93	269.0	145.0 / 41	143 / 61
E1_13C3	3.94	272.2	148.1 / 44	186 / 44

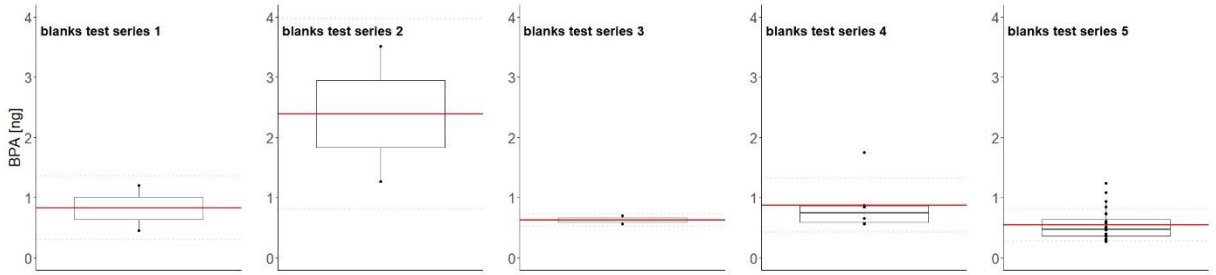
Table D. List of part of the materials and equipment used for processing and analyzing EDC in samples.

Name	Product Number	Vendor
Estrone-2,3,4-13C3	E-108-1ML, Lot FN11071402	Sigma
Bisphenol-A (Ring-13C12, 99%)	CLM-4325-1.2, Lot SDFK-015	CIL
Estrone, ≥99%	E9750	Sigma
Bisphenol A, ≥99%	239658	Aldrich
Ammoniumfluorid puriss. p.a.	30101	Sigma-Aldrich
Methanol (optima LC/MS) Fisher	A456-212	Fisher
ms-Pure septum non-pigmented multiple injection HPblack 100er	G004-HP-CS-FKSKFK10	infochroma
Agilent compatible 2 mL Screw Vial amber 100er	G004-HP-H	infochroma
ACQUITY UPLC BEH Shield RP18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm	186002854	Waters
ACQUITY UPLC BEH Shield RP18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm	186003977	Waters

10.5 Complementary information blank samples



10.5.1 BPA in full method procedure blanks across all test series (main experiment = main experiment). Note that only in test series 4 and 5 (main experiment) n ≥ 9. Means were displayed as red line, SD as fine dotted lines.



10.5.2 BPA in full method procedure blanks across all test series (main experiment = main experiment). Test series 4 without 5 ml – conditioned cartridge series. Means were displayed as red line, SD as fine dotted lines.