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The influence of microplastics and halogenated contaminants in feed on toxicokinetics and gene expression in European seabass (*Dicentrarchus labrax*)

Kit Granby, Sandra Rainieri, Rie Romme Rasmussen, Michiel J.J. Kotterman, Jens Jørgen Sloth, Tommy Licht Cederberg, Alex Barranco, António Marques, Bodil Katrine Larsen

Highlights

- The presence of microplastic particles in feed influenced the toxicokinetics and affected the liver
- k_{el} of PCBs and BFRs were significantly lower for diet with sorbed contaminants compared with other contaminated diets
- The assimilation efficiencies were lower for the contaminated diet with clean microplastics, likely due to higher elimination coefficients
- Seabass quickly metabolized PBDE99 to PBDE47 unaffected by the presence of microplastics
- Significant gene expression of livers (*cyp1a*, *il1β*, *gstα*) indicate that contaminants sorbed to microplastics increase the toxicity of some contaminants

Abstract

When microplastics pollute fish habitats, it may be ingested by fish, thereby contaminating fish with sorbed contaminants. The present study investigates how combinations of halogenated contaminants and microplastics associated with feed are able to alter toxicokinetics in European seabass and affect the fish. Microplastic particles (2%) were added to the feeds either with sorbed contaminants or as a mix of clean microplastic particle and chemical contaminants, and compared with feed containing contaminants without microplastics. For the contaminated microplastic diet, the accumulation of polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) in fish was significantly higher, increasing up to 40 days of accumulation and then reversing to values comparable to the other diets upon the end of accumulation. The significant gene expression results of livers (*cyp1a*, *il1β*, *gstα*) after 40 days of exposure indicate that microplastics might indeed exacerbate the toxic effects (liver metabolism, immune system, oxidative stress) of some chemical contaminants sorbed to microplastics. Seabass quickly metabolized BDE99 to BDE47 by debromination, probably mediated by deiodinase enzymes, and unlike other contaminants, this metabolism was unaffected by the presence of microplastics. For the other PCBs and BFRs, the elimination coefficients were significantly lower in fish fed the diet with contaminants sorbed to microplastic compared with the other diets. The results indicate that microplastics influences detoxification in the liver and lipid distribution, both affecting contaminants concentrations.

Abbreviations

α	assimilation efficiency
α -HBCD	α -hexabromocyclododecane
BFR	brominated flame retardant
$C_{control\ feed}$	contaminant concentration in control feed
C_{feed}	contaminant concentration in feed
C_{fish}	contaminant concentration in fish fillet
<i>cyp1a</i>	cytochrome-P450-1A1
DNA	deoxyribonucleic acid
<i>ef1a</i>	elongation factor α
F	feeding rate
FCR	feed conversion ratio
GC	gas chromatography
GF	growth factor
GI	gastro intestinal system
<i>gsta</i>	glutathione S transferase α
<i>il1b</i>	interleukin β
k_{el}	elimination coefficient
$k_{el\ adj}$	elimination coefficient, adjusted for contaminants from control feed
LC	liquid chromatography
HIS	hepatosomatic index
MS	mass spectrometry
PE-(HD)	high density polyethylene
PE-(LD)	low density polyethylene
PAH	polyaromatic hydrocarbons
PCB	polychlorinated biphenyls
PBDE	polybrominated diphenyl ethers
Pit-tagging	passive integrator transponder tagging
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SGR	specific growth rate
$t_{\frac{1}{2}}$	half-life
w	weight

Introduction

Since the 1950s the growth in the world's plastic production has been increasing, comprising in 2015 ~300 million tons per year (Plastics Europe, 2016), some of these plastics will end up in the marine environment and with time it degrades to microplastics through physical, chemical and biological processes. Microplastics is becoming an ecotoxicological problem of growing proportions, especially for the aquatic environment (Avio et al., 2017).

Aquatic animals can accidentally ingest microplastic particles that are suspended in water (Ivar do sul, Coasta, 2014). Marine fish drink considerable amounts of seawater to regain the water loss by osmosis. For example salmon smolts drink approximately 10%-15% of their body weight per day (Fuentes, Eddy, 1997; Usher et al., 1988; Carroll et al., 1994), hence they can ingest microplastic particles when drinking. Fish can also ingest microplastic particles when they are inadvertently mistaken by prey (Wright et al., 2013), e.g. de Sá et al. (2015) showed in a trial that fish confused microplastics by prey when exposed to polyethylene (PE)-microplastic particles of similar size and abundance as prey, causing reduced feeding efficiency.

Microplastics can cause physical damage to fish that ingest it, and the type of damage depends on the size of the particles. Microplastic particles in the environment may occur in the defined size range from <5 mm down to where they become nanoplastic particles (<100 nm). In the upper size range, microplastic may cause intestinal blockage with decreased nutrition and suffocation (Choy, Drazen, 2013), but smaller microplastic particles are expected to pass through the fish's gastrointestinal (GI) system. However, if the microplastic particles are very small (<10 µm) they may be transported across the GI system, as was shown in common mussels, *Mytilus edulis* (Browne et al., 2008). The effects of microplastic particles <10 µm and nanoplastic particles (<100 nm) on marine organism are not yet fully elucidated. However, Besseling et al. (2014) showed that feeding nano-polystyrene particles to the zooplankton *Daphnia Magna* affected their reproduction.

Microplastics can also cause damages in combination with chemicals. For example, microplastic particles can concentrate environmental contaminants and 'carry' them to fish, where they may be bioaccessible and accumulate for example in the lipid fraction of fish fillets (Teuten et al., 2007). Pedá et al. (2016) found histological alterations in the distal part of the intestine after exposing European seabass, *Dicentrarchus labrax* to 0.1% environmentally contaminated polyvinyl chloride (PVC) microplastic particles <0.3 mm via feed, with the intestinal functions in some cases being totally compromised during the 90 days exposure. Rochman et al. (2014) demonstrated that the brominated flame retardants (BFRs) polybrominated diphenyl ethers (PBDEs) can be transferred from plastic to fish upon microplastic ingestion. The affinity and sorption of chemicals with high log K_{ow} to microplastic is very high, with pre-concentration up to 10^6 times (Mato et al., 2001), the affinity being polymer dependent. Polychlorinated biphenyls (PCBs) for example revealed the highest affinity to PE, followed by polypropylene (PP), compared with other polymer types (Rochman et al. 2013a). Chemicals that exhibit the highest affinity for PE microplastics are also some of those that bioaccumulate and biomagnify in marine organisms and humans, such as PCBs and BFRs. How important microplastics is for the transfer of chemical contaminants into fish, and the extent to which they affect the bioaccessibility have not yet been fully studied. However, model systems have shown that one important

parameter for desorption of contaminants from microplastic in the intestine is the presence of digestive surfactants, such as cholesterol-derived bile salts (Teuten et al., 2007; Bakir et al., 2014).

There are currently doubts whether to consider microplastics as a food safety issue. Microplastics is generally found in the guts of aquatic animals that ingest it. For this reason, animals of which the guts are eaten (such as mussels or crabs, for example) may represent a risk to human health. However, exposure studies related to microplastics and their additives/contaminants carried out with mussels confirm that microplastics and its sorbed contaminants do not represent a risk for consumers as they would increase only in insignificant amount the concentration for Bisphenol A, PCBs and polyaromatic hydrocarbons (PAHs) (Li et al., 2016). Moreover, several authors state that the contribution of microplastics to the dispersion of chemical contaminants in the environment where they already are abundant does not seem to be significant (Lohman, 2017, Koelmans et al., 2013; 2016; EFSA 2016). Some studies, however, highlighted that the effect of chemical contaminants is enhanced by the presence of microplastics (Pedá et al., 2016; Rainieri et al., 2018). In this context, this aspect should be thoroughly considered as it might influence risk assessment evaluation of chemical compounds.

The aim of the present study was to investigate if the presence of microplastics can influence the toxicokinetics, including the transfer of contaminants from feed to fish. Furthermore, this study also estimates if microplastics can influence the adverse effects of a mixture of commonly occurring halogenated contaminants in a commercially important seafood species. Specifically, it assesses if microplastics with sorbed contaminants are able to produce distinct or exacerbated effects compared with clean microplastics in the presence of chemical contaminants.

To reach this aim we performed a bioaccumulation study with European seabass (*Dicentrarchus labrax*) by feeding them with three contaminated feeds and one control. We then evaluated and compared toxicokinetic parameters, hepatosomatic indexes and the differential expression of selected genes involved in the detoxification process and in the immune response.

Materials and methods

Seabass trial

The experiment took place between March 2016 and August 2016 at The North Sea Science Center, Hirtshals, Denmark. The approval (no. 2014-15-2934-01041) of the experiment was given by the Animal Experiment Directorate, The Ministry for the Environment and Food of Denmark. The experiment consisted of a 80 days accumulation period, where European seabass were fed with one of the four diets described

below (three contaminated diets + one control) followed by an 51 days depuration period, where all fish were fed the control diet. Juvenile seabass were obtained in February 2016 from a seabass breeder (Ecloserie Marine de Gravelines Ichtus) in Graveline, France, and kept for four weeks in a large holding tank (2 m x 2 m, 4000L) under flow-through condition with seawater (33ppt) and heated by a heat exchanger to $\sim 20^{\circ}\text{C}$. They were fed to satiation, initially using commercial diets from Biomar A/S.

After four weeks, fish of similar size were transferred to the experimental facilities, which consisted of clear thermoplastic round tanks (diameter 0.81 m, water column height ~ 1.25 m, total water volume ~ 465 L) with the lower third being conical and separated from the rest of the tanks by a grid, allowing rapid sedimentation of faeces and uneaten feed pellets. Heated seawater was pumped to a storage tank, oxygenated with pure oxygen and pumped to the eight experimental tanks at a flow rate of about 110 L h^{-1} per tank. In addition, each tank was equipped with a filter unit to which the water within the tank was pumped at a flow rate of about 500 L h^{-1} , i.e. the water was continuously recirculated within each tank. It served primarily to aerate the water further and to create a current within the fish tank. From the tank outlet the water was led to the sewage system. Seabass were kept at an automated 15 h light/9 h dark cycle, where the light was switched between 7:00 to 7:30 h (using light dimmer) and between 22:00 to 22:30 h. Feeding was done by belt feeders for a period of ~ 4 hours per day, starting at 9:00 h. Uneaten feed pellets were collected and counted each day at the end of the feeding period. The temperature was monitored on a daily basis and showed an average temperature of $20.6 \pm 1.0^{\circ}\text{C}$ throughout the experiment. Oxygen saturation varied during the day due to an increased activity level during feeding hours, where saturation could decrease to about 60%, but increase again to about 90%. Upon transfer to the experimental tanks, fish were anaesthetised in benzocaine (BENZOAK® VET, 200 mg L^{-1} , diluted as 15 mL per 100 L water), sorted and those of similar size were pit-tagged with 12 mm RFID glass tags (Loligo systems, DK) and randomly distributed to the eight tanks.

A total of 482 fish were transferred, i.e. 60-61 fish per tank, around 5.3 kg per tank (mean ~ 88 g per fish). They were allowed to recover from passive integrator transponder (pit)-tagging and to acclimatize to the experimental tanks for two weeks, during which they were fed the experimental control diet. Pit-tagging did not cause any problems, i.e. no signs of infections, scale loss, or redness of skin. The two week acclimation period was also used to estimate feed conversion ratio ($\text{FCR} = \text{feed intake/biomass gain}$) in order to be able to feed at an appropriate level during the experimental period.

At the onset of trial, two tanks per diet were randomly selected among the eight tanks. All fish were individually weighed (mean 96.5 ± 17.5 g, $N=482$) and their length was measured (mean 19.5 ± 1.1 cm). Total biomass was used to calculate feeding level, which initially was set at 1% of the expected biomass

(based on estimated FCR), but quickly increased to 1.2% during the first growth period (20 days). From 20 days to 80 days the feeding level was increased further to 1.3% of expected biomass. The feeding level had to be adjusted during the depuration period, as some feed loss started to occur, likely linked to the increase in fish size and thereby lower growth rate and feed conversion efficiency. Consequently, the feeding level was gradually decreased during this period and reached 1% during the last 32 days of the trial. The FCR used from day 20 onwards) was based on average FCR for all tanks calculated for each growth period. The chosen feeding levels were just below satiation, and essentially fish ate all pellets every day during the accumulation period. As described, some feed loss occurred at the end of the depuration period, but as all feed loss was recorded and subtracted from the amount offered, accurate feed intake per tank could still be calculated.

Weighing/sampling

All fish were weighed at 0, 20, 40, 60 and 80 days during the accumulation period, and after 26 and 51 days of depuration. Fish removed for sampling were killed by an overdose anaesthetic (BENZOAK® VET, 200 mg L⁻¹, diluted ~ 1:1500 in water), whereas fish that were only weighed were anaesthetised at the recommended dose of Benzoak, allowing a fast recovery within few minutes. Fillets were sampled from weighed fish at the same sampling points and fish were removed from tanks, weighed and sampled after 3, 6, 12, 18, 25 and 51 days of depuration, i.e. the 80 days accumulation period included five sampling points, and the following 51 days depuration period included 6 sampling points. At the first sampling (time 0), one fish was removed per tank, weighed, length measured and two fillets were collected and kept on ice during sampling. The skin was carefully removed from the flesh, and one fillet per fish was individually bagged, whereas the other fillet (from eight specimens) was pooled (two samples of four fillets each) and all samples were frozen at -20°C. For the remaining sampling points, the same procedure was followed, except that four fish were removed per tank, being one fillet from each fish individually bagged and the other pooled in one bag, i.e. two pools and eight individuals per diet group. At each sampling point, control tanks were always sampled first in order to avoid any cross-contamination from the exposed groups. Liver and intestines were sampled for gene expression analyses after 40 and 80 days during accumulation and at the end of the depuration period (51 days). Small pieces of liver and intestine (mid-section) with 100-200 mg were sampled for gene expression by preservation in *RNAlater* (Sigma R0901). Samples were kept on ice during sampling and frozen at -20°C afterwards. The samples were transported in dry ice to DTU in Lyngby, Denmark and to AZTI in Bilbao, Spain.

The specific growth rate (SGR) of individual fish (sampled and non-sampled) was calculated as: $SGR = (\ln w_2 - \ln w_1) (t)^{-1}$, i.e. for each fish, the SGR was calculated for the accumulation period, as well as for the

depuration period. FCR was calculated for individual tanks and for each growth period as: $FCR = \text{feed intake} / \text{biomass gain}$. This was used to estimate individual feed intake of fish sampled from that tank, i.e. $\text{feed intake per fish} = FCR * \text{weight gain}$. This was done for each growth period during accumulation and the total feed intake for individual fish was calculated as the sum of feed intake for each period. The average intake of contaminated feed for each sampled fish could then be calculated as the total intake divided by the number of feeding days during accumulation. The same procedure was followed for the depuration period.

Feed preparation

The experimental feed (4.5 mm extruded pellets) was processed by the Danish Technological Institute, (Kolding, Denmark). The feed formulation used for all four experimental diets (see below) was relatively simple and consisted of a nutritionally optimal mix of fishmeal (67%), fish oil (17%) and wheat binder (17%) with a mix of vitamins and minerals (Premix Dk 3, Biomar A/S). The resulting proximate composition of the four diets were (mean \pm SD, N=2) $92.5 \pm 0.9\%$ dry matter, $44.8 \pm 0.4\%$ protein, $23.2 \pm 0.2\%$ oil and $13.3 \pm 0.9\%$ nitrogen free extract (by calculation).

Four experimental diets were included in the trial: ¹⁾a *control* feed containing only background contamination and three contaminated feeds of ²⁾ *contaminants sorbed to microplastics* before incorporation of 2% into pellets (see below); ³⁾ *contaminants without microplastics*, coated with oil onto feed pellets; and ⁴⁾ with *contaminants and clean microplastics*. More specifically, the contaminated feed were prepared either ²⁾with contaminants sorbed to plastic particles before incorporation of 2% into pellets(see below);³⁾ with contaminants coated together with oil onto plastic free feed pellets, or ⁴⁾ with contaminants coated in double concentration onto feed pellets which were ingested (1:1) together with control feed pellets containing 4% plastic particles, *i.e.* the plastic particles did not “encounter” contaminants until they reached the digestive tract of fish. Overnight sorption of contaminants to microplastic particles before addition to the feed attempted to simulate environmental conditions where contaminants are bound to the low density (LD)-PE micro plastic due to their high affinity, before they are ingested by fish. The sorption may not have reached equilibrium, when incorporated in the feed, but subsequent migration into the microplastic can continue during storage. Coating contaminants to the feed pellets was done by adding contaminants to the fish oil and spray coating the extruded pellets. Coating plastic particles with contaminants was done by solubilising those contaminants in ethanol, then mixing them with plastic particles overnight at ambient temperature to allow ethanol evaporating, whereby contaminants adhered or absorbed to the plastic particles. The particles were clean, LD-PE plastic particles (125-250 μm) used to mirror the composition of marine litter. Care was taken not to include too small

particles in the diet (<20-30 μm), as they could possibly induce histological changes, inflammatory responses or cross the digestive tract, neither to choose too large particle sizes that could obstruct the gut system. The LD-PE plastic particles were made from RIBLON 30, milled to plastic particles size < 400 μm , which were sieved to the size range of 125-250 μm . A “pre-wash” with octane and pentane was done in order to extract any solvent-soluble plastic monomers as well as to remove very fine attached plastic particles.

The three contaminated diets/diet mix had the same distribution of contaminant concentrations, i.e. α -HBCD at $\sim 27 \text{ ng g}^{-1}$ lipid weight (lw), 2,4,6-tribromophenol at $\sim 26 \text{ ng g}^{-1}$ lw, a technical mixture of polybrominated biphenyl ethers (PBDEs), DE71 (with congener composition as described by La Guardia et al., 2006), including PBDE 47, 99, 153, 154 at concentrations of $\sim 80, 130, 14$ and 10 ng g^{-1} lw, respectively; and PCB congeners 28, 52, 101, 118, 138, 153, 180 at concentrations of $\sim 33, 36, 43, 60, 140, 73, 8 \text{ ng g}^{-1}$ lw, respectively. All BFR and PCB chemicals used for the feed were solids. Furthermore the feed contained $\sim 50 \text{ ng g}^{-1}$ weight weight (ww) methyl mercury, and 5-10 ng g^{-1} ww of perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorosulphonamide (PFOSA) and perfluorononanoic acid (PFNA) (results not included in the present study). The concentrations of contaminants added to the feed are environmentally relevant, as described by Aznar-Alemany et al. (2017), Law et al. (2006, 2014) and Liu et al. (2011) for PBDEs; EFSA (2012) and Rüdél et al. (2011) for PFCs and Bełdowska and Falkowska (2016) for mercury.

Chemical analyses

Chemicals: acetone and methanol were from Rathburn (Walkerburn, UK); hexane, ethanol, acetic acid and concentrated sulphuric acid were from Merck (Darmstadt, Germany); anhydrous sodium sulphate from VWR (Radnor, US-PA); the water was from a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). The solutions α -, β -, γ -Hexabromocyclododecane ($50.0 \pm 2.5 \mu\text{g mL}^{-1}$) as well as $^{13}\text{C}_{12}$ - α -HBCD were from Wellington Laboratories (Ontario, Canada). Native and $^{13}\text{C}_{12}$ -labelled PCB and PBDE congeners were from Cambridge Isotopes Laboratories Inc. (Andover, MA, USA).

Sample preparation

When received at Technical University of Denmark, National Food Institute, each sample of four fillets was registered with an ID number. Then samples were homogenised in a blender while partially thawed. Subsamples of the homogenised materials were weighed for chemical analyses and kept at -20°C until chemical analyses.

Analyses: A 4 g homogenised fish or 1 g homogenised feed sample were mixed with 60 g anhydrous sodium sulphate and soxhlet extracted for six hours with 150 mL acetone hexane 1:1. The extract was evaporated using a Kuderna Danish evaporator until 10 mL remained of which a subsample of 2.5 mL was taken for lipid determination and 2.5 mL for PCB and PBDE analyses. The remaining 5 mL extract was further evaporated until only the lipid fraction remained and then dissolved in 2 mL ¹³C-labelled α -HBCD internal standard solution (1 ng mL⁻¹) in hexane and cleaned in 10 mL of concentrated sulphuric acid by turning by hand the centrifuge tube 20 times. After centrifuging, the cleaned hexane phase was further cleaned with 10 mL of milliQ water and evaporated using a gentle stream of nitrogen until reaching less than 300 μ L, added methanol and then further evaporated to a volume of 200 μ L and transferred to HPLC vials. The 2.5 mL extract for PCB and PBDE analyses was evaporated by a Kuderna Danish evaporator to remove the solvent, dissolved in 2 mL of hexane added ¹³C-labelled internal PCB (CB 28, 52, 101, 118, 138, 153, 180) and PBDE (BDE47, 99, 153, 154) standards of 0.75 ng mL⁻¹ and 0.55 ng mL⁻¹, respectively, and cleaned up with concentrated sulphuric acid and water as for the HBCD-analyses. The clear solvent layer was evaporated to 200 μ L using a gentle stream of nitrogen and transferred to GC-vials. The lipid content was determined by weighing after drying the 2.5 mL subsample at 70°C to constant weight.

Detection: The LC-MS/MS detection of α -HBCD was performed on an Agilent HPLC (Agilent Technologies, Santa Clara, CA, US) coupled with a Micromass Quattro Ultima tandem MS (Waters, Hertfordshire, UK). The separation of 15 μ L of the analytes was made on a Kinetex C18-HPLC column 2.6 μ m particle size, 100 mm x 2.1 mm (Phenomenex, Allerød, Danmark) with an Ultra HPLC in line filter using a gradient of 0.01% acetic acid (A) and methanol (B) at a flow of 0.2 mL min⁻¹: at 0-10 min. 50% B, 10-23 min. 95% B, 33 min. 50% B. The 'MS source' temperature was set to 120°C and the 'MS desolvation' temperature to 380°C. The collision energy was 35 eV and the argon collision gas pressure 2.3 e⁻³ mbar. The multiple reaction monitoring (MRM) traces were for α -HBCD $m/z > m/z$: 642.7 > 81.0 and for ¹³C₁₂- α -HBCD $m/z > m/z$: 653.6 > 81.0. Quantitation of samples was performed from calibration curves with concentrations 0, 0.25, 0.5, 1, 5, 10 ng mL⁻¹ α -HBCD. The GC-high resolution MS detection was performed on a Waters Autospec Premier magnetic sector instrument in electron impact ionisation mode (EI) and monitoring in selected ion recording (SIR) at 10,000 resolution. Two ion fragments were monitored for each of the analysed PCB and PBDE congeners from the chlorine and bromine isotope cluster, respectively. The MS conditions were ion source temperature 260°C, electron energy 40 eV, trap 650 μ A and GC/MS- interface temperature 270°C-280°C. The GC column was a DB-5MS (Agilent Technologies), 60 m, i.d.: 0.25 mm, film thickness: 0.25 μ m. The temperature programme was 90° C for 2 min; 20°C min⁻¹ to 180°C; 2°C min⁻¹ to 260°C; 5°C min⁻¹ to 320°C; 320°C kept for 10 min. Quantitation of samples was performed from calibration curves with

concentrations 2, 6, 16, 42 ng mL⁻¹ of PCB congeners 28, 52, 101, 118, 138, 153, 180 and 0.6, 4, 14, 40 ng mL⁻¹ of PBDE congeners 47, 99, 153, 154.

Quality of the chemical analyses: The laboratory of the National Food Institute is accredited according to ISO_17025. The institute is the National Reference Laboratory for PCB in food and feed and participates regularly in proficiency tests arranged by the European Reference Laboratory. The recoveries of PCB and PBDE spiked to a seabass sample in the analytical batch at 4 ng g⁻¹ were 93% to 101% for the six PCB congeners and 91% to 95% for the four PBDE congeners. The HBCD method No. 412.1 is accredited (DANAK, 2015). The method was proven satisfactory in the UNEP 'Biennial Global Interlaboratory Assessment on Persistent Organic Pollutants' IL2016-POP L208. The recoveries of the HBCD spiked to seabass samples at 4 ng g⁻¹ (~40 ng g⁻¹ lipid) run with the analytical batches were 102 ± 4 % (n=8). The standard deviations of double determinations of seabass samples in the range from 0.6-20.0 ng g⁻¹ lipid were 0.6 ± 0.6 ng g⁻¹ lipid (n=7). To keep analytical blanks low, glassware and sodium sulfate were heated, and soxhlet thimbles were cleaned by soxhlet extraction. Reagent blanks contained only CB28 at 0.2 ng g⁻¹ lw. Limit of quantification (LOQ)(~6 times S/N), estimated as 2·LOD (limit of detection ~ 3 times S/N) given by the MS masslynx software, corresponded in fish fillet to: CBs 0.2 ng g⁻¹lw , BDE47, BDE99 0.6 ng g⁻¹lw , BDE153 1.0 ng g⁻¹lw, BDE154 0.8 ng g⁻¹lw and α-HBCD 1.0 ng g⁻¹lw .

Modelling the toxicokinetics

The carryover of contaminants from feed to fish was modelled based on the assumption that the pathway for uptake of contaminants in fish is via the ingestion of contaminated feed (Bruggeman et al., 1981; Fisk et al., 1998). The high octanol-water partition coefficients and polyethylene-water partition coefficients of the same orders indicate that HBCD, PBDEs or PCBs sorb to feed or microplastic, and uptake via the aqueous phase is of minor importance (Lohmann, 2012; Wardrop et al., 2016).

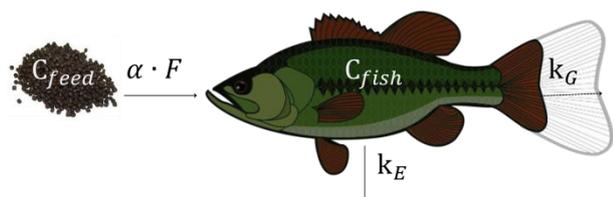


Fig. 1. Model of fish concentration (C_{fish}) as a function of assimilation(α), feeding rate (F), feed concentration (C_{feed}), elimination (k_E) and growth dilution (k_G).

The model for obtaining the toxicokinetic parameters: the elimination coefficient, the half-life and the assimilation efficiency include the pathways in Fig. 1, where the contaminant concentration in fish is described as a function of time:

$$\frac{dC_{fish}}{dt} = \alpha \cdot F \cdot C_{feed} - k_{el} \cdot C_{fish} \quad (1)$$

where C_{fish} is the contaminant concentration for each sample of four seabass fillets multiplied with a growth factor (GF) = (1 + SGR · t) to compensate for the growth dilution. The concentrations are calculated based on lipid weight of the individual samples; hence the mean feeding rates (F) of accumulation and elimination of each diet are also based on lipid weight. The elimination coefficient k_{el} is calculated by assuming first order kinetics and plotting the natural logarithm of the contaminant concentration versus time, with the slope equalling the elimination coefficient:

$$\ln C_{fish} = constant - k_{el} \cdot t \quad (2)$$

$$t_{1/2} = \ln 2 / k_{el} \quad (3)$$

The half-life ($t_{1/2}$) is derived from k_{el} (equation 3). When knowing k_{el} and C_{feed} , the assimilation efficiency (α) can be modelled from the regression line of the growth corrected concentration data of fish sampled during the accumulation period. This was performed in an iterative calculation using Excel Solver Tool. The assimilation efficiency (α) was fitted (iteratively modelled) using equation (4), so that the plot of the measured concentrations of C_{fish} during accumulation is best fitted to the corresponding modelled concentrations (to the smallest sum of the squared deviations between modelled and measured concentrations),

$$C_{fish} = (\alpha \cdot F \cdot C_{feed} / k_{el}) \cdot (1 - \exp^{-k_{el}t}) \Rightarrow \alpha = \frac{C_{fish} \cdot k_{el}}{F \cdot C_{feed} \cdot (1 - \exp^{-k_{el}t})} \quad (4)$$

The modelled α is based on concentrations of contaminants in the feed of the respective diets. To be comparable, C_{fish} data is normalized in the graphical presentation of the accumulation lines, relative to C_{feed} and F for feed without microplastics.

The control feed contained low concentrations of contaminants, which slightly influence the estimate of k_{el} , i.e. the slope of the linear elimination regression lines, where fish were fed the control feed.. This was adjusted by subtracting the concentration in fish derived from the control feed ($C_{fish, adj} = C_{fish} - \alpha \cdot F \cdot C_{control feed}$) and iterating a new $k_{el, adj}$ and then α based on equation (5,6):

$$\ln (C_{fish} - \alpha \cdot F \cdot C_{control feed}) = constant - k_{el, adj} \cdot t \quad (5)$$

$$C_{fish,adj} = (\alpha \cdot F \cdot C_{feed} / k_{el,adj}) \cdot (1 - \exp^{-k_{el,adj} \cdot t}) \Rightarrow \alpha = \frac{C_{fish} k_{el,adj}}{F \cdot C_{feed} \cdot (1 - \exp^{-k_{el,adj} \cdot t})} \quad (6)$$

Gene expression assays

Gene expression analysis was performed in samples of liver and intestine extracted from seabass at different time points. The time points were chosen to evaluate the effect at mid stage of bioaccumulation (t = 40 days), at the end of the bioaccumulation (t = 80 days) and at the end of the depuration stage (t depuration = 51 days). The following genes were tested: *cyp1a*, encoding for the cytochrome-P450-1A1 enzymes; *il1b* (interleukin β) and *gst α* (glutathione S transferase α), encoding for the glutathione s transferase enzyme. Gene expression of liver and intestine extracted from fish fed with the three contaminated feeds were compared with gene expression of liver and intestine extracted from fish fed with the control feed at each time point. In this way, we could test the effect of the feed contamination and not of the feed itself.

RNA from livers and intestines of exposed fish were extracted with TRIzol® (Sigma-Aldrich), following the instructions from the manufacturer. RNA concentration and quality was determined with a BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). Reverse transcription reactions were carried out on 20 ng of total RNA by the use of a TaqMan Reverse Transcriptase Reagents kit (Applied Biosystems, Carlsbad, CA, USA). Reaction conditions were as follows: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The corresponding cDNA was used as a template for qRT-PCR. Quantitative PCR was carried out with a LightCycler® 480 Real-Time PCR System (Roche, Mannheim, Germany). Three genes were tested and gene elongation factor 1 α (*ef1 α*) was used as a constitutive control to normalize all samples. The PCR primers used were designed in previous studies; specifically, *ef1 α* and interleukin 1 β (*il1b*) were taken from Mazurais et al. (2015), whereas *cyp1a1* and glutathion S transferase (*gst α*) were taken from Vannuccini et al. (2015). Each reaction was performed in a 10 μ L solution, containing 300 mM of each pair of primers, 5 μ L SYBR Green PCR master mix (Roche) and 4 μ L of the cDNA obtained in the previous step. Reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A final cycle at 95°C for 1 min and 65°C for 1 min, followed by 40°C for 30 s was performed. Expression levels of RNAs were calculated according to Livak and Schmittgen (2001). Gene expression data of liver and intestine were the result of four independent replicates (i.e. two samples x two tanks).

Statistics

The differences between diets of slopes of the linear elimination regressions representing k_{el} and those of the normalized linear accumulation regressions were tested using a statistical programme working together with excel (www.real-statistics.com/regression/hypothesis-testing-significance-regression-line-slope/comparing-slopes-two-independent-samples/theory; Paternoster et al., 1998). The t-test of slopes was performed using data of two samples of four fish fillets, and on the mean result of two samples of four fish fillets per sampling time. As curves for accumulation of the diet with contaminants sorbed to microplastics were all quadratic, and it was relevant to compare the difference in accumulation around day 40, at which point the diets with contaminants sorbed to microplastic showed the highest difference to the other diets, comparisons of the linear regressions up to 40 days accumulation were performed.

Paired t-tests of elimination coefficient and assimilation efficiencies between the three contaminated diets (i.e. data presented in table 1 and fig. 5a,c) were performed by Excel Analysis ToolPak. Linear regression analyses were also performed by Excel Analysis ToolPak. The confidence limit of the linear accumulation regression used for modelling α , was used as estimate for uncertainty of α , by determining the relative 95% confidence limit and convert to absolute uncertainty of α .

A one-way analyses of variance (SigmaPlot) was used to test differences in hepatosomatic index (HSI). The significance of expression levels of RNAs was measured using the Relative Expression Software Tool (REST) of Qiagen (Hilden, Germany; <http://www.REST.de.com>) based on the pair wise fixed reallocation randomization test (Pfaffl et al., 2002).

Results and Discussion

Growth, growth factors and feeding rates of seabass

No fish mortality was registered in the trial. The mean value (\pm standard deviation) of fish weight of the four different diets was at the start 98 ± 1 g and at the end of the trial 338 ± 28 g.

The mean growth factor ($GF = 1 + SGR \cdot t$) showed for all diets the same pattern increasing linearly during the accumulation period and afterwards with a smaller growth rate during the elimination period (supplementary Fig. S1). The growth factors revealed no differences between the diets and particularly up to day 40 they were very similar.

Furthermore, the fillet lipid contents were comparable between the four diets, increasing from 4.9 % at the start to 10.1 ± 0.8 % at the end of the trial (supplementary Fig. S2). The feeding rates (F) during accumulation were 0.0428, 0.0388 and 0.0417 g feed lipid g fish⁻¹ day⁻¹ for the feed with contaminants

sorbed to microplastics; contaminants and clean microplastics; and contaminants without microplastics, respectively.

Feed contaminant concentrations

The concentrations of α -HBCD, PBDEs and PCBs were at the same levels in the three contaminated feeds (Fig. 2). However, for modelling the assimilation efficiencies, the specific feed concentrations were used in the equations. The control feed contained a background level of contaminants, corresponding mostly to 10% of the concentrations of contaminants spiked to the contaminated feeds, except CB28 and CB180 \sim 20% and CB 138 \sim 3%. The lipid concentrations of feeds ranged from 19.6-20.4 %.

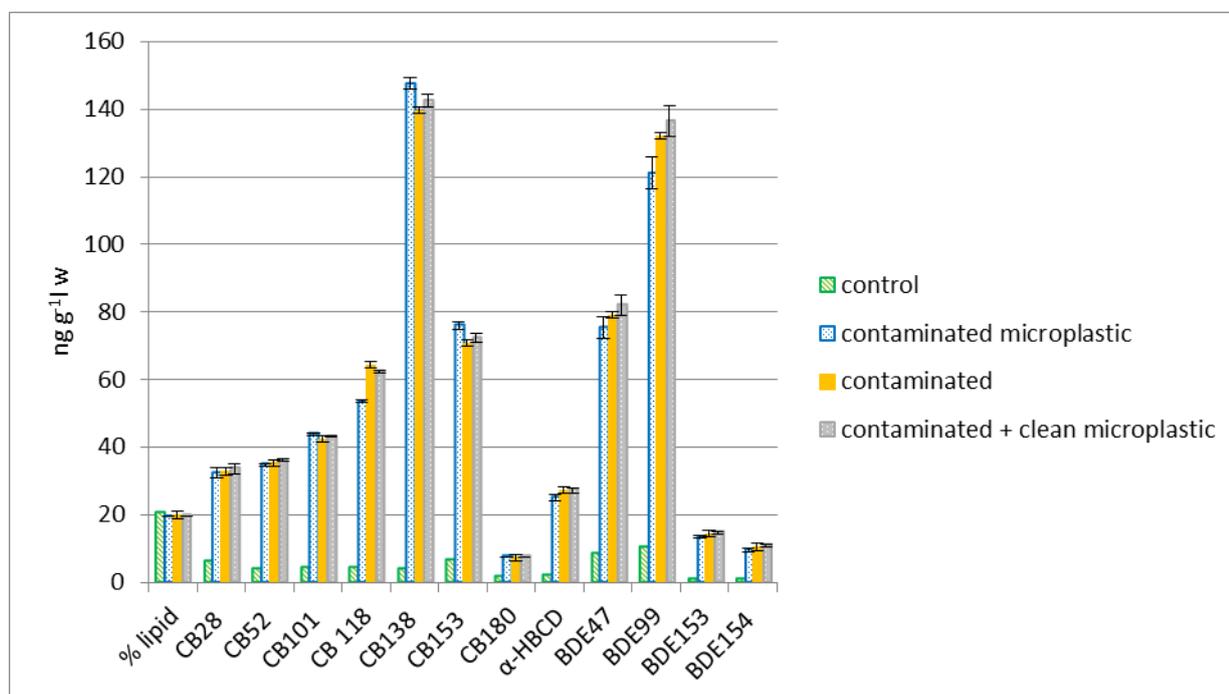
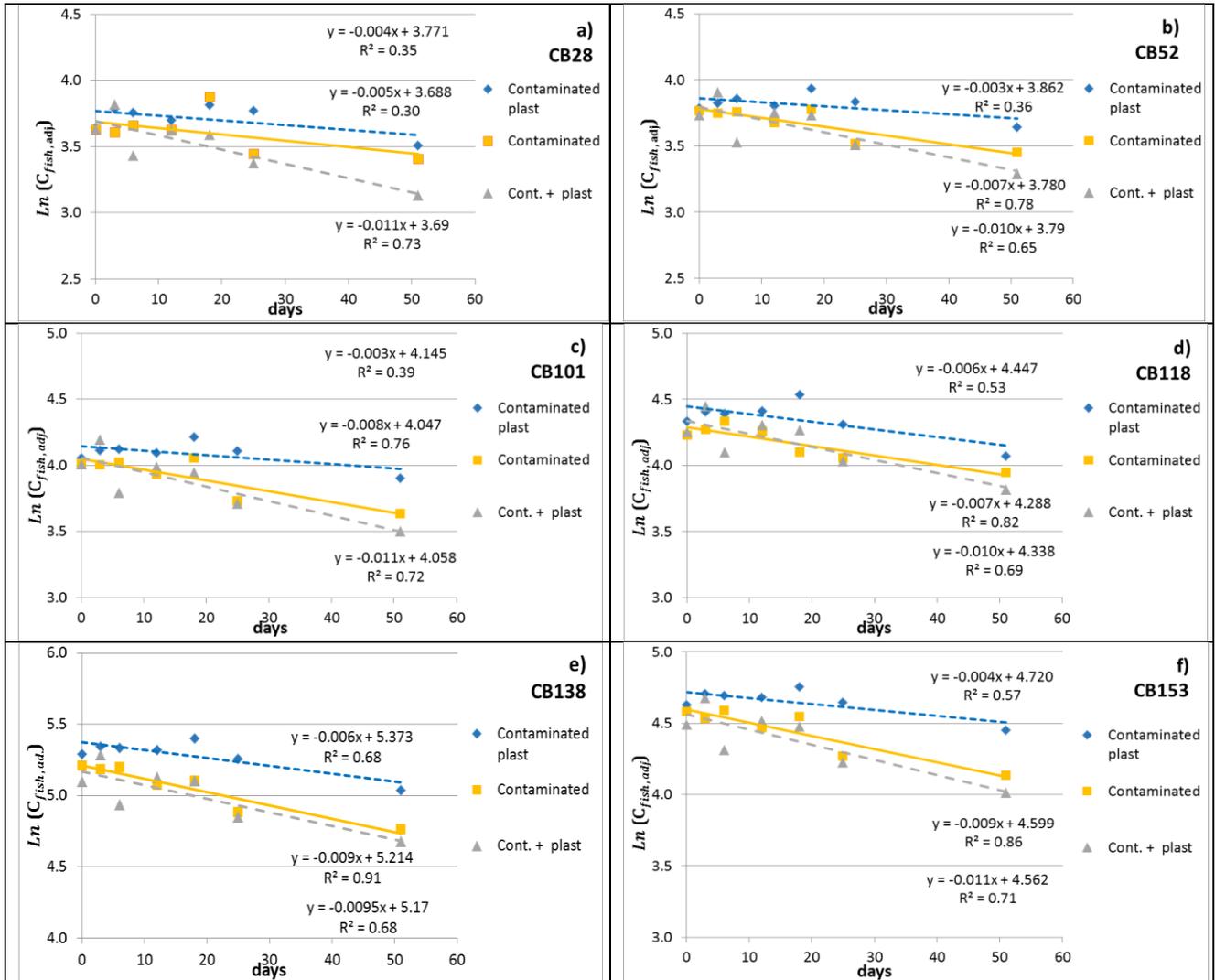


Fig. 2. Contents of lipid (%) and contaminants (ng g⁻¹ lipid) in control feed and in the three contaminated feeds with: *contaminants sorbed to microplastics; contaminants and clean microplastics and contaminants without microplastics.*

Seabass PCB and brominated flame retardant (BFR) elimination coefficients and assimilation efficiencies



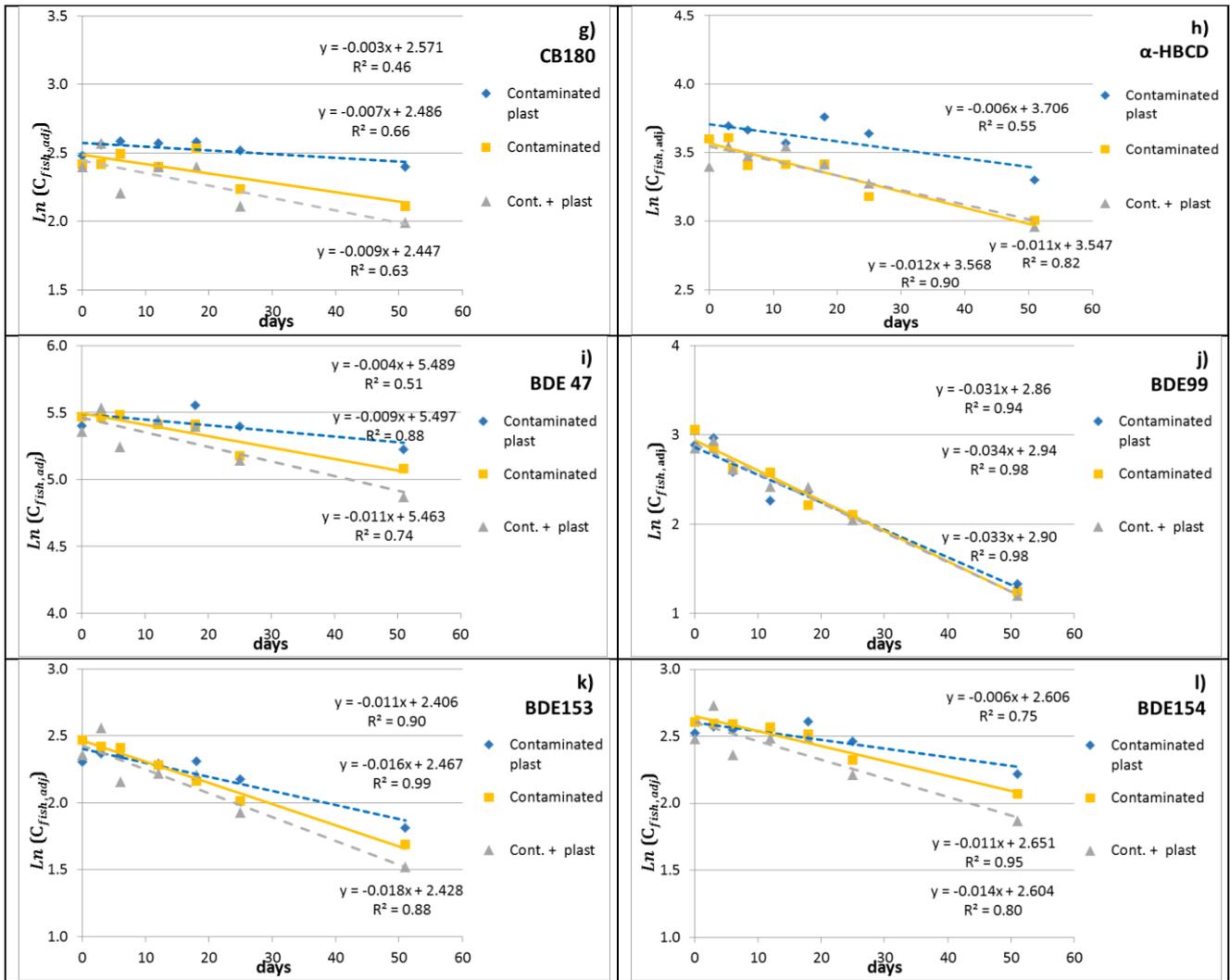
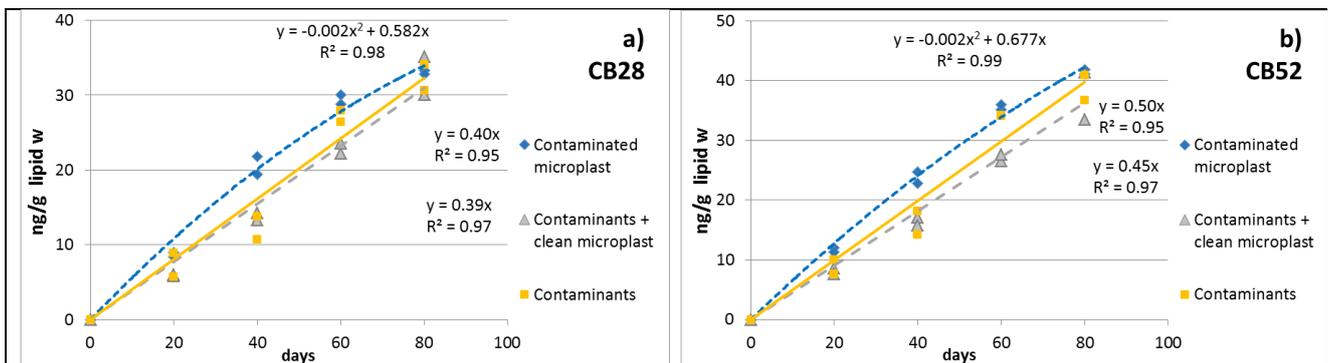
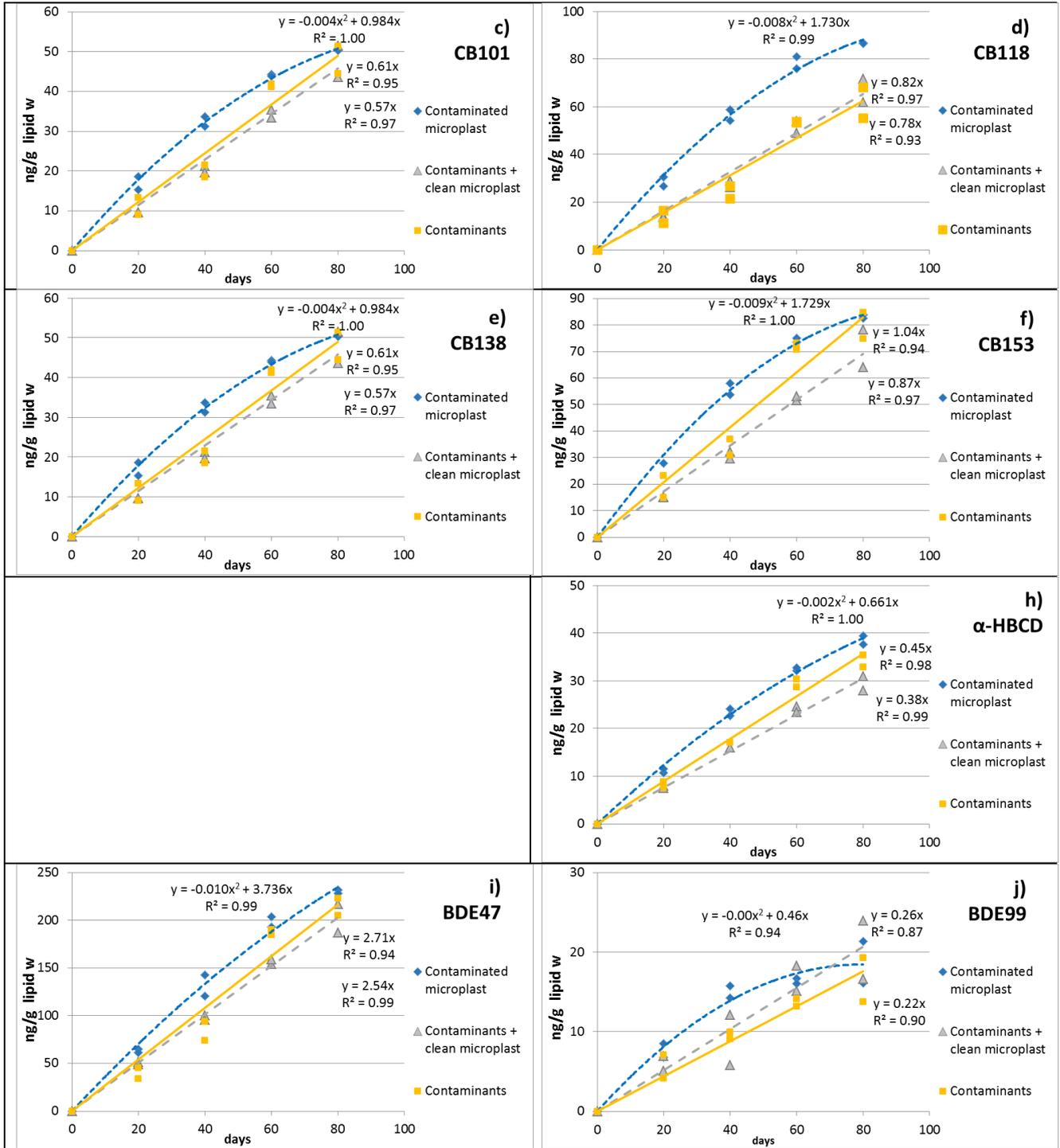


Fig. 3. Elimination regression lines (k_{el}), of $\ln(C_{fish\ adj})$ in ng g^{-1} lw versus time for seabass fillets of the three contaminated seabass diets (data points are means of two samples of four fillets).





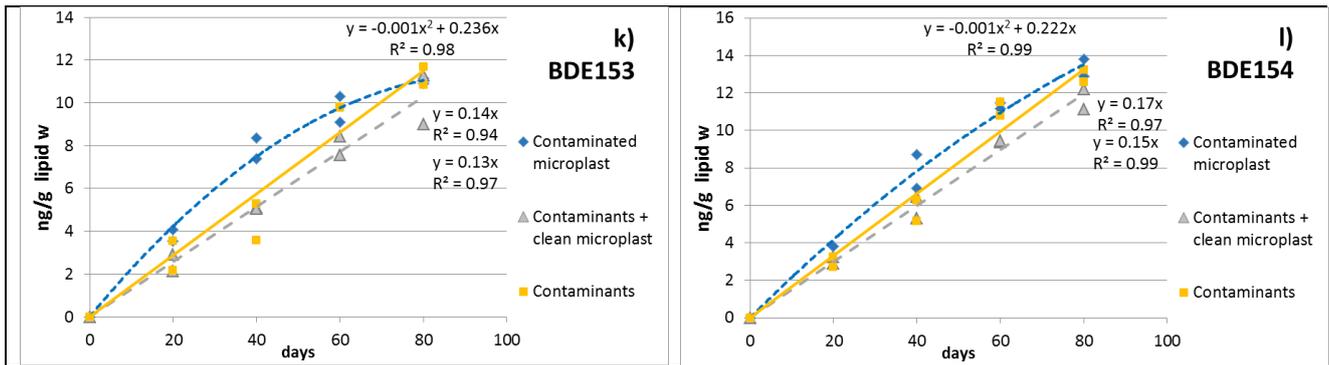


Fig. 4. Accumulation regression lines of the contaminants in pooled samples of four seabass fillets of the three contaminated feeds (data subtracted t_0 -concentration and normalized to C_{feed} and F relative to feed without microplastics).

Table 1. Results of assimilation efficiencies, elimination constants and half-lives of PCBs and BFRs in seabass fillets.

	Assimilation efficiency (a)			$k_{el}, \cdot 10^{-2} (d^{-1})$			$t_{1/2} (d)$		
	Contaminants sorbed to microplastics	Contaminants and clean microplastics	Contaminants without microplastics	Contaminants sorbed to microplastics	Contaminants and clean microplastics	Contaminants without microplastics	Contaminants sorbed to microplastics	Contaminants and clean microplastics	Contaminants without microplastics
α -HBCD	54 ± 4	54 ± 3	63 ± 5	0.62	1.06	1.17	112	65	59
PCB28	38 ± 3	40 ± 3	39 ± 5	0.36	1.08	0.48	194	64	145
PCB52	42 ± 3	41 ± 3	47 ± 5	0.30	0.95	0.67	230	73	104
PCB101	43 ± 4	45 ± 3	50 ± 5	0.34	1.10	0.81	204	63	85
PCB118	55 ± 6	42 ± 4	42 ± 5	0.59	0.98	0.72	118	70	97
PCB138	52 ± 6	42 ± 3	53 ± 6	0.55	0.95	0.94	126	73	74
PCB153	47 ± 6	39 ± 3	50 ± 6	0.42	1.06	0.93	166	65	75
PCB180	42 ± 14	33 ± 3	39 ± 6	0.27	0.91	0.68	258	76	101
BDE47+99	55 ± 3	43 ± 3	48 ± 6	0.70	1.18	0.96	98	59	72
BDE47	(107 ± 7)	(111 ± 6)	(121 ± 15)	0.42	1.10	0.86	166	63	81
BDE99	12 ± 3	12 ± 2	9 ± 1	3.07	3.32	3.40	23	21	20
BDE153	37 ± 5	35 ± 3	40 ± 5	1.06	1.78	1.59	66	39	44
BDE154	54 ± 4	49 ± 3	57 ± 5	0.65	1.38	1.12	107	50	62

The elimination coefficients (k_{el}) appear from the slope of the linear regression of $\ln C_{fish, adj}$ (adjusted for contribution from background feed according to equation 5) versus time of depuration (Fig. 3). For all individual contaminants except BDE99, i.e. seven PCBs and four BFRs, the elimination coefficients seem to have the lowest values for seabass fed the feed with *contaminants sorbed to microplastics*. Generally, for this feed seabass fillets also contained higher contaminant concentrations at the start of the depuration period. Seabass fed the feed with *contaminants and clean microplastics* showed the highest elimination

coefficients. Especially for the lowest congeners, the elimination was faster for this diet (Fig. 5a). Seabass fed the feed with *contaminants without microplastics* were intermediate and these data showed the best correlations of the linear regressions (Fig. 3). Statistical comparison between k_{el} of individual contaminants (slopes of the linear regressions) of the three contaminated feeds showed for CB28, CB101, CB180, BDE154 significant differences ($p < 0.05$) and for CB52, CB153, BDE47, BDE153 p values < 0.1 , between the diet with *contaminants sorbed to microplastics* and that with *contaminants and clean microplastics*. Furthermore BDE153 and BDE154 showed significant differences ($p < 0.05$) and CB138, CB153, BDE47 and α -HBCD p values < 0.1 between the diet with *contaminants sorbed to microplastics* and that with *contaminants without microplastics*.

The elimination coefficients of the seven PCBs (PCB 28, 52, 101, 118, 138, 153, 180) were significantly different between the three contaminated diets (tested by paired t-test), and ranged from $0.27-0.59 \cdot 10^{-2} \text{ d}^{-1}$ for seabass fed the feed with *contaminants sorbed to microplastics* compared with $0.48-0.94 \cdot 10^{-2} \text{ d}^{-1}$ for those fed the feed with *contaminants without microplastics* and $0.91-1.10 \cdot 10^{-2} \text{ d}^{-1}$ for seabass fed the feed with *contaminants and clean microplastics* (Table 1). This is evident from Fig. 5a showing k_{el} of PCBs as function of $\log K_{ow}$. Accordingly, the half-lives ($t_{1/2}$) of these diets had the reverse order, i.e. the diet with *contaminants sorbed to microplastics* had the greatest $t_{1/2}$ values and that of *contaminants with clean microplastics* showed the smallest $t_{1/2}$ values (Fig. 5b).

In the case of BFRs, BDE99 was quickly metabolised (Fig. 3j) as discussed later. However, the elimination coefficients of α -HBCD, BDE47, BDE153 and BDE154 from seabass fillets of fish fed with the feed containing *contaminants sorbed to microplastics* were significantly lower ($0.42-1.06 \cdot 10^{-2} \text{ d}^{-1}$) compared with those fed with the feed containing *contaminants and clean microplastics* ($1.06-1.78 \cdot 10^{-2} \text{ d}^{-1}$) and those with the feed containing *contaminants without microplastics* ($0.86-1.59 \cdot 10^{-2} \text{ d}^{-1}$). The lower elimination coefficients of both PCBs and BFRs from fish fed with the feed containing *contaminants sorbed to microplastics* were also found in a previous feeding trial of salmon, performed with exactly the same feeds but at different tank feeding facilities (Granby et al., submitted). In general, seabass had higher elimination coefficients compared with what was found for the same diets fed to salmon (Granby et al., submitted). This may indicate that seabass detoxification and/or excretion were more efficient. However, the difference may also reflect that the seabass study was performed at higher temperature ($\sim 20^\circ\text{C}$) compared with the salmon study ($< 10^\circ\text{C}$).

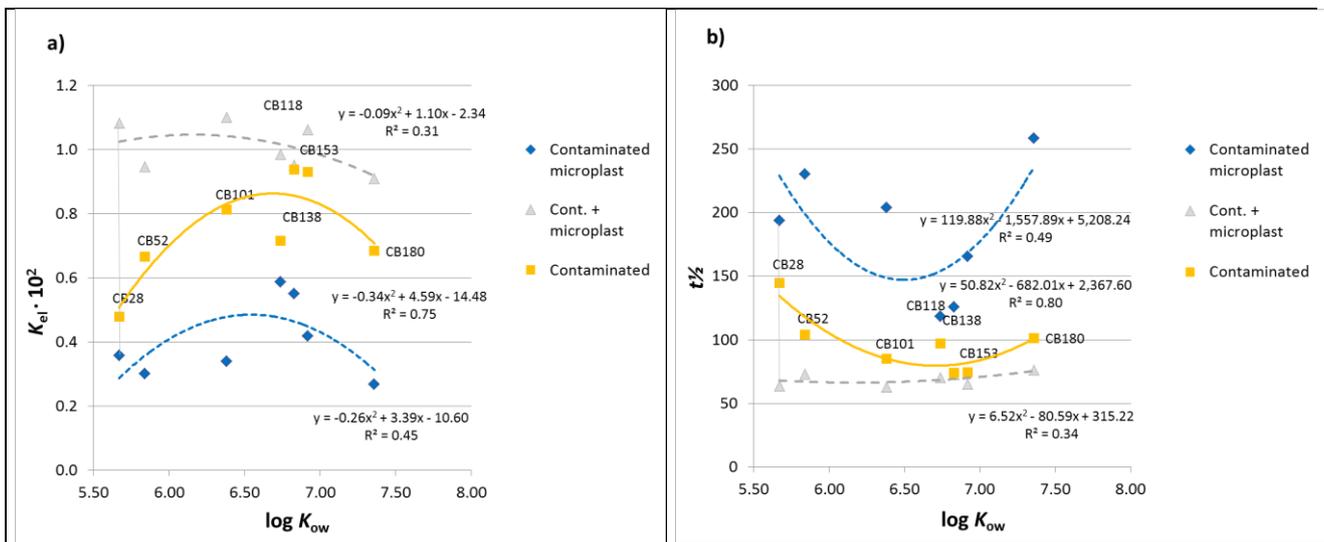
The growth corrected and normalized accumulation lines of the individual contaminants in seabass fillets of fish fed with the three contaminated feeds (Fig. 4) show that for all contaminants, the concentrations (C_{fish}) are generally higher for seabass fed with the feed containing *contaminants sorbed to microplastics*. A

characteristic for the accumulation lines of this diet is also that they better fit quadratic than linear curves. At the end of the accumulation period, these differences in C_{fish} between the fish fed with the feed containing *contaminants sorbed to microplastics* and the other feeds seem less pronounced. Due to the quadratic form of the diet with *contaminants sorbed to microplastics*, we tested differences between slopes of the linear regression lines of the three diets only using data up to day 40, where the relative differences to the other diets were highest, and the gene expression in liver was also affected, as it will be shown later. For all twelve contaminants, the slope of the linear regression (up to day 40) of the diet with *contaminants sorbed to microplastics* was significantly higher than that of the *contaminants without microplastics*; and for all contaminants except BDE99 and BDE154, the diet with *contaminants sorbed to microplastics* was also significantly higher than the diet with *contaminants and clean microplastics*. The higher values obtained for the diet with *contaminants sorbed to the microplastics*, might at least partly be explained by the lower elimination rates for that diet. Fish fed the feed with *contaminants and clean microplastics* showed accumulation lines more comparable with those fed with the feed containing *contaminants without microplastics*, both showing linear regression lines. It was our original assumption that contaminants that are sorbed to PE microplastic particles, for which they have very high affinity, would to some extent pass through the GI system along with the microplastic particles and thereby reduce bioaccessibility. However, from the results of the present study it is now apparent, that the presence of microplastic particles with sorbed contaminants rather increased bioaccessibility of contaminants. The higher accumulation of contaminants in fish fed with the feed containing *contaminants sorbed to microplastics* can be explained by a corresponding slower elimination of the chemicals. Additionally, it may also be that those contaminants which were sorbed to particles were transferred to the circulatory system from a more distal part of the intestine compared with the other diets where contaminants were added to the oil. The contaminants sorbed to or absorbed in the microplastic particles have to migrate from the microplastics into the intestinal fluid before they can then be transported across the intestinal epithelium, hence that process may be delayed and occur in the more distal intestine. Pedá et al. (2016) found histological alteration of the intestine of European seabass exposed to feed with 0.1% <300µm polyvinyl chloride (PVC) microplastic particles, especially in the distal intestine. As their study is indeed comparable with this study, it is possible that in the present seabass study the intestinal epithelium might also be affected and perhaps be more vulnerable to contaminants uptake from the intestine to the circulatory system. This situation may contribute to an increased accumulation of contaminants in fish fed with the diet with *contaminants sorbed to microplastics*.

The assimilation efficiencies (a) modelled from the slopes of the regression lines of the accumulation phase of growth corrected concentrations (C_{fish}) using diet specific feeding rates (F) and feed contaminant

concentrations (C_{feed}) are shown in Table 1. The assimilation efficiencies (α) of the seven PCBs were 38-55%, 39-46%, 39-53% for the feed containing *contaminants sorbed to microplastics*, the one containing *contaminants and clean microplastics* and the one containing *contaminants without microplastics*, respectively. Figure 5c shows the assimilation efficiencies (α) of PCBs from the three contaminated feeds to seabass fillets as function of $\log K_{ow}$. It appears from this figure that the curves are comparable to those of Fisk et al. (1998), who found a positive relationship of $\log \alpha$ (assimilation efficiency) with $\log K_{ow}$ up to $\log K_{ow} \sim 7$ for PCBs in rainbow trout, and then the polynomial curve is deflected. Fish fed with the feed containing *contaminants and clean microplastics* showed significantly lower assimilation efficiencies in comparison with fish fed with the other two contaminated feeds, which in turn did not show significant differences between them (paired t-test analysis). The reason for that may be, that the fish fed with the feed containing *contaminants and clean microplastics* showed faster elimination, hence PCB metabolism or excretion was higher, thereby reducing the assimilation efficiencies of contaminants from feed to fish fillet.

For the BFRs α -HBCD, BDE153 and BDE154 the assimilation efficiencies (α) were 37-54%, 35-54%, and 40-63% for the fish fed with the feed containing *contaminants sorbed to microplastics*, for those fed with the feed containing *contaminants and clean microplastics* and for those fed with the feed containing *contaminants without microplastics*, respectively. The paired t-test showed significantly lower α for BFRs in fish fed with the diet containing *contaminants and clean microplastics* compared with the fish fed with the feed containing *contaminants without microplastics*.



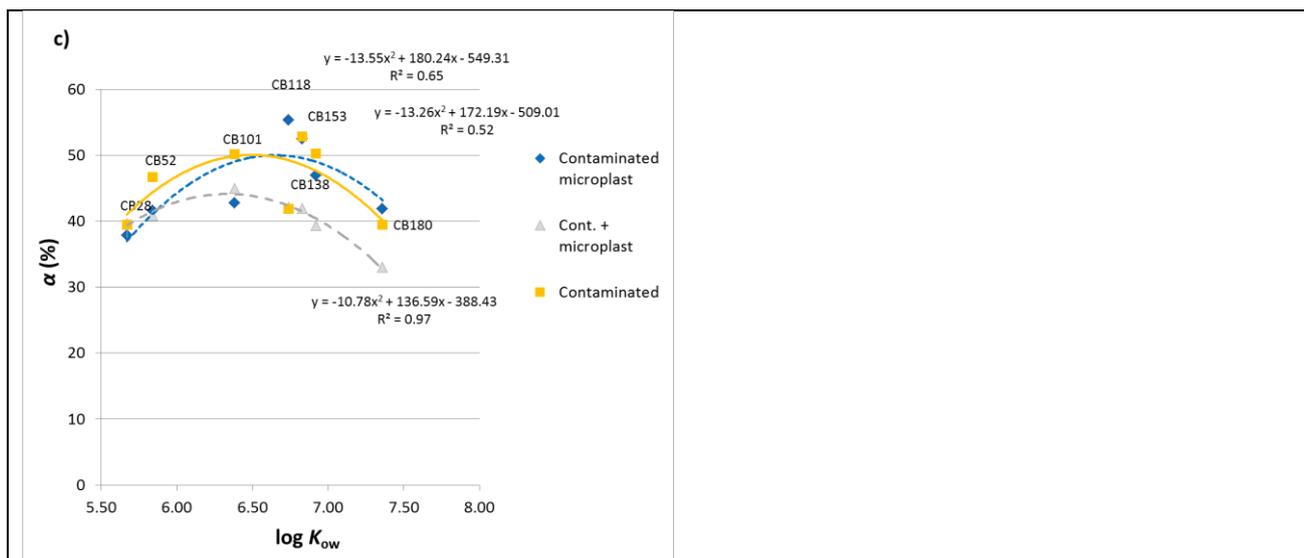


Fig. 5. ^{a)} Elimination coefficients (k_{el}); ^{b)} half-lives ($t_{1/2}$) and ^{c)} assimilation efficiencies (α) of PCBs in seabass fed with the three contaminated diets in relation to the octanol water partitioning coefficients ($\log K_{ow}$) (Hawker and Connell 1988; Li et al., 2008; Braekvelt et al., 2003)

Metabolism of BDE99 in the seabass in relation to microplastics in the diet

BDE47 and BDE99 were both present in contaminated feeds in relatively high concentrations of $\sim 80 \text{ ng g}^{-1}$ lipid and $\sim 130 \text{ ng g}^{-1}$ lipid, respectively. However, in seabass fillets, unlike other contaminants and different from a former salmon study (Granby et al., submitted), BDE99 did not accumulate as expected, only low BDE99 concentrations were detected in seabass fillets and the assimilation efficiencies were only 9-12%. It also appears from Fig. 3j, that BDE99 eliminates much faster than the other contaminants, i.e. the elimination coefficients of BDE99 were in the range between $3.07\text{-}3.40 \cdot 10^{-2} \text{ (d}^{-1}\text{)}$ and the half-lives in the range between 20-23 days in fish fed with the three contaminated diets (Table 1). On the other hand, the content of BDE47 in seabass fillets was approximately twice the expected concentration (Fig. 3i, Fig. 4i), implying that the calculated assimilation efficiency for BDE47 was 107%-121%. The reason for this result is that seabass metabolizes 2,2,4,4,5-pentabromodiphenyl ether (BDE99) by a reductive debromination to 2,2,4,4-tetrabromodiphenyl ether (BDE47), whereby a bromine atom is selectively removed (i.e. debrominated) from the meta-position of the diphenyl ring. Stapleton et al. (2004) observed this in a study with carp. However, to our knowledge the present study reveals for the first time this phenomenon in seabass. Roberts et al. (2011) suggested that in carp the debromination was due to thyroid hormone deiodinase activity and that also BDE153 debrominate to BDE47. In the present study, next to BDE47 the highest elimination coefficient was detected for BDE153 and the assimilation efficiency of BDE153 was

lower than that of the structural analogue BDE154 (Table 1), indicating that also in this case debromination may contribute to the metabolism.

The accumulation of BDE99 revealed the same behaviour as the other contaminants, with higher C_{fish} for seabass fed with the feed containing contaminants sorbed to microplastics, especially at 40 days accumulation (Fig. 4j). However, unlike other contaminants, the elimination of BDE99 from the seabass fillet was similar in fish fed with the three diets as well as fast: $3.3 \pm 0.1 \cdot 10^{-2} \text{ d}^{-1}$ (R^2 : 0.94-0.98) (Fig. 3j), indicating that during the elimination phase, the debromination, presumably by deiodinase, was not influenced by the presence of microplastics and not necessarily taking place in the liver, as deiodinase activity may also occur in muscles and plasma, for example (Benedict et al., 2007; Gabrielsen et al., 2015).

Hepatosomatic index of seabass

Fish uses liver for energy storage either as glycogen or fat, and in seabass, liver seems to be the main site of lipid storage (Dias et al., 1999). In the present study, the hepatosomatic index (HSI) increased over time and to the same extent in all diet groups, indicating that the nutritional status of fish was good (Fig. 6). However, at the end of the depuration period, the two groups exposed to microplastic particles during the accumulation period showed a significantly reduced hepatosomatic index compared with the *control* group and the group exposed to *contaminants without microplastics* ($p=0.015$). It appears that both groups, which until day 80 were exposed to feed containing microplastics mobilize the lipid/energy reserves in the liver leading to a decrease in liver size. Why this occurred only during the depuration period and not when fish were actually exposed to plastic remains unknown. However, it could possibly explain or contribute to the slower elimination of some contaminants observed in these diet groups, as it could be expected that lipid soluble contaminants stored in the liver would be released when the lipid was mobilized (La Merrill et al., 2013). The lipid contents of seabass fillets, which generally increased throughout the trial, were at the same level for all diets at the end of the trial (mean \pm SD between diets $10.1 \pm 0.8 \%$, supplementary Fig. S2). However, in the first 26 days of elimination lipid contents in seabass fillets of fish fed with the feed containing *contaminants sorbed to microplastics* during accumulation were lower than in the other diets. This could support the hypothesis that seabass exposed to microplastics during accumulation, mobilized energy reserves in the body.

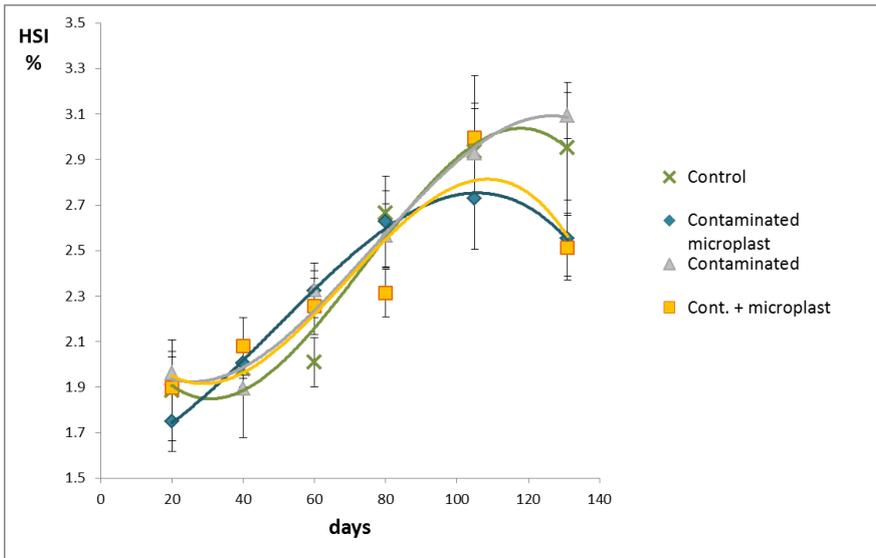


Fig. 6. The hepatosomatic indexes of seabass during the trial (mean \pm S.D. n=8 per diet)

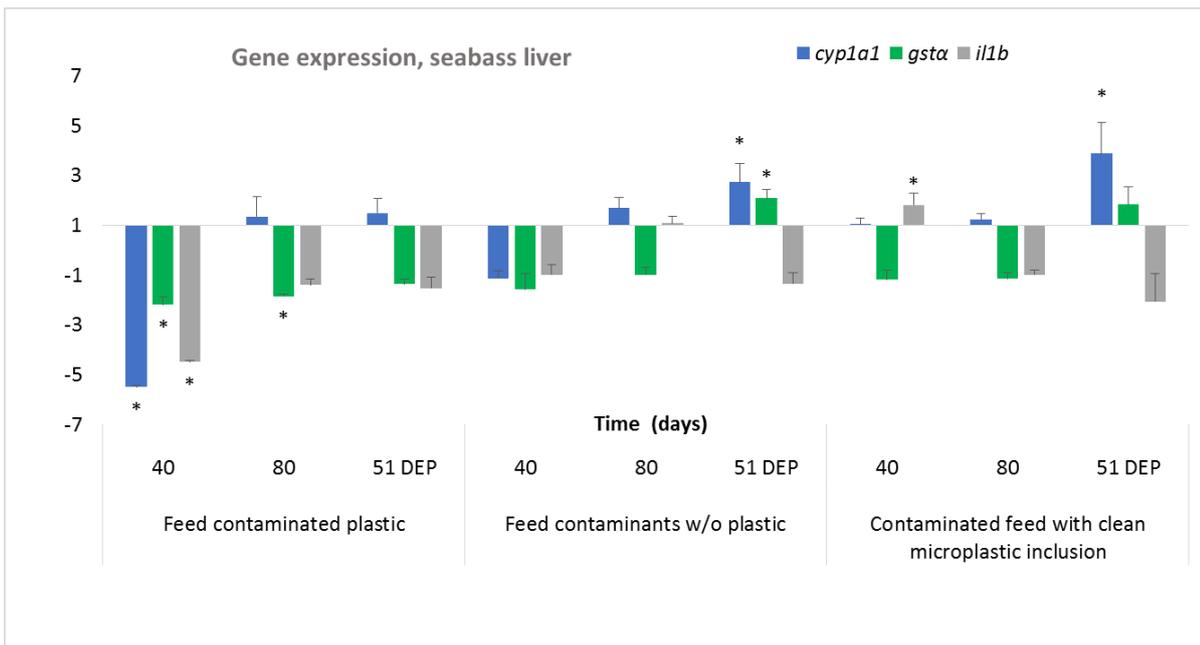


Fig.7. Differential gene expression of *cyp1a1*, *gsta* and *il1b* in livers of seabass fed with the three contaminated feeds after 40 and 80 days of accumulation and 51 days of depuration (DEP) (normalized with the housekeeping gene elongation factor 1- α (*ef1a*)). Bars represent means \pm SD from four replications. The asterisks represent significant differences compared with the expression of livers of fish fed with the control feed ($p < 0.05$).

Gene expression of seabass livers and intestines

Gene expression analysis was performed in liver (Fig. 7) and intestine samples from seabass. As expected, the most prominent results were detected in the liver. The feed producing the most relevant changes in gene expression was the one containing *contaminants sorbed to microplastics*. In this case, after 40 days of exposure, down-regulation of all tested genes was detected. The down-regulation of *cyp1a1* and *gsta* during the first phase of bioaccumulation indicates an inhibition of the detoxification process (Maradonna et al., 2014). Genes *cyp1a1* and *gsta* encode in fact for phase I (cytochrome-P450-1A1) and phase II (glutathione S transferase α) detoxification enzymes, respectively. Additionally, the down-regulation of both genes is an indicator of oxidative stress, and several studies confirm a role of reactive oxygen species in the down-regulation of at least some CYP isoforms (see Reynaud et al., 2008). Prolonged exposures to PCBs revealed a negative effect on CYP1 system by down-regulation of the *cyp1a1* gene, as demonstrated by Celander et al (1996) in rainbow trout (*Oncorhynchus mykiss*).

HBCDs exposure also produced an inhibitory effect on CYP1A enzyme activity in rainbow trout (Ronisz et al., 2004). In zebrafish livers, HBCDs produced the down-regulation of *cyp1a* after 7 and 21 days of exposure, proportionally to the contaminant concentration and exposure time (Du et al., 2015). Hong et al. (2014) tested the protein expression profile in marine medaka (*Oryzias melastigma*) exposed to HBCDs and detected the suppression of proteins involved in key cellular processes, such as proteolysis, transcription, translation, nucleotides and lipid metabolism, calcium binding and signaling, all leading to developmental toxicity.

Additionally, the down-regulation of *cyp1a1* has been reported in association with the presence of estrogenic compounds (Maradonna et al., 2014). Both PCBs and PBDEs, present at high concentration in contaminated plastics, possess estrogenic effects (Brower et al., 1999; Siddiqi et al., 2003) and can therefore be responsible for the detected effect. The down-regulation of glutathione S transferase genes can indicate the onset of oxidative stress as detected in zebrafish and rodents (Lu et al., 2017; Deng et al., 2017) and the down-regulation of *il1b* (interleukin β enzymes) triggering the alteration of a proper immune response (according to Ogryzko et al., 2014, *il1b* is involved in the immune response driving the early response to injury in several organisms including fish species). After 80 days of accumulation only the repression of *gsta* could be detected, and at the end of the depuration phase (51 DEP), no differential gene expression occurred.

Rochman et al. (2013b) also reported liver stress in histological sections of medaka (*Oryzias latipes*) exposed to microplastics contaminated with PCBs, PAHs and PBDEs. However, those authors did not find any effect at gene expression level. This discrepancy could be due to the fact that histopathological effects were detected after two months exposure, while in the present study gene expression effects were

detected at an earlier stage (40 days). Gene expression provides an early sign of toxicity that was probably not detected in Rochman et al. (2013b) study due to the later analysis of exposed fish. Indeed, gene expression after 80 days was hardly detected for all types of feeds tested in the current study. However, lower hepatosomatic indexes were found in fish fed with feeds containing microplastics. This may indicate that gene expression detected after 40 days of feeding with contaminated feeds anticipates the physiological effect that can be detected at a later stage.

The down-regulation of *cyp1a1* or *gsta* does not probably explain the debromination of PBDE99 to PBDE47 detected in this work, as this phenomenon seems to be due to the induction of deiodinase genes (Benedikt et al., 2007, Brown et al., 2009; Roberts et al., 2011). In this work, we did not test the differential expression of deiodinase genes. However, it is possible that their differential expression would be detectable towards the end of the accumulation phase rather than at the beginning.

Livers of fish fed the feed with *contaminants without microplastics* did not produce effects on genes tested during the accumulation phases. This occurred in spite of the fact that contaminants were the same and at the same concentrations as those added to the feed containing microplastics that produced the down-regulation of all tested genes. We speculate that microplastics can enhance the effect of the mixture of chemical contaminants added to the feed. This enhancing effect of microplastics on chemical contaminants was also detected in livers of zebrafish fed with the same feeds (Rainieri et al., 2018)) and in livers of medaka (*Oryzias latipes*) (Rochman et al. 2013b). A slight induction of *cyp1a1* and *il1b* was detected at the end of the depuration phase in fish fed with the feed containing *contaminants without microplastics*.

The feed containing *contaminants and clean microplastics* slightly induced *il1b* after 40 days of exposure and the induction of *cyp1a1* at the depuration phase. This indicates that the way contaminants enter in contact with fish can greatly affect their adverse outcome and that clean microplastics does not significantly influence the effect of chemical contaminants. PFOS and PFOA have shown to induce the cytochrome P-450 detoxification system, as PFOS induce the *cyp1a1*, but PFOA induce another mechanism (Manzetti et al., 2014; Lui et al., 2009). In the present study PFOS and PFOA were added to the feed, but relatively low concentrations were detected in seabass fillets, the highest being PFOS at <3 ng g⁻¹ ww. A significant inhibition of *cyp1a1* was observed for the diet with *contaminants sorbed to microplastics* after 40 days accumulation. However, for the other two diets, significant induction of *cyp1a1* was seen at the end of depuration, for which PFOS may also contribute.

Gene expression in the intestine was not affected after 40 days, with the exception of a slight repression of *gsta* in fish intestine fed with feed containing *contaminants sorbed to microplastics*. The repression of this gene was also detected after 80 days of feeding fish with this feed. At the depuration stage, *gsta* was

induced in fish fed with feed containing *contaminants sorbed to microplastics* and being slightly repressed in fish fed with feed containing *contaminants and clean microplastics* (data not shown). These results indicate the onset of oxidative stress in fish fed with *contaminants sorbed to microplastics* in the accumulation phase and the activation of a response against this stressor in the depuration stage, confirmed by the induction of *gstα*. Pedá et al. (2016) reported histological alterations of European seabass intestine exposed to contaminated PVC pellets, indicating that contaminated microplastics can lead to physiological effects also in the intestine. In the present work, histopathological analysis was not performed, but it cannot be excluded that histological alterations of seabass intestine occurred due to oxidative stress produced during feeding with contaminated microplastics. The presence of PFOA and PFOS may also have contributed to such oxidative stress (Liu et al., 2007).

Gene expression results indicate that microplastics might indeed potentiate the toxic effect of some chemical contaminants as also confirmed in a preliminary study performed with zebrafish (Rainieri et al., 2018).

Altogether, our gene expression results confirm that in fish PCBs and BFRs sorbed to microplastics cause an alteration of the detoxification process and the set in of oxidative stress at the earliest stages of exposure. With time, such effects revert to normality. These results are in agreement with the bioaccumulation results. Significantly lower elimination coefficients of PCBs and PBDEs were in fact obtained in fillets of fish fed with the feed containing *contaminants sorbed to microplastics* compared with the other feeds. The slower elimination of the seven PCBs, the four PBDEs and α -HBCD can be explained by an inhibition of the detoxification process demonstrated by the down-regulation of genes encoding for cytochrome-P450-1A1 and glutathione S transferase enzymes detected in livers of seabass fed the diet with *contaminants sorbed to microplastics*. This down-regulation, especially after 40 days of accumulation, supports a slower metabolism and thereby higher concentrations of PCBs and BFRs found in sea bass at that stage of accumulation. As it appears from the accumulation lines of figure 4, the shapes of regression lines for the diet with contaminants sorbed to microplastics are quadratic, while the other diets are linear.

Conclusion

Conclusion

To the authors knowledge this is the first study showing the influence of microplastic particles in feed on the toxicokinetics of halogenated contaminants in fish. Gene expression data supported the results obtained.

Feed containing *contaminants sorbed to microplastics* produced the greatest effects on seabass toxicokinetics. Seabass fed with this feed showed a quadratic accumulation of all the 12 contaminants sorbed to microplastic with significantly higher concentrations up to day 40 compared with seabass fed with the other feeds. At the end of the accumulation period no differences were detected among fish fed with the different feeds. In other words, microplastics increased the bioavailability of sorbed contaminants until the end of the accumulation period. This accumulation was supported by gene expression results in seabass liver, showing significant down-regulations of phase I and phase II detoxification enzymes (*cyp1a1* and *gsta*) and interleukin β (*il1b*), involved in the immune response driving the early response to injuries at 40 days accumulation. Such down-regulation was neither observed for the diet with *contaminants and clean microplastics* nor for the diet of *contaminants without microplastics*.

For the first time, this work showed that the metabolism of BDE99 to BDE47 in seabass is rather fast and the elimination of BDE99 from seabass fillets was unaffected by the presence of microplastics.

For the other contaminants, the presence of clean microplastic particles in contaminated feed increased the elimination coefficient from seabass fillets, while the presence of the same microplastic particles with sorbed contaminants decreased the elimination coefficient compared with the feed containing only contaminants.

Gene expression data confirm this finding. In fact, during depuration, the detoxification enzymes (*cyp1a1* and *gsta*) were up-regulated in fish fed with the feed containing *contaminants and clean microplastics* and in those fed with the feed containing *contaminants without microplastics*. This up-regulation may partly explain the faster elimination for those two diets compared with the diet with *contaminants sorbed to microplastics*.

Overall, the results indicate that microplastics influence contaminants' bioavailability, inhibit or induce detoxification in the liver, influence lipid distribution and also affect contaminant concentrations. Gene expression results indicate that microplastics might indeed potentiate the adverse effect of some chemical contaminants.

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SUPPLEMENTARY

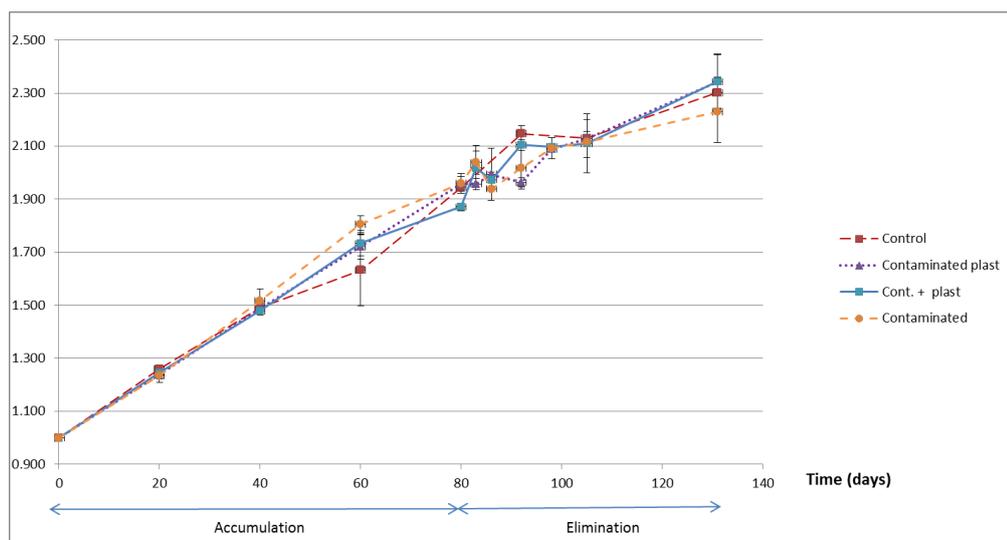


Fig. S1 . The growth factor ($GF = 1 + SGR \cdot t$) mean per seabass diet (mean of two samples of four fish fillets), where SGR is the specific growth rate per day in relation to the initial weight.

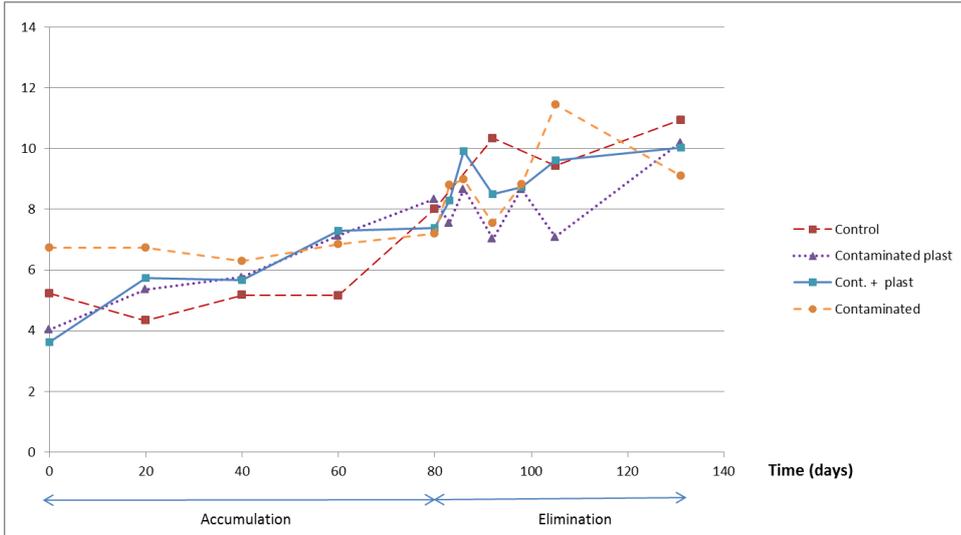


Fig. S2. The mean lipid contents (%) per diet in seabass fillets sampled during the trial