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Effects of Plastic Additives on Precision Cut Liver Slices (PCLS) from Atlantic cod (*Gadus morhua*)

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Environment and natural resources

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Hilde Andersen

ABSTRACT

A vast amount of the plastic we use end up in the ocean and estimates suggest a doubling of plastic in the ocean, to 250 million tonnes by 2025. Plastic contain additives such as phthalates, and these are known to have adverse health effects on living organisms. The additives may leach from products into the environment. Fish liver is a target organ for contaminants, due to its crucial role in biological functions such as metabolic homeostasis and detoxification processes, and precision-cut liver slices (PCLS) is a promising ex vivo system that is utilized within toxicology, using slices of complete liver tissue (Eide et al. 2014). The overall aim of the present study was to determine if exposure to plastic additives, such as phthalates, bisphenol A (BPA) and benzotriazoles (BT), have the potential to promote adverse effects in Atlantic cod (*G. morhua*). PCLS from six male juvenile Atlantic cod were exposed to 4 concentrations of mono-(2-ethylhexyl)-phthalate (MEHP), BPA and BT both singly and in mixtures ranging from 0.1-100 µM (MEHP), 0.022-22 µM (BPA) and 0.042-42 µM (BT). Histology and transmission electron microscopy (TEM) were used to assess pathological changes and ultrastructure of the exposed liver tissue. Vitellogenin produced by the hepatic tissue were analysed using ELISA, and the transcription levels of selected biomarker genes (*vtg1, esr1, cyp1a, scdb, aclya, fabp1a, acox1, hnf4a and cebp*) were measured using Q-PCR. A satisfactory assessment of the ultrastructure was not possible due to incomplete fixation of the PCLS with resulting lack of membranes and organelle structure. Histological evaluation did not show any pathological changes. An estrogenic effect was observed with a significant increase in vtg protein synthesis and upregulation of the *vtg1* and *esr1* genes following exposure to BPA and a mixture of the selected compounds. *Hnf4a* showed a significant downregulation following mixture exposure, where the BPA were suspected to be the main driver for this response however not inducing a significant downregulation in the single component exposure. A possible antagonistic mixture effect of the selected compounds might be questioned regarding the vtg protein, *vtg1* and *esr1* as well as a possible additive or synergistic effect regarding the *hnf4a,* and further investigation is warranted.

SAMMENDRAG

Effekten av plasttilsetningsstoffer på finkuttede leversnitt (PCLS) fra Atlantisk torsk (G. morhua).

En betydelig mengde av plasten som brukes i dag ender opp i havene, og det anslås at vi innen 2025 kan komme til å se en dobling av denne mengden til 250 millioner tonn. Plast inneholder tilsetningsstoffer som for eksempel ftalater, som har vist seg å ha negative helseeffekter på levende organismer. Tilsetningsstoffene som brukes i plast kan lekke fra produktene og ut i miljøet. På grunn av sin avgjørende rolle i biologiske prosesser som metabolisme og avgiftning, er fiskelever et målorgan for forurensende stoffer. Dette gjør *ex vivo*-systemet med finkuttede intakte leversnitt spesielt lovende innen toksikologi (Eide et al. 2014). Det overordnede målet med dette studiet var å bestemme om eksponering for plastadditiver som ftalater, bisfenol A (BPA) og benzotriazoler (BT) har potensiale til å utløse uønskede helseeffekter hos Atlantisk torsk (*G. morhua*). For å undersøke dette, ble finkuttede leversnitt fra seks hanntorsk eksponert for 4 ulike konsentrasjoner av mono-(2-ethylhexyl)-ftalat (MEHP), BPA og BT både som enkelt-eksponering og i mikstur i konsentrasjoner på 0.1-100 µM (MEHP), 0.022-22 µM (BPA) og 0.042-42 µM (BT). Histologi og transmisjons elektronmikroskopi ble benyttet for å vurdere eventuelle patologiske forandringer og ultrastrukturen til det eksponerte levervevet. Vitellogeninprotein produsert i levervevet ble analysert med ELISA, og genekspresjonen av enkelte utvalgte biomarkør-gener (*vtg1, esr1, cyp1a, scdb, aclya, fabp1a, acox1, hnf4a and cebp*) ble målt ved hjelp av Q-PCR. På grunn av ufullstendig fiksering med resulterende tap av membraner og organell-struktur, var det ikke mulig å vurdere leversnittenes ultrastruktur tilfredsstillende. Ved histologisk vurdering ble det ikke funnet patologiske forandringer i levervevet. I leversnittene eksponert for BPA og mikstur, ble det funnet en østrogeneffekt med signifikant økning i vitellogenin-produksjonen og en oppregulering av *vtg1* og *esr1* i leversnittene. Genekspresjonen av *hnf4a* ble signifikant nedregulert som følge av mikstur-eksponeringen. Til tross for at BPA kun grenset mot å gi en signifikant nedregulering av dette genet i enkelt-eksponeringen, antas det at BPA var den viktigste driveren, Det ses en tendens til antagonistisk effekt av stoffene i miksturen når det gjelder vitellogenin-produksjonen og ekspresjonen av *vtg1* og *esr1*, i tillegg til en mulig additiv eller synergistisk effekt på ekspresjonen av *hnf4a*. Videre undersøkelser av den samlede effekten av stoffene i mikstur anbefales.

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AOP	Adverse Outcome Pathway
AR	Androgen receptor
BBP	Benzyl butyl phthalate
BPA	Bisphenol A
BT	Benzotriazole
(CCl_4)	Carbon tetrachloride
Cd	Cadmium
cDNA	Complementary DNA
Cq	Quantitation cycle
DBP	Dibutyl phthalate
DEHP	Diethylhexyl phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DMSO	Dimethyl Sulfoxide
E2	Estradiol
EAS	Estrogen, androgen and steroidogenesis
ED	Endocrine disruptor
EDCs	Endocrine disrupting chemicals
EE2	17α-ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FHM	Fathead Minnows

GDB	Glycol Dibenzoate
ILAB	Industrial and Aquatic Research Laboratory
K_{ow}	Octanol water partition coefficient
LDH	Lactate dehydrogenase
MEHP	Monoethylhexyl phthalic acid
µM	Micromolar
MOA	Mode of Action
NMBU	Norwegian University of Life Sciences
NMDR	Non-monotonic dose response
NSB	Non-Specific Binding
PPAR	Peroxisome proliferator activated receptor
PAEs	Phthalate esters
PCLS	Precision cut liver slices
PCR	Polymerase chain reaction
PFOA	Perfluorooctanoic acid
POPs	Persistent Organic Pollutants
PVC	Polyvinyl chloride
Q-PCR	Quantitative real-time polymerase chain reaction
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEM	Transmission Electron Microscopy
VTG	Vitellogenin
WHO	World health Organization

1. INTRODUCTION

1.1. Plastic pollution

The oceans are increasingly challenged by human activities. Since 1950s, almost 6 billion tonnes of fish and other seafood have been retrieved from the world oceans (Steffen et al., 2015). The marine environment is continuously influenced by fishery, aquaculture, shipping, oil and gas activity, industry, agriculture and draining. In addition, water- and air currents transport pollutants over long distances.

An important contribution to the marine pollution is the soaring amount of plastic both from land-based sources, including via rivers, and from ocean-based sources such as fisheries, aquaculture and commercial cruise or private ships. On a global scale, the quantities of plastics leaking to the oceans are largely unknown, but it is suggested that the ocean may already contain over 150 million tonnes of plastic (Conservancy, 2015). Additionally, it is estimated that the global quantity of plastic in the ocean might double to 250 million tonnes by 2025 (Jambeck et al., 2015) (Fig. 1). This probably also represents a pollutant load of millions of tonnes of potential harmful chemicals added to plastic (Gallo et al., 2018).

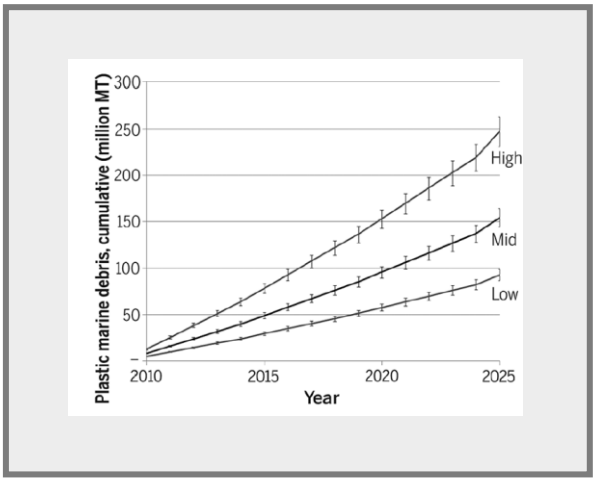


Fig. 1. Estimated mass of mismanaged plastic waste (millions of metric tons) input to the ocean by populations living within 50 km of a coast in 192 countries, plotted as a cumulative sum from 2010 to 2025. Estimates reflect assumed conversion rates of mismanaged plastic waste to marine debris (high, 40%; mid, 25%; low, 15%). Error bars were generated using mean and standard error from the predictive models for mismanaged waste fraction and percent plastic in the waste stream (Jambeck et al., 2015).

Plastics have transformed everyday life, and undoubtedly brings many economical and practical benefits. It is light, easily shaped, strong, and inexpensive. Without using plastic food packaging, the shelf life of most fresh foods would decrease considerably, and food waste

would be an even bigger problem. However, there are big concerns regarding usage and disposal of plastics. The durability of plastic that makes it such an attractive material to use also makes it highly resistant to degradation, thus disposing of plastic waste is problematic (Barnes et al., 2009; Sivan, 2011). We are continuously learning more about physical hazards for wildlife resulting from ingestion or entanglement in plastic, but also about the "cocktail of chemicals", which are released from plastic products to the environment representing a potential toxicological hazard to animal and human health. This includes both chemicals accumulated to the plastic debris from surrounding marine environment, and the chemicals added or produced during manufacturing (Lithner et al., 2011). During manufacturing, polymerization reactions often is incomplete, and unpolymerized residual monomers can drift off the plastic (Lithner et al., 2011). The additives are usually not covalently bound to the plastics, and these chemicals might leach into the environment (Engler, 2012; Lithner et al., 2011). Release of hazardous substances, like phthalates and bisphenol A (BPA), from plastic products have been shown (Crain et al., 2007; Lithner et al., 2012). Studies have also assessed the toxicity of the mixture of chemical ingredients in leachates. It was demonstrated that leached chemicals from plasticized PVC and epoxy products were acutely toxic to *D. magma* (Lithner et al., 2012), and exposure of Japanese medaka (*Oryzias latipes*) to a mixture of polyethylene with chemical pollutants sorbed from the marine environment resulted in bioaccumulation of the pollutants in the fish. The authors concluded that liver toxicity and pathology was induced by both the sorbed contaminants and the plastic materials (Rochman et al., 2013). Because of the vast evidence that both macro (whales) and microplastics as well as chemical plastic additives may pose a significant health hazard to the environment and humans, there is an urgent need to reduce the amount of plastic emissions. However, completely ceasing the use of plastic is unrealistic and not feasible. The use of plastics is not unconditionally a threat, but rather the emissions during production and use, in addition to the disposal. Focus on recirculation and preventing emissions should be supplied with increased knowledge on the occurrence of adverse effects related to the different types of plastic components. This knowledge is necessary for the manufacturers and policy makers to make qualified assessments regarding this issue.

An important shortage in our knowledge is the gaps in our understanding of the cocktail of chemicals. Exposure to mixtures can have additive, synergistic or antagonistic effects as a result of different types of chemical interactions, and these interactions are typically overlooked when each chemical's safety-levels are being defined. Therefore, there is a risk for unforeseen adverse health effects with emerging new chemicals (Celander, 2011). Some studies have been

conducted on mixture effects of certain plastic compounds. A study on Japanese medaka (*Oryzias latipes*) showed early-warning signs of endocrine disruption in fish exposed to a mixture of plastic and sorbed contaminants (Rochman et al., 2014). Christen et al. (2012) demonstrated that the antiandrogenic activities of phthalate mixtures and BPA displayed additive interactions, and Li et al. (2017) found that the combined toxicity of BPA, dibutyl phthalate (DBP) and cadmium enhanced cytotoxicity, oxidative stress and genotoxicity compared to the mono-exposures in HepG 2 cells.

1.2. Atlantic cod as an indicator

Management plans are developed to mitigate overload of the marine ecosystems, both in the Norwegian marine management and through international agreements like The Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR). An important contribution to this management is the surveillance of the coastal areas. One of the indicator species that has been utilized in surveillance for several years, is the Atlantic cod (*Gadus morhua)*. The Atlantic cod is economically, ecologically and culturally important to the Norwegian fjords and coastal areas. In addition, Atlantic cod is suitable for such purposes due to their habitation in areas covered by the management plans (van der Meeren et al., 2015) where high levels of pollutants also are known to exist. Due to their bottom dwelling and their opportunistic feed behaviour, the Atlantic cod are known to ingest a wide selection of prey (Hansen et al., 2016). This makes them disposed to ingesting anthropogenic matter from both pelagic and benthic habitats. Microplastic is widespread and found in both pelagic and benthic environments (reviewed in e.g. Cole et al. (2011)) and it is known that the Atlantic cod ingest microplastic (Foekema et al., 2013). One study on plastic ingestion by Atlantic cod identified plastics in the stomachs from two out of six locations along the coast of Norway. Of the 302 fish stomachs examined, 3% contained plastic items (Bråte et al., 2016) (Fig. 2).

The genome of the Atlantic cod was published in 2011, as the first genome of a commercially important teleost (Star et al., 2011). The availability of a sequenced genome has promoted the use of toxicogenomic analyses of this species as a model of ecotoxicological effects of pollutants (Karlsen et al., 2011; Yadetie et al., 2013). The present study is a part of the dCod-project (NFR nr 248840), where the goal is decoding the systems toxicology of Atlantic cod, and the sequenced genome is central. The project is associated with the centre for digital life and hosted by The Department of Biology at the University of Bergen. A transdisciplinary approach with biologists, mathematicians and statisticians, among others, is aiming to develop

a deep knowledge of systems responses of the Atlantic cod to various types and combinations of environmental stress. Large and complex data sets are generated and integrated into models and systems toxicological understanding, beneficial in environmental monitoring.

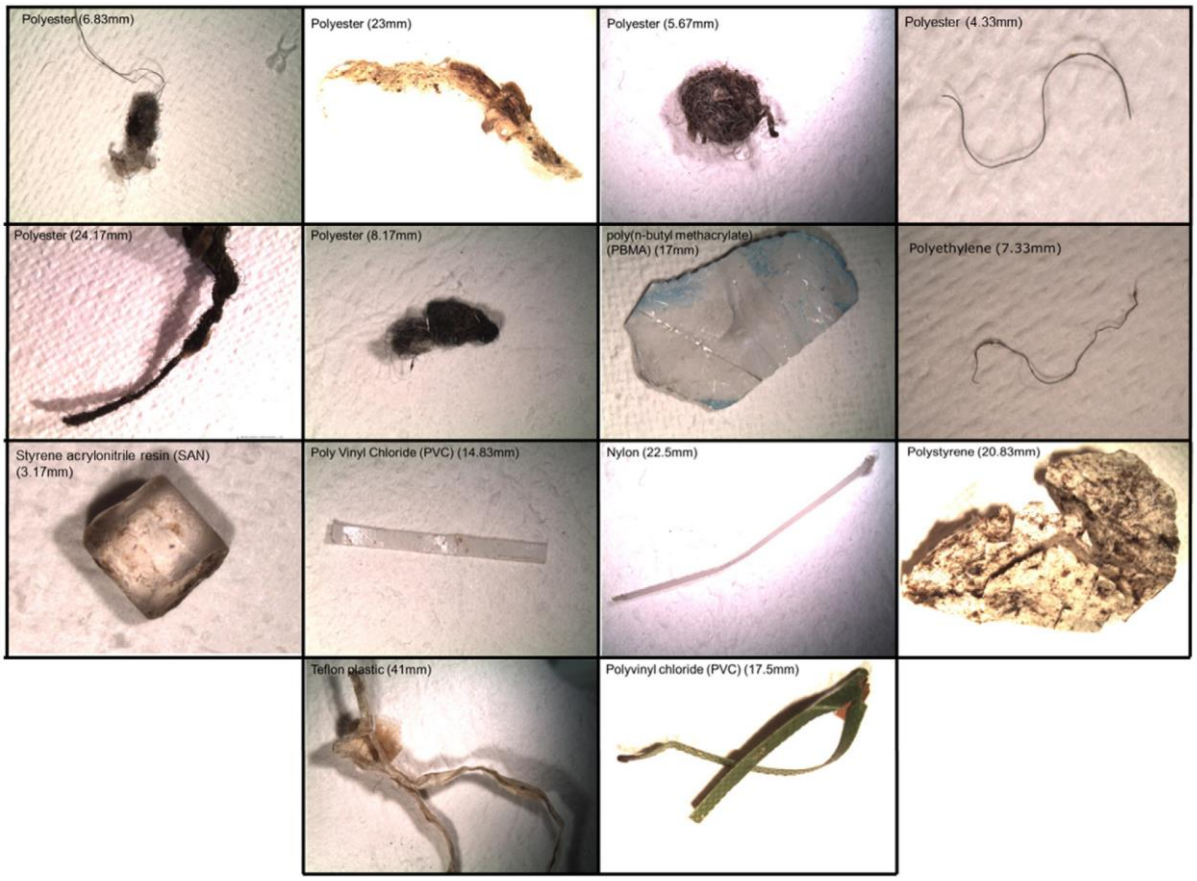


Fig. 2. Plastic polymers found in fish (from Bergen City Harbour): polymers found in 8 fish individuals (Bråte et al., 2016).

1.3. Precision cut liver slices (PCLS)

The liver is an essential metabolic organ that expresses important components of the biotransformation as well as the endocrine system, hence it is an important target organ for toxicants (Eide et al., 2014).

For many animals such as large fish species, toxicological studies using *in vivo* methods are often cumbersome, expensive and with low throughput, and can be ethically challenging (Yadetie et al., 2018). Therefore efficient *in vitro* methods, computational models and systems biology approaches that can replace or minimize the use of animal models are needed (Krewski et al., 2010). Cell cultures are the most used *in vitro* model; however, they do not provide a

complete liver model. The liver contains several cells in addition to hepatocytes, and all these cells communicate and interact with each other in a complex way (De Graaf et al., 2010). Although few drug-metabolizing enzymes have been reported to be expressed in these other hepatic cells (Koop et al., 1991; Lerche et al., 1996; Piscaglia et al., 1999; Schrenk et al., 1991; Vandenberghe et al., 1990), their role in drug-induced toxicity has been demonstrated with many compounds, via direct or indirect mechanisms (Edwards et al., 1993; Nastevska et al., 1999). PCLS provide an integrative representation of the mechanisms occurring *in vivo* (Olinga & Schuppan, 2013; Subramanian et al., 2008), hence using complete liver tissue like PCLS is suggested to give a better overview of the complex multicellular liver functions and reactions to toxic compounds. Also, they can be maintained for longer periods than the primary cell monolayer cultures (Boess et al., 2003; Olinga & Schuppan, 2013). The cell differentiation condition is preserved, as the PCLS are composed of all cell and tissue types present in the liver, avoiding the common dedifferentiation processes observed in isolated cell cultures (Boess et al., 2003; Olinga & Schuppan, 2013).

It has been shown that compared to primary hepatocyte cultures, gene expression patterns in liver slices is more similar to *in vivo* liver gene expression patterns (Boess et al., 2003). PCLS combined with omics technologies can be used to generate high throughput data for a large number of chemical exposures (Yadetie et al., 2018). The produced data may lead to further mechanistic studies and generate computational models using systems biology approaches and the adverse outcome pathway (AOP) framework to facilitate chemical risk assessment (Ankley et al., 2010; Brockmeier et al., 2017). The AOP of the compound describes the pathway from the effect on the molecular through cellular level, and adverse effects on organisms, populations and eventually ecosystems.

Eide et al. (2014) proved for the first time that the cod liver slices were viable in culture for several days and responded to contaminants in a dose- and time-specific manner. Also, a regeneration of Atlantic cod liver slices following 24hr of culture was shown, with healed edges and an increase in intact cells after 48hr of culture. This demonstrated that PCLS is a promising *ex vivo* system, which can be utilized in toxicological studies. There are however some limitations to the liver slice system. Even if the slices can stay viable for up to several days, this may not be enough to study the chronic toxicity of compounds (De Graaf et al., 2010). Also, the *ex vivo* system lack the absolute competence of a whole organism and may not uncover biological processes such as metabolism and toxicological responses mediated through other indirect pathways.

Further details and illustrations on the PCLS method is described in the methods and materials chapter.

1.4. Vitellogenin as a biomarker for endocrine disruptors (EDs) in fish

According to the widely accepted World health Organization (WHO) definition, an endocrine disruptor is an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations,". Endocrine disruptors are chemicals that interfere with the hormone systems even at low doses and produce adverse reproductive, developmental, neurological, and immunological effects. Vitellogenin (vtg) is a well-known biomarker for estrogenic and anti-androgenic effects of EDs in fish. It is a precursor of the egg yolk proteins and is encoded by the vtg gene family. During sexual maturation, estradiol (E2) is synthesized in the female gonads. The E2 circulates and is subsequently taken up by hepatocytes where it binds to E2 receptors leading to the transcription of the vtg genes (Nilsen et al., 2004). The vtg proteins are secreted from the hepatocytes into the blood, further transported to the ovaries and finally modified by developing eggs to form the egg yolk. It also plays a vital role in embryogenesis.

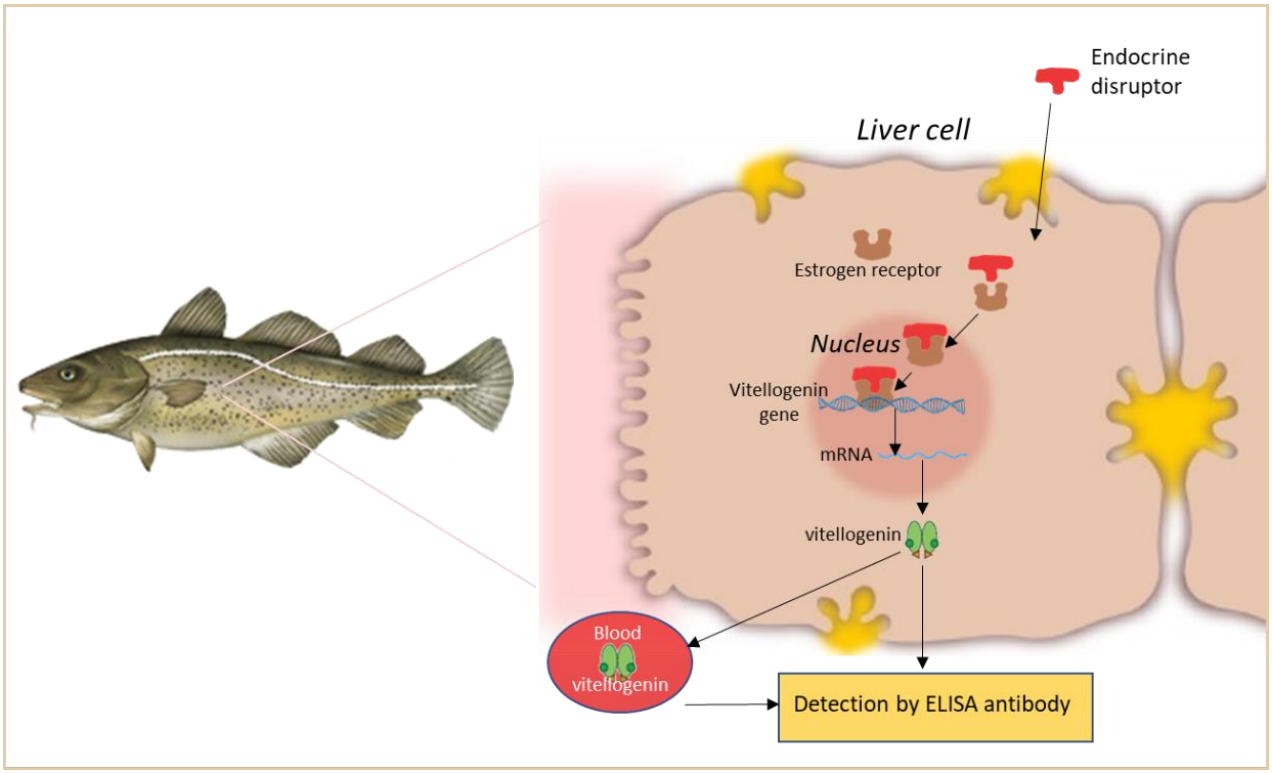


Fig. 3. Vitellogenin production in fish, and effect of endocrine disruptors. Illustration made by Hilde Andersen.

Vtg is almost undetectable in the plasma of male and juvenile fish (Crain et al., 2007). If exposed to estrogens or xenoestrogens, the hepatocytes are induced to produce and release vtg, and changes in vtg protein levels and mRNA levels may be detected (Dang, 2016) (Fig. 3). Vtg in male fish is thus, considered as a sensitive fish biomarker indicating exposure to estrogen agonists. Thus, changes in vtg can be used for the detection of chemicals interfering with estrogenic signalling according to the OECD test guidelines (OECD, 2012). It has also been shown that chemicals interfering with the androgen receptor mediated pathway and chemicals disrupting steroidogenesis activities can induce vtg changes (Dang, 2016).

1.5. Diethylhexyl phthalate (DEHP) and monoethylhexyl phthalic acid (MEHP)

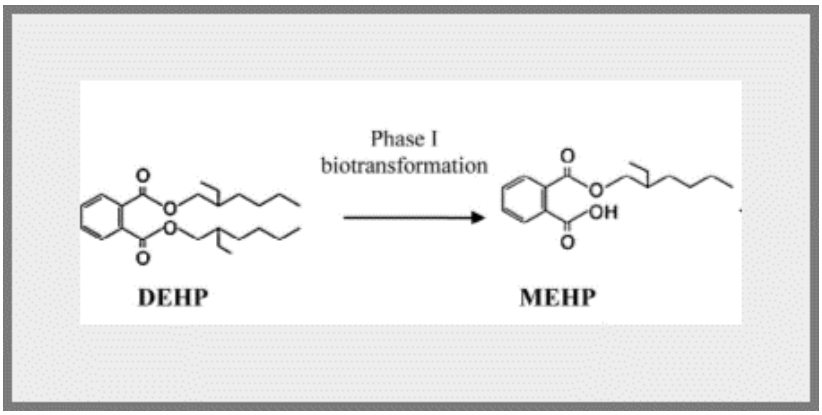


Fig. 4. Metabolism of DEHP to MEHP (Modified illustration from Kumar and Sivaperumal (2016)).

DEHP is the diester of phthalic acid and the branched-chain 2-ethylhexanol. It is the most used member of the class of phthalates, a group of plasticizers with an approximate annual production of one million tons in Europe (Engel et al., 2017).

Phthalates with high molecular weights, such as DEHP are used as additives and plasticizers in polyvinyl chloride plastics (PVC). Their main applications are flexible materials used in food packages, building, toys, medical devices such as blood storage bags, intravenous fluid bags and other products (Sunny et al., 2004). DEHP is documented to be ubiquitously present in the global marine environments. A reported concentration of phthalate esters (PAEs) in surface marine water, surface marine sediment and marine organisms were 0–300 µg/L, 3 µg/g and 4.07 ng/g, respectively (Liu et al., 2009). Because DEHP is ubiquitous in the environment, it is expected that its metabolites, like MEHP, are also ubiquitous (Ye et al., 2014).

Following oral ingestion, DEHP undergo rapid cleavage into their monoester metabolites (MEHP) (Fig. 4) by nonspecific esterases and lipases in the gastrointestinal tract. Following absorption, the monoesters are further metabolized by various oxidation and hydroxylation reactions, resulting in secondary metabolites, which are excreted via urine (Lyche et al., 2009). This formation of the monoester prior to absorption from the gastrointestinal tract is an important mechanistic step for phthalate toxicity. It has been indicated that MEHP induces endocrine disrupting effects in marine aquatic organisms and the observed toxic effects of DEHP were induced by both DEHP itself and DEHP metabolites, including MEHP (Ye et al., 2014).

Documented adverse effects of phthalates include fetal development and reproductive anomalies in addition to insulin resistance and obesity (Stahlhut et al., 2007). Phthalate esters are suggested to have endocrine disrupting properties (Latini, 2005; Veeramachaneni & Klinefelter, 2014). Experimental animal studies have shown that the perinatal period is the most sensitive life stage, and the best documented effect is the "phthalate syndrome", which constitute of different male genital developmental anomalies, increased sterility and testicular cancer (Foster, 2006; Sharpe & Skakkebaek, 2008). DEHP has also been characterized as a developmental and reproductive toxicant in various aquatic organisms including fish (Ye et al., 2014). However, the available data on the effect of MEHP in aquatic organisms are scarce, especially marine fish. In experimental studies, endocrine disrupting effects of MEHP has been indicated in the laboratory fish medaka (Ye et al., 2014).

Engel et al. (2017) found in an *in vitro* study that primary and secondary phthalate metabolites appear to exert different mechanism of action compared to the parent compounds, with the metabolites leading to endocrine effects via indirect mechanisms as deregulation of steroid hormone production, while the parent compounds affect the activity of steroid hormone receptors via direct protein-ligand interaction. Despite the different molecular mechanisms of action, they both seem to contribute to adverse effects regarding reproduction and development (Engel et al., 2017).

Even though association between adverse human health effects and exposure to phthalates has been reported, no clear cause-effect relationships are documented to date. However, based on the animal data, there are clear indications that phthalates pose certain health hazards, and the Environment Directorate-General of the European Commission categorized DEHP, DBP, and benzyl butyl phthalate (BBP) as "reproductive-toxic" (Lyche, 2017).

1.6. Bisphenol A (BPA)

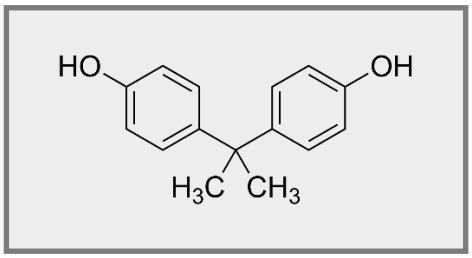


Fig. 5. Bisphenol A molecule

BPA is a synthetic chemical that is used as a monomer to manufacture polycarbonate plastics, as well as an intermediate in the synthesis of epoxy resins (Vandenberg et al., 2007). It is a phenol with a hydroxyl residue directly bound to an aromatic ring (Fig. 5). It may convert to ethers, esters and salts. (Flint et al., 2012; Vandenberg et al., 2007).

BPA is one of the highest volume chemicals produced worldwide, with more than 10 million tons per year (Fenichel et al., 2013). BPA is used to manufacture plastics, epoxy resins, hard plastic bottles and metal-based food and beverage cans (Cuomo et al., 2017) thus the main source for human exposure to BPA is food and liquid storage containers. It is also found in other applications including thermal papers, dental materials, medical devices, and personal care products (Geens et al., 2012). It is known that BPA can leach from plastic, several studies have identified BPA in freshwater, marine water and ground water (Flint et al., 2012). In the environment, sewage effluent and landfill leachates are significant point sources of BPA, and fragments of plastic debris entering the watershed through runoff are non-point sources (Crain et al., 2007). Plastics are also dumped directly to the ocean through fishery and maritime activities. BPA leaching could be a concern at marine sites where plastic waste has accumulated, as BPA leach from plastic to water is more rapid in marine than in freshwater systems (Crain et al., 2007; Sajiki & Yonekubo, 2003). In the environment, BPA is degraded through both microbial biodegradation and photodegradation and has a low potential to bioaccumulate in animals (Staples et al., 1998). In humans, the elimination of BPA into urine largely occurs within 24 h (Thayer et al., 2015). Although aquatic organisms readily excrete BPA, they may be chronically exposed to the compound and the excretion may be overwhelmed (Canesi & Fabbri, 2015).

BPA is an estrogenic compound (Dodds, 1936). It has a similar structure as the highly potent estrogen receptor (ER) agonist, diethylstilbestrol (DES), and binds classical nuclear ER alpha and beta (Seachrist et al., 2016). Thus, BPA is expected to have effects on ER function in addition to other nuclear hormone receptors and most of the studies on BPA action have focused on hormone sensitive tissues (Seachrist et al., 2016).

No causal relationship has been confirmed, but a lot of health studies of BPA have been published during the last years, and extensive reviews of these studies has been presented (Gore et al., 2015; Rochester, 2013; Vandenberg et al., 2013). These indicates associations between BPA exposure and adverse human health outcomes, including reproductive and developmental effects. BPA has also been associated with metabolic disease, and other health effects like abnormal thyroid function and immune function. In fish, BPA has been shown to cause developmental and reproductive effects, and disturbed immune function and metabolism (Canesi & Fabbri, 2015).

1.7. Benzotriazole (BT)

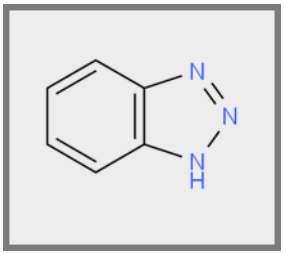


Fig. 6. Chemical structure of benzotriazole

BT is a heterocyclic compound featuring two fused rings containing three nitrogen atoms (Fig. 6). BT and its derivatives are considered emerging contaminants and some of them have the characteristics typical for the persistent organic pollutants (POPs) (Cantwell et al., 2015). It is reported that the annual production of BTs is in the range of 9000 tons per year worldwide (Reemtsma et al., 2006). BTs have been widely used in numerous applications including dishwashing agents, antifoggant in photography, industrial compounds such as anticorrosive and de-icing agents, and used as UV stabilizers for plastics.

BTs are highly water-soluble, resistant to oxidation under environmental conditions and to UV radiation (Giger et al., 2006). One of the major environmental concerns regarding BT is that it

is also resistant to biodegradation and highly persistent in the aquatic environment (Liu et al., 2012). As a result, BTs are dispersed in various water systems like surface water, wastewater, runoff from airfields (especially during the de-icing season) (Breedveld et al., 2003) and ground water (Giger et al., 2006). Widespread occurrence of BTs in freshwater and marine environments have been reported (Seeland et al., 2012; Wang et al., 2016), occasionally at high levels of µg/L or even mg/L (Cancilla et al., 1998; Giger et al., 2006).

Previous biomonitoring studies have demonstrated the occurrence of BTs in a variety of human tissues and body fluids (Asimakopoulos et al., 2013b; Wang et al., 2015). Due to the high polarity and hydrophilicity, BTs has been assumed to mainly excrete through urine as free and conjugated forms (Asimakopoulos et al., 2013a). The metabolic transformation of BT was found to be relatively low (<5% product formation) (DECOS, 2000).

Despite the widespread occurrence and high production, current knowledge is limited on the modes of action and the toxicological effects, especially regarding the chronic toxicity (Fent et al., 2014). This applies for both the BTs and their derivatives. However, toxicities of BTs to fish have been reported, e.g., endocrine disrupting effects, oxidative stress, and hepatotoxicity (Duan et al., 2017; Fent et al., 2014; Harris et al., 2007; Liang et al., 2017; Tangtian et al., 2012a).

1.8. Objectives

The main objectives of this study are 1) To determine if exposure to certain plastic compounds promote adverse health effects in Atlantic cod using changes in mRNA expression of target genes and vitellogenin as biomarkers of effect; 2) Examine possible mixture effects, including antagonism and synergism. 3) Investigate whether histopathology or transmission electron microscopy (TEM) could be applied as a method to evaluate ultrastructure and cell morphology in toxicology studies on PCLS.

2. MATERIAL AND METHODS

2.1. The fish

The experiment was done on liver slices from six male juvenile 1-year old Atlantic cod (*G. morhua*), delivered from Nofima/Havbruksstasjonen in Tromsø and kept at the Industrial and Aquatic Research Laboratory (ILAB; Bergen, Norway). To avoid potential gender difference, only males were selected in this experiment. The fish were kept in 500 L tanks in natural seawater at 9°C with a 12:12 h light:dark cycle regime. They were fed about 0,5-1% of bodyweight daily, with a special formula suited for cod (with about 51-54% protein, and 18% lipid) based on rinsed fish oil with lowest possible level of environmental toxicants and antioxidants in the fishmeal, and preferably only natural antioxidants.

2.2. Precision-cut liver slices (PCLS)

This protocol was based on the procedure described by Eide et al. (2014) with a slight modification. All consumables were autoclaved or rinsed with 70% ethanol, and both PCLS buffer and culture medium were sterilized. Working surfaces were cleaned with 70% ethanol before each liver was sliced, and gloves were used and changed between different steps of the process to keep the environment as sterile as possible. PCLS buffer was prepared in advance according to the recipes (Table 1), calibrated to pH 8,4 and sterile filtered into autoclaved flasks.

Table 1. PCLS buffer recipes (Ellesat et al., 2011).

Compound	Concentration	Amount
NaCl	122 mM	14,26 g
KCl	4,8 mM	715,6 mg
$Na_2HPO_4(H_2O)_2$	11 mM	3915,6 mg
$MgSO_4(H_2O)7$	1,2 mM	591,6 mg
$NaHCO_3$	3,7 mM	621,6 mg
H_2O solvent		2 L

Culture medium was prepared from Leibowitz-15 medium (Life TechnologiesTMGibco®, Paisley, UK) supplemented with 10% charcoal-stripped and heat-inactivated foetal bovine serum (Gibco®) and 1% penicillin–streptomycin–amphotericin (10,000 U/mL potassium

penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B; Sigma-Aldrich), as described by Søfteland et al. (2010). The PCLS buffer and culture medium were always maintained in an incubation cabinet at 3°C, and the culture medium was used within the same or following day of preparation.

The fish were euthanized by a blow to the head, and weight and length were measured. The gender was determined, and males were selected for the experiment. Prior to dissection, the cod were rinsed with 70% ethanol and opened through the ventral side. The following steps of the slicing procedure is illustrated in Fig. 7.

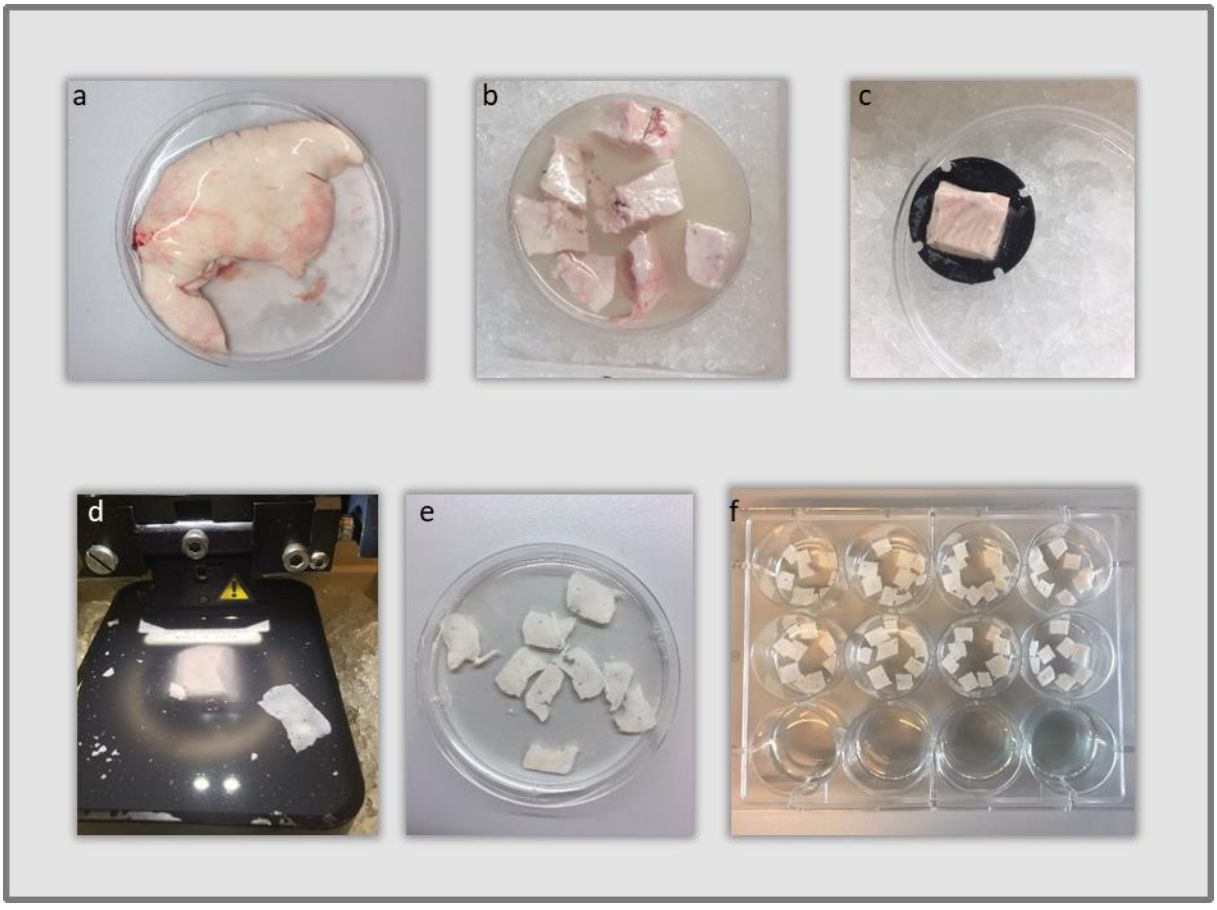


Fig. 7. The PCLS preparation process from liver to exposure ready slices. (A) The dissected liver in PCLS buffer (B) Liver cut in blocks of tissue 3x2x2 cm, (C) Liver block mounted for vibratome (D) Slicing into 250 µm slices by vibratome (E) slices about 2x3 cm, and eventually (F) the slices further divided to about 4x4 mm pieces distributed in 12 well plates before the exposure. Photos by Hilde Andersen.

The liver was excised, immediately transferred to a large petri dish containing cold (4°C) PCLS buffer and weighed. Within ice-cold culture medium, blocks of liver tissue (approximately 3

cm x 2 cm, height 1-2 cm) were carefully cut out from the central liver, avoiding the larger hepatic vessels and intrahepatic bile canaliculi. For attachment, superglue was used to glue the liver tissue on the specimen plate of Leica vibrating blade microtome VT1200 (Leica, Wetzlar, Germany). The plate with the specimen was assembled into the buffer tray containing PCLS buffer, and the buffer tray was placed in the prefilled ice tray that was mounted on the microtome. A razor blade was mounted and positioned, and the upper part of the core was trimmed to avoid damaged tissue. Slices of 250 µm was cut at a speed of 0,9 mm/s and amplitude 3 mm. During slicing, the tissue was kept submerged in ice-cold (4°C) PCLS buffer.

The sliced strips were collected in petri dishes containing culture medium at 4°C. With a razor blade, the strips were split into smaller slices (approximately 4x4 mm). These were distributed into each well of 12-well plates containing 1 ml culture medium (9 small slices per well), and pre-incubated under orbital shaking (50 rpm) at 10°C for 2 hours before exposure.

2.3. Exposure of PCLS

After 2 hours of acclimatization in the incubator, the wells were supplemented with 1 ml of medium containing four selected concentrations of the contaminants MEHP (CAS nr 4376-20-9), BPA (CAS nr 80-05-7) and BT (CAS-nr 95-14-7), all purchased from Sigma-Aldrich in powder. Prior to the exposure, the powder was dissolved in dimethyl sulfoxide (DMSO) (CAS-nr 67-68-5) (Sigma-Aldrich), as main stock solution, and further diluted into 4 different concentrations. The final concentrations of all compounds are shown in Table 2, with environmentally relevant concentrations based on previous experiments performed on the current compounds (Bizarro et al., 2016; Careghini et al., 2015; Seeland et al., 2012; Welshons et al., 2006; Zhang et al., 2014). Since the DEHP is rapidly metabolized in the intestinal tract and mainly absorbed as MEHP (Lyche, 2017), the metabolite is of especial interest in a PCLS study where the hepatic tissue is directly exposed via immersion, bypassing gastrointestinal metabolization of DEHP to MEHP prior to the exposure of the hepatic tissue. The compounds were also combined in mixtures for each level of concentration, named mix x-low, low, high and x-high. As solvent control, a group of PCLS was exposed to 0,1% of DMSO.

Table 2. Final concentrations of each compound used in the exposure.

Compound	1	1:10	1:100	1:1000
MEHP	100 µM	10 µM	1 µM	0,1 µM
BPA	22 µM	2.2 µM	0.22 µM	0.022 µM
BT	42 µM	4.2 µM	0.42 µM	0.042 µM
Mixture of MEHP/BPA/BT	X-high	High	Low	X-low

The 12-well plates were maintained in an incubation cabinet at 10°C, under orbital shaking during the exposure. After 48 hours of exposure, the slices were harvested in separate Eppendorf tubes for further analyses (Fig. 8). From each fish and exposure, one slice was put in formaldehyde for histopathologic examination, two slices in tubes containing TEM fixative for transmission electron microscopy and three slices were put in empty tubes and directly frozen in liquid nitrogen for later Q-PCR. The latter were stored at - 80 °C until further analysis. Three slices were also collected and frozen in liquid nitrogen for the possibility of proteomic analysis, but the proteomics were not performed in the time frame of this thesis.

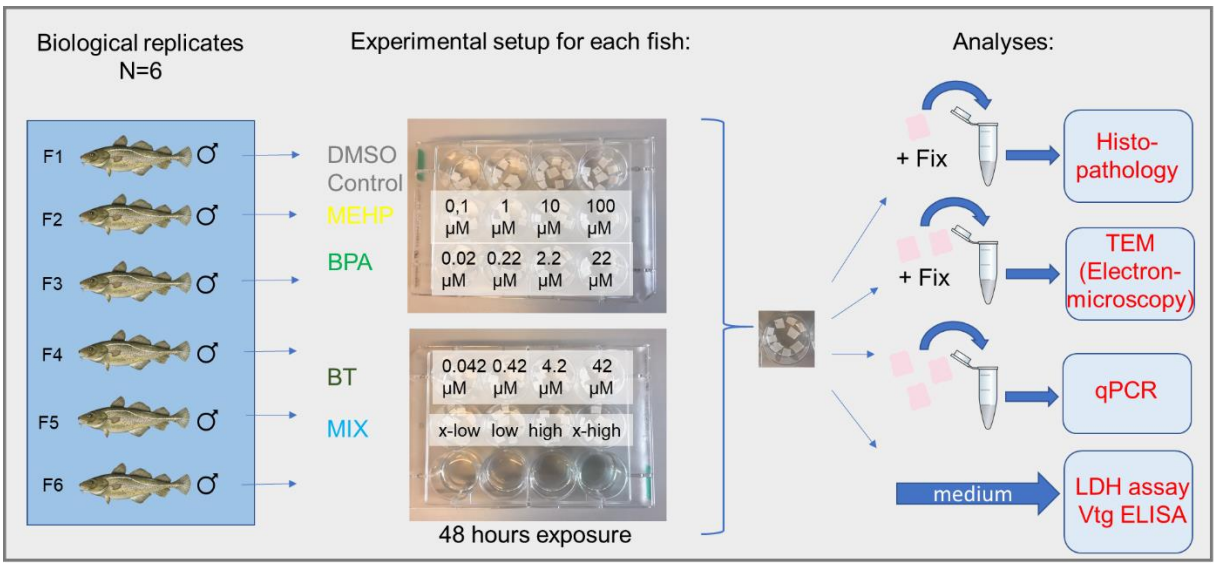


Fig. 8. Study design; 6 biological replicates exposed to DMSO, MEHP, BPA, BT and a mixture of all three compounds at four different concentrations, followed by the selected analyses.

2.4. Analyses:

The US EPA's definition of adverse effect is "a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism or reduces an organism's ability to respond to an additional environmental challenge." To determine adverse effects, microscopy, vtg production and change in expression of certain target genes were employed in this study. For the Lactate dehydrogenase (LDH) assay and vitellogenin ELISA, media from all concentrations of the compounds and mixture were analysed. For histopathology, TEM and Q-PCR, analyses were performed only on the PCLS exposed to the highest concentrations of each compound and mixture.

2.4.1. Lactate dehydrogenase (LDH) assay for viability testing of PCLS

After exposure, an LDH assay was performed to verify viability of the PCLS, thus confirming that the cells were functional, and that toxicity of the chosen compounds was not a limitation to the experiment. LDH is a cytosolic enzyme present in many different types of cells. When the cell membranes are damaged, LDH is released into the growth medium, hence this enzyme is an indicator of cellular toxicity. The released LDH was quantified by using a lactate dehydrogenase kit (Cytotoxicity Detection Kit; Roche Applied Sciences cat no 11644793001, Basel, Switzerland). The assay was done according to the manufacturer's instruction. After 48 hours of exposure, growth medium was pipetted in triplicates of 50 µl of each sample to a clear 96-well plate, and the plates was stored at 4°C. Fresh medium was used as a blank control. Prior to LDH activity determination, cells were removed by centrifugation at about 250 x g for 10 minutes. 250 µl from bottle 1 was mixed with 11 250 µl of bottle 2. From the reaction mixture 50 µl was added to each well and incubated for about 10 minutes protected from light, eventually the plate was read with the EnSpire Multimode Plate Reader, Perkin Elmer, Waltham, USA.

The LDH activity is determined in a two-step enzymatic test (Fig. 9). An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant directly correlates to the amount of formazan formed during a limited time period. Therefore, the amount of colour formed by formazan dye is proportional to the number of lysed cells. Absorbance at 490 nm (measures enzymatic activity) and 650 nm (reference measurement) were measured using the program called "Cytotoxicity detection (LDH) Roche" in the EnSpire. LDH activity values were obtained by subtracting the reference absorbance (A490-A650 nm).

Changes in LDH activity (relative to DMSO controls) were used to assess viability of the slices during the experiments.

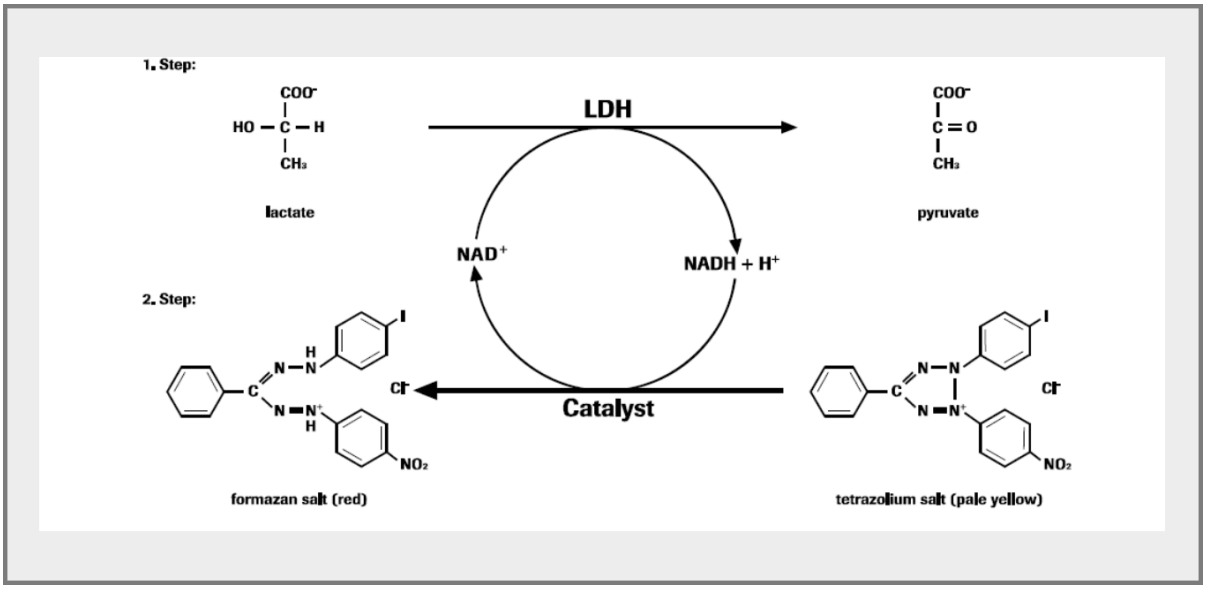


Fig. 9. In the first step, released lactate dehydrogenase (LDH) reduces NAD+ to NADH+ H+ by oxidation of lactate to pyruvate. In the second enzymatic reaction 2 H are transferred from NADH+ H+ to the yellow tetrazolium salt INT (2-[4-iodophe-nyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) by a catalyst. Taken from the manufacturer's instruction.

2.4.2. Quantitative real time polymerase chain reaction (Q-PCR)

Quantitative real-time polymerase chain reaction (Q-PCR) was performed for nine different target genes: *vtg1*, *esr1*, *cyp1a*, *scdb*, *acly*, *fabp1a*, *acox1*, *hnf4a*, *cebp* (see Table 3 for functions), and in addition, two housekeeping genes (*rpl22l1*, *actb2*) were tested.

TRIzol solubilization and extraction is a general method for deproteinizing RNA (Rio et al., 2010). All samples were added 500 µl of TRIzol reagent (15596-026 Invitrogen, USA), 25 MagNA Lyser ceramic Green Beads (Roche, 03358941001) and homogenized 15s by Fastprep-24 (MP Biomedicals, 6933050). Additionally, 200 µL chloroform (Invitrogen) were used for phase separation, which is where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase (Rio et al., 2010)). Samples were centrifuged with 14000 g in 15 min, added 1 µl glycolblue (Ambion, AM 9515) and 200 µl isopropanol for precipitation. Furthermore, they were centrifuged for 14000 g in 15 minutes. The pellet was dissolved in 17 µl H_2O, and to clear the RNA from DNA contamination, it was treated with

TURBO™ DNase (Ambion, AM2238) according to manufacturer. Total RNA was quantified with a Qubit 3 fluorometer (Thermo Fisher Scientific, Waltham, U.S.A.). cDNA was prepared from 230 ng of total RNA, using Superscript III reverse transcriptase (18080-044 Invitrogen) and random hexamer primers (Thermofisher scientific) according to product specifications. cDNA samples were diluted 10 times and Q-PCRs was performed on a *LightCycler®96* System (Roche) using SYBR Green I master (Roche, *4887352001*).

Table 3. Selected target genes and their function.

GENE	MARKER FOR
vtg1	Established biomarker for estrogenic compound exposure (ER-pathway)
esr1	Established biomarker for estrogenic compound exposure in fish liver (ER-pathway)
cyp1a	Established biomarker for response to xenobiotic exposure (activation of Ahr pathway)
scdb	May be a marker for lipogenic effects (possibly PPAR ɣ pathway)
aclya	May be a marker for lipogenic effects (possibly PPAR ɣ pathway)
fabp1a	May be a marker for lipogenic effects (possibly PPAR ɣ pathway)
acox1	May be a marker for lipogenic effects (possibly PPAR α pathway)
hnf4a	May be a marker for lipogenic effects (possibly PPAR α pathway)
cebp	May be a marker for lipogenic effects (possibly PPAR ɣ pathway)

Q-PCR primers were designed using Primer3Plus software[1] (details in Table 4). Single stranded nucleic acid sequences may have secondary structure (hairpin loops and primer dimer) due to the presence of complementary sequences within its length. Hairpin loops greatly reduce the efficiency of the reaction by limiting its availability and ability to bind to the target site (Singh et al., 2000). In this analysis, the PCR primer pair candidates were analyzed to test for possible hairpin loops and primer dimer formations using Vector NTI (Life Technologies). To avoid amplification of genomic DNA, amplicons spans over to exons.

The PCR cycling parameters were 300 s pre-incubation time at 95 ºC, 40 cycles of amplification at 95 ºC for 10 s, 60 ºC for 10 s and 72 ºC for 6 s, followed by a melting curve from 60 ºC to 95 ºC to assess the specificity of the Q-PCR products. Relative gene expression was measured on duplicate samples, three non-template controls and three positive controls (calibrator)

[1] http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

consisting of equal amounts of all cDNA samples in all Q-PCR plates. To perform accurate normalization of the Q-PCR, the stability of two candidate reference genes was investigated. The analyzed reference genes were Ribosomal Protein L22 Like 1 (*rpl22l1)* and actin beta 2 (*actb2*).

Table 4. Selected target genes and reference genes with their according primers and efficiency.

Gene	Forward primer	reverse primer	Efficiency %	Efficiency
vtg1	5´-TCTGCCCGCCGATTCTAAC	5´-CCAGGTGGGCTTCGGTGT	103	2,06
esr1	5´-CGCTTTCGGATGCTCCAG	5´-ACGAGAAGGCCCCAGAGTTG	95	1,9
cyp1a	5´-CACCAGGAGATCAAGGACAAG	5´-GCAGGAAGGAGGAGTGACGGAA	100	2
scdb	5´-CCCTTGCATGGACTGTAGTGTG	5´-CATGGAGTTAGCCAGAGCCAGGA	100	2
aclya	5´-CTGCGGTGGATTTACACGAGATGA	5´-CTTCTGGTCCAGGTAGTGTCCGATGA	100	2
fabp1a	5´-GTCAACACTTTCATCGTCGG	5´-GATTCCCTTGAGGGAGACCT	100	2
acox1	5´-ATGCCATCGTGTTGGCTC	5´-TGTCCCCGATCACAATACCT	100	2
actb2	5´-CGACGGGCAGGTCATCACCATCG	5´-CCACGTCGCACTTCATGATGCTGT	101	2,02
rpl22l1	5´-AGAAGTCCAAAAAAGGAGCTTCCT	5´-GTTACCGGTCTTCCCGTTGA	100	2
hnf4a	GCTGGCATGAAGAAAGAAGC	TGGAGGGTAAACTGCTGTCC		2,03
cebp	GGCCAAAGACTTCTGCATGT	AGGAAATCGGAGAACACGTC		1,96

In this assay, the amplified DNA is fluorescently labelled by SYBR Green I fluorescence signal, and the amount of the fluorescence released during amplification is directly proportional to the amount of amplified DNA. Fluorescence is monitored during the whole PCR process. The higher the initial number of DNA molecules in the sample, the faster the fluorescence will increase during the PCR cycles. Hence, if a sample contains more targets, the fluorescence will be detected in earlier cycles. The cycle in which fluorescence can be detected is termed quantitation cycle (Cq) and is the basic result of Q-PCR: lower Cq values mean higher initial copy numbers of the target. Cq values of all reference genes and target genes, were recalculated to values representing 100% PCR efficiency (E = 2). Average adjusted Cq values of reference genes *rpl22l1* and *actb2* in each sample were calculated and used for normalization of relative gene expression values using the following equation.

Relative expression:

$$E_{target}^{\Delta Cq\ target\ (calibrator-\ sample)} \times E_{reference}^{\Delta Cq\ reference\ (sample\ -\ calibrator)}$$

where:

E$_{\textbf{target}}$: Amplification efficiency of the target gene (e.g. *vtg1*).

E$_{\textbf{reference}}$: Mean amplification efficiency of the reference genes (*actb2+ rpl22l1*)

ΔCq $_{\textbf{target}}$ **(calibrator-sample)**: Cq of the target gene (e.g. *vtg1*) at calibrator concentrations - Cq of the target genes (e.g. *vtg1*) at a certain concentration.

ΔCq $_{\textbf{reference}}$ **(sample-calibrator)**: Cq of the mean of reference genes at a certain concentration - Cq of the mean of the reference genes at calibrator concentrations.

2.4.3. Analysis of vitellogenin levels by enzyme-linked immunosorbent assay (ELISA)

Vitellogenin was measured directly from the growth medium of the exposed liver slices, applying the ELISA Vitellogenin kit for cod (Biosense Laboratories AS) according to the manufacturer's instructions. The assay has been developed for quantification of vtg in plasma samples, but instructions for samples prepared otherwise is also included. *In vivo*, the vtg proteins are secreted from the hepatocytes into the blood (Arukwe & Goksøyr, 2003), hence it is assumed that vtg will be released from the liver slices into the surrounding growth medium.

Plates containing liver slice growth medium collected after 48 hours of exposure were thawed on ice, and dilutions of standards and samples were prepared. The ELISA utilizes specific binding between antibodies and vtg to quantify vtg in samples from cod (Fig. 10).

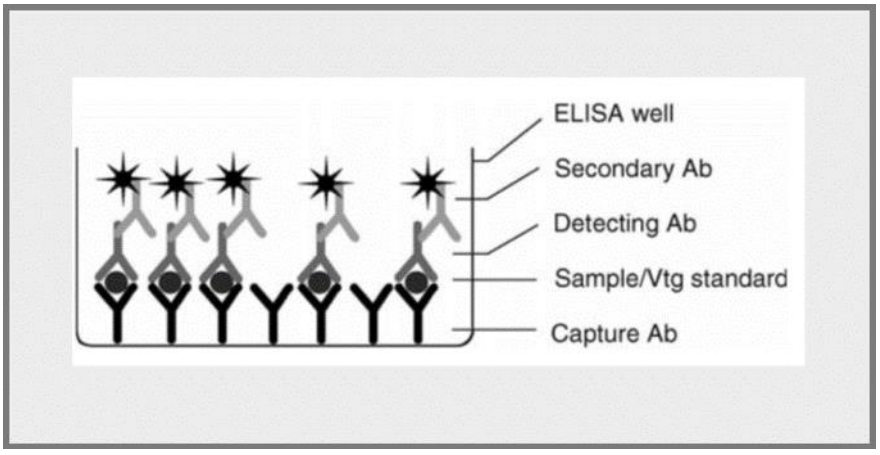


Fig. 10. Format for the sandwich ELISA assays. Taken from the manufacturer's instruction.

Pre-coated plates were added duplicates of 100 µl dilution buffer to the NSB (Non-Specific Binding) wells. 100 µl of diluted standards and samples were added in duplicates to the remaining wells which were pre-coated with a specific capture antibody that binds to vtg, and the plates were incubated at 37°C for 1 hour. The plates were subsequently washed 3 times with 200 µl washing buffer per well, and 100 µl of diluted vtg-specific detecting antibody was added to all wells creating a sandwich of vtg and antibody, before incubated for 1 hour at 37°C. The plates were washed 3 times with 200 µl washing buffer per well, and 100 µl of diluted enzyme-labelled secondary antibody were added to all wells before incubated for 1 hour at 37°C. Following this, the plates were washed 5 times with 200 µl washing buffer per well.

To determine the enzyme activity, colour development was initiated by adding 100 µl of substrate solution to all wells after the final washing step. The colour intensity is directly proportional to the amount of vtg present. The plates were further incubated in the dark at room temperature and the reaction was stopped after 30 minutes by adding 100 µl of 0.3 M H_2SO_4 to the wells. After 5 minutes the colour absorbance was measured at 450 nm using an EnSpire plate reader (Perkin Elmer, Waltham, USA). The standard and sample values were corrected for NSB, and vtg concentration in the samples were calculated based on the adjusted standard curve from the standard dilutions.

2.4.4. Histological analysis

Following the collection from the growth medium after 48 hours of exposure, slices from each well were fixed in 4 % formaldehyde. Histological assessment was performed on a total of 30 liver sections (one slice from each of the highest concentration exposure of each compound and mixture, and one slice from the DMSO control). These were processed following standard histological methods at the pathology department, Norwegian Veterinary Institute, and the sections were cut into about 4 µm with a HM 355S Automatic Microtome (Thermo Fisher, Massachusetts, USA) and stained with haematoxylin and eosin (H&E).

The tissue sections were scanned using the MicroVisoneer Manual Scanning Software (MKS Instruments, spectra products, Cheshire, U.K.) and an IDS UI3260-CP-C-HQ R2 USB3 camera (IDS Imaging Development Systems, Obersulm, Germany) mounted on an Olympus BX43 light microscope (Olympus Life science, Massachusetts, USA) using the x20 objective lens. The sections were evaluated with focus on any degeneration, inflammatory and/or other pathological changes. Additionally, the liver tissue was also assessed for any autolytic changes

during the 48-hour incubation in the medium preparations containing the test substances. The degree of change for the different morphological characteristics evaluated were graded using a scoring system with a scale of 0-4 where 0 represented normal; 1, mild changes; 2, moderate changes; 3, marked changes, and 4, severe changes. Assignments of individual samples to the test compound groups was obtained after the evaluation was completed.

2.4.5. Transmission Electron microscopy

Transmission Electron Microscopy (TEM) was used to evaluate the ultrastructure of the tissue. After the exposure, the liver slices designated for TEM were immediately fixed at 4 °C in a fixative according to protocol used at the Imaging Centre, Norwegian University of Life Sciences (NMBU). The fixative mixture contained 50 ml 4 % paraformaldehyde $(CH_2O)_n$, 25 ml 0.4 M cacodylic acid sodium salt trihydrate ($(CH_3)_2AsO_2Na \cdot 3H_2O$), 5 ml 25% glutaraldehyde ($C_5H_8O_2$) and 20 ml distilled H_2O. One slice from each exposure were placed in Eppendorf tubes containing 1 ml of this fixative over-night. Consequently, the slices were washed twice in 0,1M CaCodylat buffer (pH 7.4) and stored at 4 °C until further processing was done at the Imaging Centre, NMBU. The liver slices were post-fixed in 1% osmium tetroxide for 1 hour, followed by CaCodylat buffer washing, three times repeated. The specimens were dehydrated in a graded series of ethanol 70%, 90%, 96% and 100%. Further they were infiltrated with LR White/EtOH before embedding in 100% LR White resin at 60°C over-night for polymerization. The high fat content resulted in some difficulties in washing the osmium tetroxide, and it was necessary to repeat the LR White/EtOH infiltration several times before the embedding. Sections of 60 nm were cut with a diamond knife mounted on an ultra-microtome (LEICA EM UC 6). The sections were stained with 4% aqueous uranyl acetate and 1 % $KMNO_4$ for 10 min. After staining, the grids were washed intensively in freshly distilled water. The sections were examined in a FEI MORGAGNI 268 transmission electron microscope (FEI Company, Eindhoven, The Netherlands), and photographs were recorded on VELETA camera (EMSIS GmbH, Muenster, Germany).

2.4.6. Statistical analyses

STATA/SE 14.1. software was used for statistical analyses. The normality of the data was tested using the Shapiro-Wilk test. Histology results could not be analyzed statistically due to the equal scores for controls and exposed groups (score 0= normal). Responses following non-normal distribution were analyzed for group-dependent statistical differences ($p<0.05$) using

quantile regression. Responses following normal distribution (only vtg protein production) were analyzed for group-dependent statistical differences ($p<0.05$) using linear regression. Spearman's rank correlations coefficient was used to examine the interrelationship between the parameters. To visualize the results, the investigated parameters were displayed in a spider plot. All values were rescaled to [0, 1] before calculating mean values by group.

3. RESULTS

3.1. LDH Assay:

According to the LDH cytotoxicity test, the PCLS exposed to the selected compounds displayed no significant difference in toxicity compared to the DMSO exposed control groups. This confirms that the PCLS were viable throughout the experiment.

3.2. Morphological findings from the histological assessment

The histological evaluation revealed that the PCLS exposed to the highest concentrations of each compound and mixture had a normal and healthy morphological appearance with scores being 0 for "normal" in all samples. The histological appearance of the PCLS showed that the tissue had remained viable and comparable to liver tissue sampled from a freshly euthanized fish (Fig. 11). No changes, either pathological or autolytic, were observed in the liver sections regardless of exposure (Fig. 12).

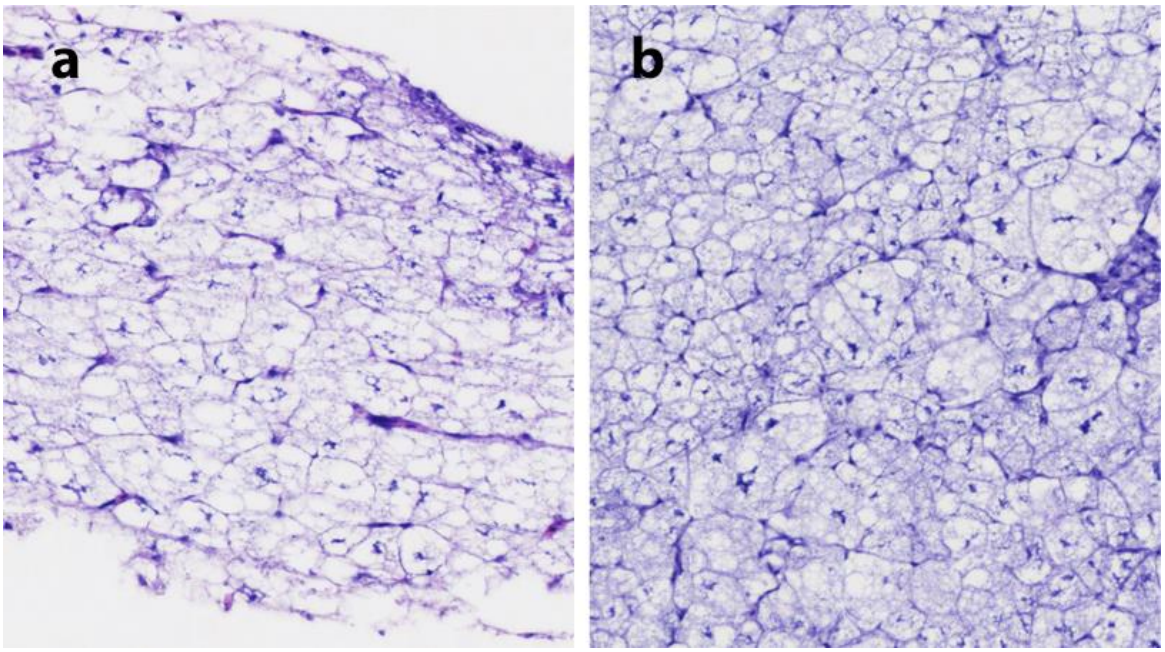


Fig. 11. Representative image of the morphological appearance of the liver sections from the *ex vitro* experiment; (A) showing normal and viable hepatocytes closely comparable in histological appearance to the liver section, (B) sampled from a freshly euthanized Atlantic cod individual.

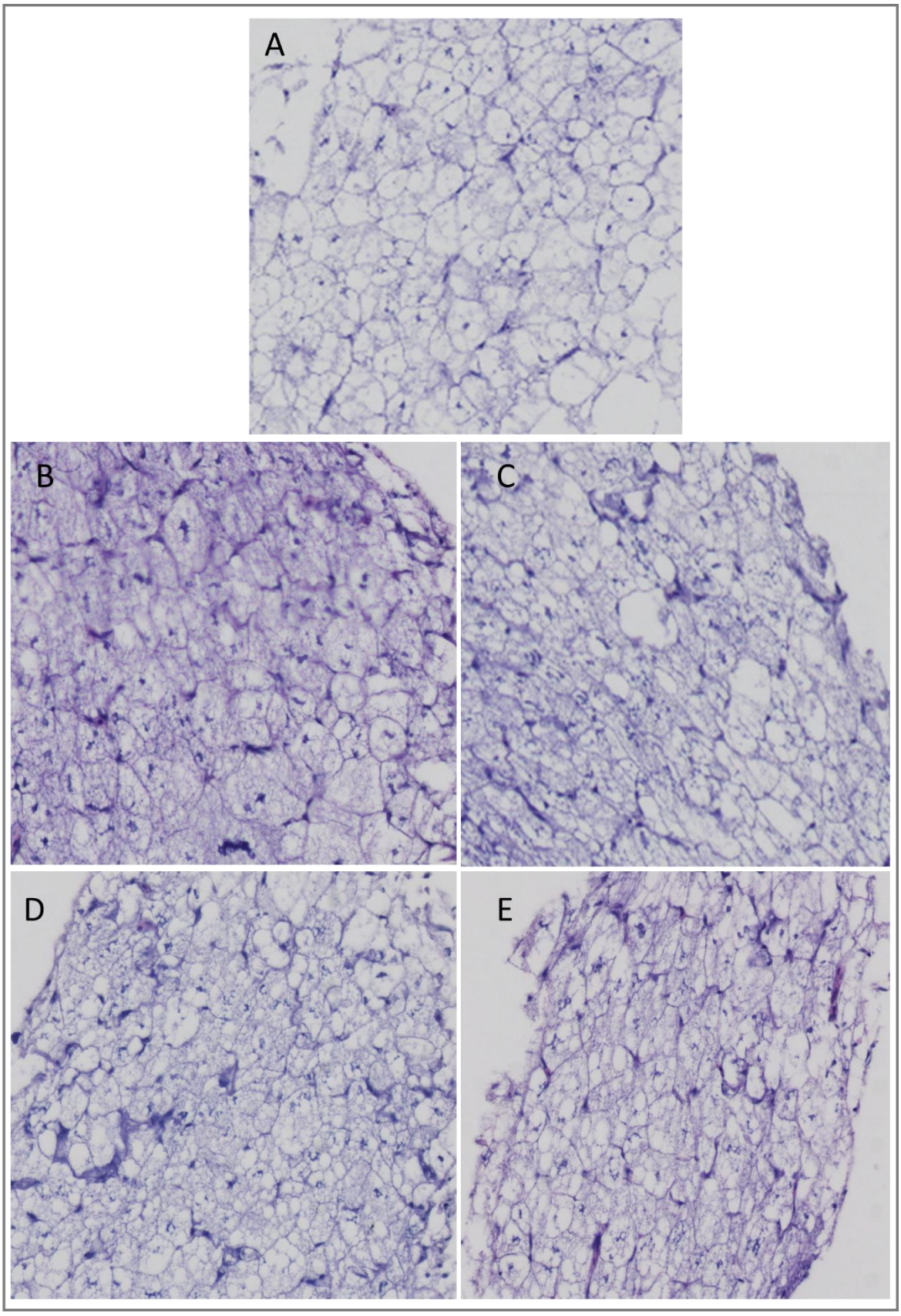


Fig. 12. Histology of cod liver slices. Normal morphological appearance was found in all samples, exemplified by PCLS exposed to (A) DMSO control (B) MEHP 100 µM (C) BPA 22 µM (D) BT 42 µM and (E) Mixture x high.

3.3. Transmission electron microscopy (TEM)

When examining the PCLS sections by TEM, a general lack of organelle structure and membranes were observed. These findings were consistent both within and between fish, including the control (Fig. 13). Consequently, it was difficult to clearly identify and assess the frequency and morphology of mitochondria, peroxisomes and other organelles. The liver samples showed a high degree of fat content as expected in cod liver. With this relatively small fraction of hepatocytes compared to fat, and a general loss of membranes and organelle structure in the present hepatocytes, it was not possible to do a satisfactory assessment of the ultrastructure.

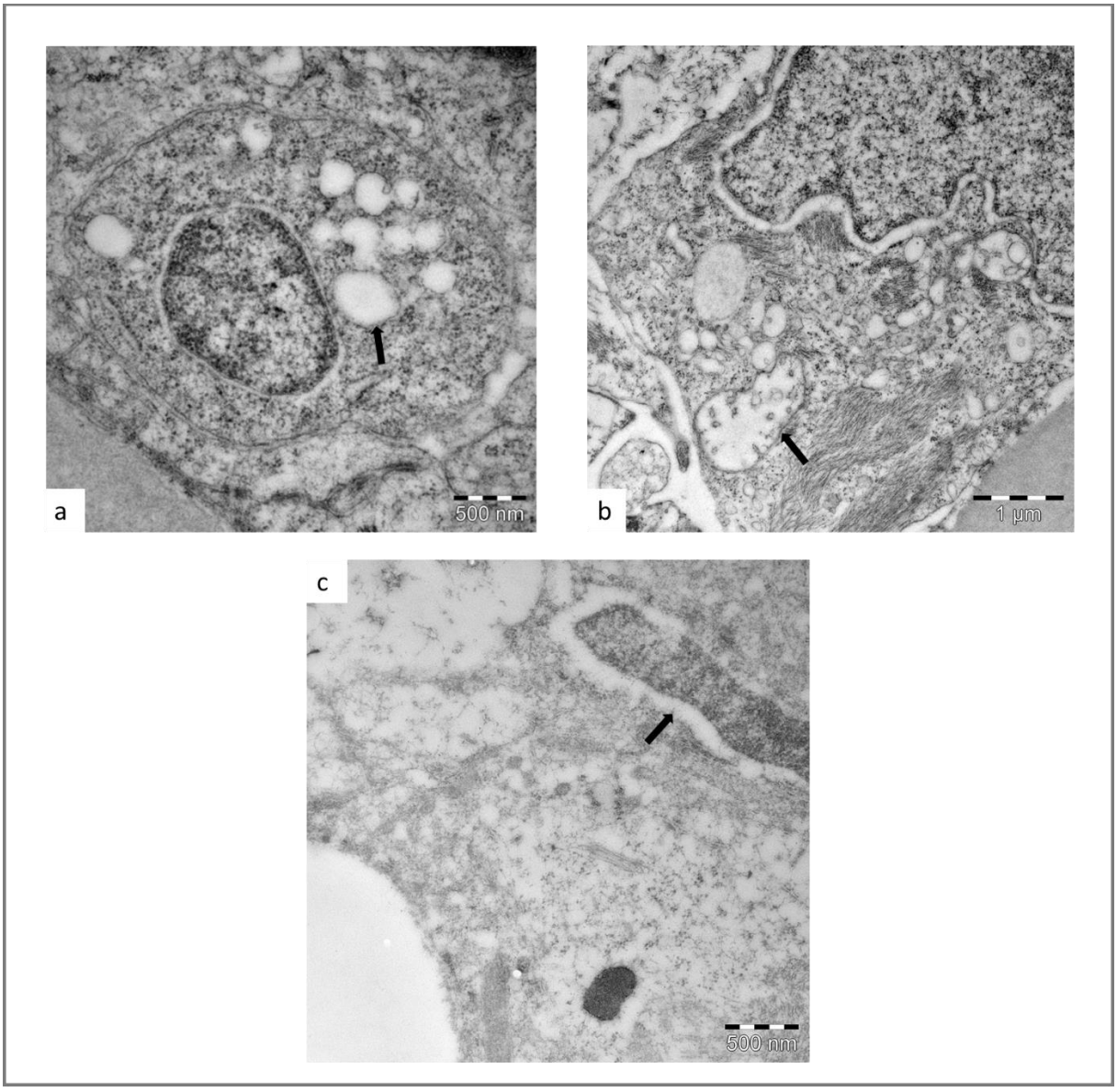
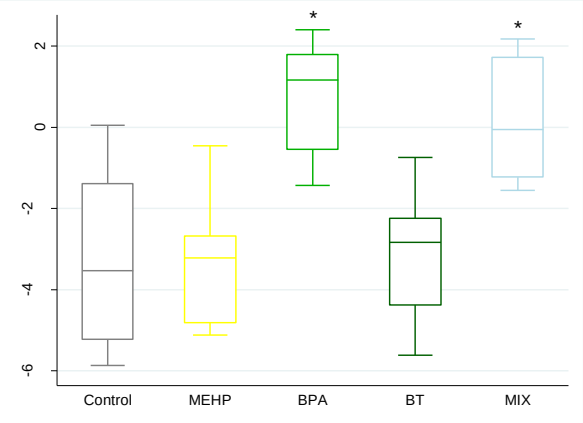


Fig. 13. Lack of organelle structure and cell membranes (arrows) in (A) PCLS exposed to BT 42 µM (B) PCLS exposed to DMSO control (C) PCLS exposed to MEHP 100 µM.

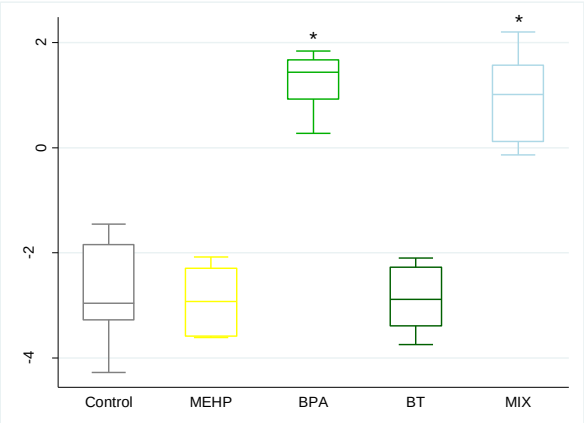
3.4. Quantitative real time polymerase chain reaction (Q-PCR)

The transcription levels of the selected genes were statistically tested with quantile regression, with the results for all groups presented in Fig. 14. For those reference genes where a significant difference was found in any of the groups compared to the control, the coefficients, the confidence intervals and the p-values are illustrated in Table 5-7. Transcription levels of *vtg1* and *esr1* were significantly upregulated in the PCLS exposed to BPA 22 µM with p=0.000 and p=0.000, respectively, when compared to controls (Fig. 14A-B, Table 5-6). A significant up-regulation was also found for *vtg1* and *esr1* in the x-high mixture exposure with p=0.041 and p=0.000, respectively, when compared to controls (Fig. 14A-B, Table 5-6). A significant downregulation was found for *hnf4a* in the mixture exposure compared to controls (p=0.025) (Fig. 14I, Table 7). The *vtg1*, *esr1* and *hnf4a* expressions were not significantly different between the BPA and mixture exposure. The exposure of PCLS to BT and MEHP did not significantly alter transcription levels of the assessed genes.

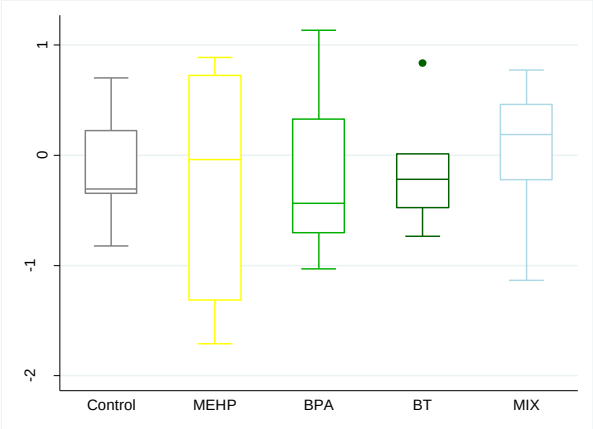
A



B



C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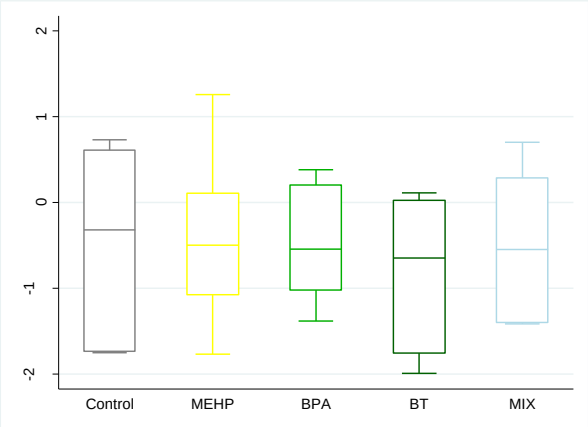
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MEHP
BPA
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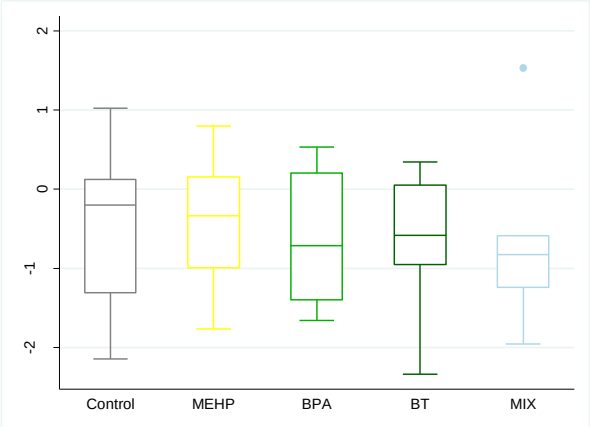
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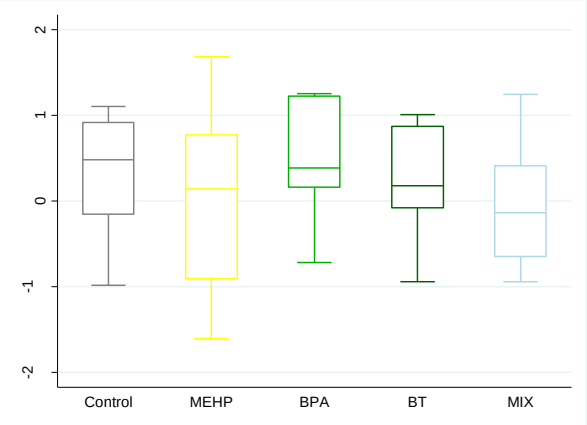
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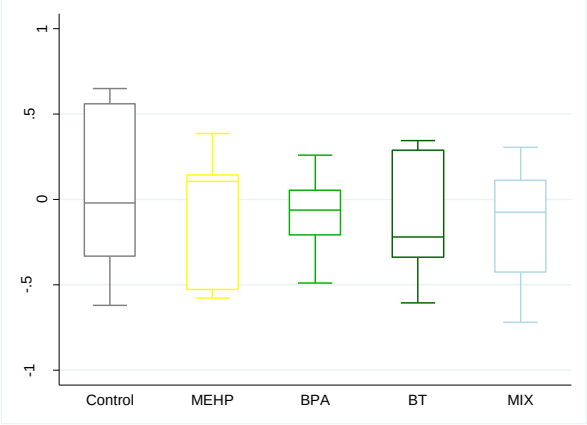
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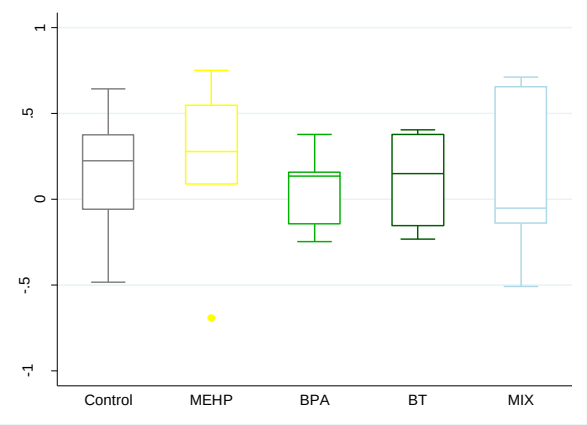
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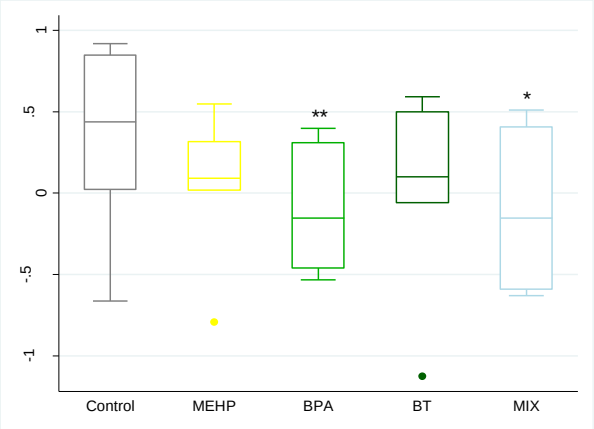

Fig. 14. Box plots presenting log transformed relative expression of (A) *vtg1*, (B) *esr1*, (B) *cyp1a*, (D) *scdb*, (E) *aclya*, (F) *fabp1a*, (G) *acox1*, (H) *cepb*, (I) *hnf4a*. The reference genes were assayed in PCLS exposed to control DMSO (grey), MEHP (100 µM) (yellow), BPA (22 µM) (green), BT (42 µM) (dark green) and mixture exposure (x-high) (blue). N=6 biological replicates for each group. Asterisk (*) and (**) indicates statistical significance ($p<0.05$) and ($p<0.1$) respectively, when comparing exposure groups to the DMSO control. (The central band represents the median, the upper and lower end of the box represents the third and first quartiles, respectively, and whiskers are the highest/lowest 1.5 × interquartile range. Observations outside these intervals are drawn as outliers (circles)).

Table 5. Quantile regression analysis for *vtg1* gene expression. Data for the coefficient, 95% confidence interval and p-value for the different treatments compared to control.

Treatment	Coefficient	95% conf. interval		P-value
MEHP 100 µM	0.0041442	-2.114609	2.122897	0.997
BPA 22 µM	5.228226	3.109474	7.346979	0.000
BT 42 µM	0.0405261	-2.078227	2.159279	0.969
Mixture x-high	2.213353	0.0945998	4.332105	0.041

Table 6. Quantile regression analysis for *esr1* gene expression. Data for the coefficient, 95% confidence interval and p-value for the different treatments compared to control.

Treatment	Coefficient	95% conf. interval		P-value
MEHP 100 µM	-0.0123167	-1.298341	1.273707	0.984
BPA 22 µM	3.523636	2.237612	4.80966	0.000
BT 42 µM	0.0001231	-1.285901	1.286147	1.000
Mixture x-high	4.510573	3.224549	5.796597	0.000

Table 7. Quantile regression analysis for *hnf4a* gene expression. Data for the coefficient, 95% confidence interval and p-value for the different treatments compared to control.

Treatment	Coefficient	95% conf. interval		P-value
MEHP 100 µM	-0.6362185	-1.406767	0.1343298	0.101
BPA 22 µM	-0.7325581	-1.503106	0.0379902	0.061
BT 42 µM	-0.6039623	-1.374511	0.1665861	0.119
Mixture x-high	0.8895089	-1.660057	-0.1189605	0.025

3.5. Vitellogenin ELISA

The vtg protein production in the PCLS were normally distributed. Hence these data were statistically tested with regression analysis, and the results for all groups are presented in the box plot in Fig. 15 and in Table 8. No significant difference was found in any of the groups at the three lowest concentrations of compounds compared to the control. In the BPA 22 µM and x-high mixture exposure groups, a significantly higher vitellogenin production was induced compared to the control with p=0.002 and p=0.027, respectively (Table 8). The PCLS exposed to the highest concentrations of the MEHP and BT showed no significant difference compared to the control. Regarding a possible mixture effect, the x-high mixture was compared to the BPA 22 µM exposure. However, no significant difference was found.

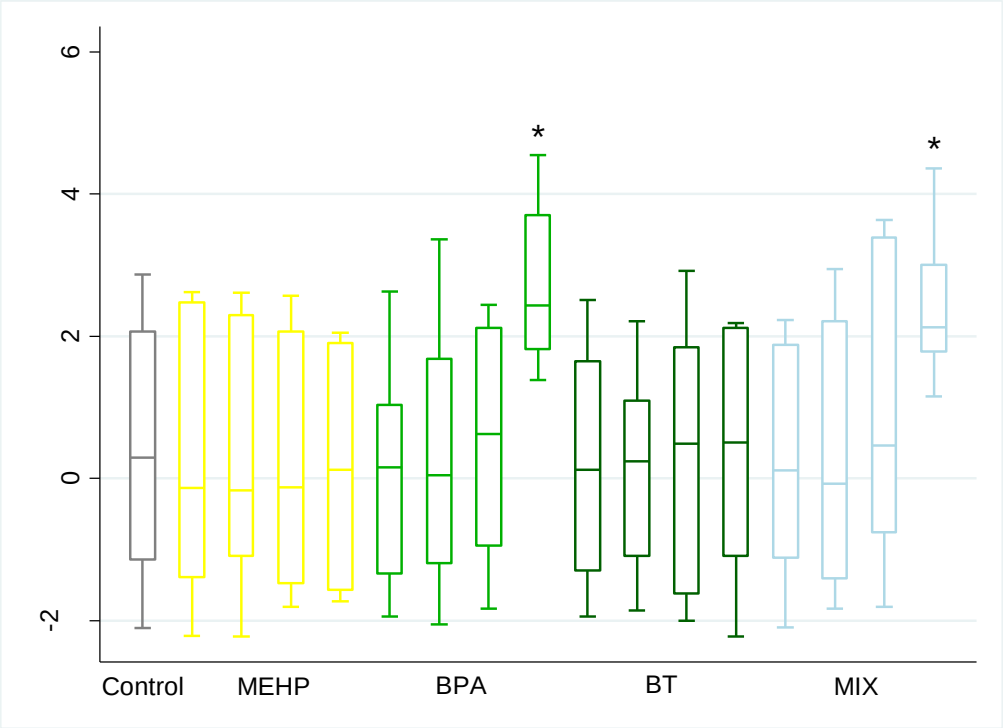

Fig. 15. Box plot of log transformed values for vtg protein production of PCLS in media (ng/ml). Values are presented for each exposure (separated by colours), with increasing concentration of the exposure compound from left to right: MEHP (0.1, 1.0, 10, 100 µM), BPA (0.022, 0.22, 2.2, 22 µM), BT (0.042, 0.42, 4.2, 42 µM) and MIX (x-low, low, high, x-high). N=6 biological replicates for each box. Regression analysis were applied, and asterisk (*) indicates statistical significance ($p<0.05$) when comparing exposure groups to the DMSO control. (The central band represents the median, the upper and lower end of the box represents the third and first quartiles, respectively, and whiskers are the highest/lowest 1.5 × interquartile range. Observations outside these intervals are drawn as outliers (circles)).

Table 8. Regression analysis for vtg production. Data for the coefficient, 95% confidence interval and p-value for the different treatments compared to control.

Treatment	Coefficient	95% conf. interval		P-value
MEHP 100 µM	-0.6340024	-14.1216	12.8536	0.924
BPA 22 µM	17.08897	3.601365	30.57657	0.015
BT 42 µM	-0.8260345	-14.31363	12.66157	0.901
Mixture x-high	8.34749	-5.14011	21.83509	0.214

3.6. Correlations between variables

Spearman correlations between the studied variables showed strong correlations between vtg protein production and *esr1* for PCLS exposed to BPA 22 µM (r=0.89), between vtg and *vtg1* for BT 42 µM (r=0.89) and between vtg and *vtg1* (r=0.94) and vtg and *esr1* (r=0.89) for the x-high mixture. The measured parameters were combined in a spider plot to better visualize exposure-specific patterns (Fig. 16). The plot shows that the BPA and mixture exposures are clearly separated from control, BT and MEHP exposures regarding expression of the *vtg1* and *esr1* genes and the vtg protein production.

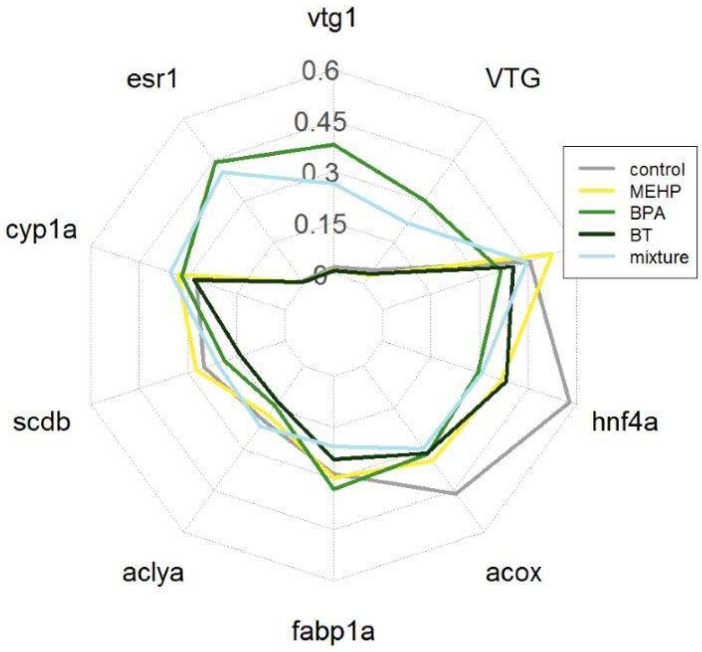

Fig. 16. Spider plot showing combined gene expression and vtg measurements in PCLS exposed to the highest concentrations of compounds and mixture. The values for all PCLS were rescaled to [0, 1] and the mean values by exposure compounds are shown.

4. DISCUSSION

4.1. PCLS as method

The PCLS method has its advantages in higher throughput and lower costs than *in vivo* experiments. Compared to hepatocyte cell-culture, the PCLS provide an integrative representation of the mechanisms occurring *in vivo* and grant an overview of the complex multicellular liver functions and response to toxic compounds that mimics the *in vivo* situation more than cell studies (Olinga & Schuppan, 2013). Another advantage of using the PCLS's instead of *in vivo* experiments is the ethical issue. In science, The Three Rs (replacement, reduction and refinement**)** are guiding principles for more ethical use of animals in testing. The PCLS *ex vivo* study implements all three Rs, by replacing of the *in vivo* study, reduction by minimising the number of animals used per experiment and refinement by minimising animal suffering and improve welfare. It should be kept in mind that *ex vivo/in vitro* systems lack the absolute competence of a whole organism and may not uncover biological processes such as metabolism and toxicological responses in other organs and systems than those studied. The 48 hours exposure limits investigation of potential chronic adverse effects, however, molecular changes found after 48 hours might point towards chronic effects that might be investigated further, by *in vivo* testing. In addition, the effects at the molecular level might contribute to characterising the mode of action (MOA) for the adverse effect of certain compounds. This could eventually be utilized to describe the adverse outcome pathway (AOP) of the compound, which describes the pathway from the effect on the molecular level, through cellular level, and adverse effects on organisms, populations and eventually ecosystems.

As observed by others (Bizarro et al., 2016), a high intra-group variability was observed in this PCLS study. A higher number of biological replicates could have contributed to a stronger statistical power in the present experiment, a conclusion also made by Bizarro et al. (2016). The variability may be influenced by a variation of tissue-mass between the slices. Also, the presence of blood vessels or the differential proportion of liver components (hepatocytes, endothelium, biliary epithelium, fat-storing cells, connective tissue) could contribute to increased response variability (Schmieder et al., 2004). In the present study, the tissue slicing was standardized using 250 um thickness and the pieces were cut to 4x4 mm. Weighing of each slice for normalization were not implemented due to risk of tissue degradation. In comparison, weighing for normalization was not reported in a previous PCLS study on Atlantic cod (Bizarro et al., 2016). Furthermore, liver blocks from the central area avoiding vessels were consistently

selected. Thus, the tissue slicing was standardized as much as possible to reduce the contribution of response variability from these factors.

4.2. Histology

In general, the liver histopathological alterations are not specific to pollutants (Salamat & Zarie, 2016), but studying the specific target organs is one of the most important benefits of using histopathological biomarkers in environmental screening. It should be highlighted that histopathology is able to assess the initial effects of exposure to chemical stressors (Salamat & Zarie, 2016). Several liver pathological alterations such as inflammatory lesions and neoplastic lesions have been used as reliable biomarkers in toxicological studies (Stentiford et al., 2003) and histopathologic categories like "early non-neoplastic toxic pathologic lesions" and "foci of cellular alteration" has been used in many national marine biological monitoring programs in Europe and the USA (Feist et al., 2004).

Apart from some pyknotic cells in the cut surface on all slices, no histological changes were observed in any of the exposures or solvent control in the current study (Fig. 12). Thus, the compounds in the present experiment did not display any acute toxicity at the chosen concentration. Moreover, the histopathology revealed that the cells were viable and healthy, as also indicated by the LDH assay.

English sole (*Parophrys vetulus*) injected intraperitoneally with Carbon tetrachloride (CCl_4) showed subcapsular hepatocellular coagulation necrosis and increased cytoplasmic eosinophilia, with associated sinusoidal congestion and edema already 4 hours post injection. After 48 hours, zones of hepatocellular necrosis and degeneration in the internal hepatic tissue were seen in 100% of the injected fish (Casillas et al., 1983). In histopathologic examination of liver from adult zebrafish 5 days following 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) intraperitoneal (i.p.) injection, lipidosis and hepatocyte hypertrophy as well as an apparent decrease in the number of hepatocyte nuclei per field were observed (Zodrow et al., 2004). However, the histology was done 5 days after exposure as opposed to the 2 days following the present PCLS study, but some more time needed for absorption and distribution could be expected from i.p. injection compared to the direct exposure to hepatic tissue in the PCLS study. The effects of a sublethal dose of the organophosphate methyl parathion on the freshwater fish *Corydoras paleatus* exposed via water or food were analysed by histopathology of the liver (Fanta et al., 2003). Four hours after exposure, the shape of the hepatocytes became irregular, some cells exhibited cloudy swelling and focal necrosis already occurred 8 h after

contamination suggesting that histological changes may occur in short time after exposure depending on the toxicant and dose. Yang et al. (2012) found that MEHP decreased the viability of HepG2 cells at the indicated concentrations (25.00–100.00 μM, thus comparable to the concentrations used in the present study) at 24 and 36 h after treatment, and apoptosis at higher concentrations at 36 h. The cells were not assessed by histopathology, but this indicates that apoptosis occurs, and cell viability could possibly be observed after 36 hrs treatment with MEHP at high concentrations. Liang et al. 2017 found histopathologic changes in rare minnows exposed to BT compared to controls. Notable, histological damage in the liver was observed following exposure to 0.05, 0.5 and 5 mg/L BT for 42 days (Liang et al., 2017) .

In conclusion, no acute toxic effects of the selected compounds were observed in the present study, while chronic effects are more likely to be observed after more than 48 hours after exposure.

4.3. Transmission Electron microscopy (TEM)

There is a lack of literature on TEM analyses in PCLS, and the present experiment should be considered as a contribution to the development of this method, hence making it applicable for the PCLS procedure. Changes at the ultrastructural level, such as a change in peroxisome numbers, could be utilized as early biomarkers of adverse effects of contaminants in toxicological studies. Due to lack of membranes and organelle integrity, change in ultrastructure following exposure could not be assessed in the current experiment (Fig. 13). The lack of structure could theoretically be caused by loss of viability during the slicing procedure or exposure. The cell viability was however considered satisfactory by LDH testing and histology, making loss of viability a less likely cause. The fixation procedure seems to be a more liable cause for the unsuccessful TEM images. Both the choice of fixative, amount and timing could influence the result. The golden standard of fixation is perfusion rather than immersion (Wisse et al., 2010). Due to the nature of this experiment, with slicing of the liver tissue prior to fixation, perfusion procedure is not feasible. Thus, in this experiment the immersion fixation was the only option. During the penetration of the fixative, its constituents react differently with cellular components, thereby changing the composition of the fixative on its way to the centre of the block. The particularly high lipid content of the cod liver tissue may lead to incomplete fixation compared to less lipid-rich tissue. The glutaraldehyde does not fix lipids, but the osmium does, and it may be better to implement a secondary osmium fixation shortly after sampling with such lipid-rich tissue. In the present study the slices were stored for 2-3 months in buffer before

osmium fixation was performed. Also, the use of buffer post-fixation should be re-evaluated. According to Prof Norbert Roos, leader at the electron microscopy-lab at the University of Oslo (pers. comm.), the slices could have been off better quality without storage in buffer after fixation, because of reversibility of the fixation. Consequently, it is likely that the choice of fixation protocol influenced the outcome, and that a different approach should be chosen for this lipid-rich tissue. Further investigation on fixation protocol is warranted to enable TEM in the PCLS studies.

4.4. Vtg production and gene expression

4.4.1. MEHP

Toxicological studies have been conducted mostly on the parent phthalates, only few toxicological data are available for the secondary phthalate metabolites, such as MEHP. In the present study, MEHP did not induce any significant effect on hepatic vtg production (Fig. 15, Table 8) or the *vtg1* (Fig. 14A, Table 5) and *esr1* (Fig. 14B, Table 6) gene expression. In contrast, Zhu et al. (2016) found a significant increase in plasma vtg level on male zebrafish exposed to 37.5mg/L MEHP for 81 days relative to the solvent controls. Both DEHP and MEHP exposure has been found to significantly increase the liver vitellogenin level in marine medaka males (Ye et al., 2014). Engel et al. (2017) however, found that the parent phthalates (DEHP) either stimulated or inhibited ERα and ERβ activity and inhibited AR activity *in vitro*, whereas the phthalate metabolites (MEHP) had no impact on the activity of these human hormone receptors. This led to the suggestion that phthalates and their metabolites may have different mechanisms of action, and that only the unmetabolized phthalates can bind to steroid receptors (Engel et al., 2017). A lack of estrogen receptor (ER) and androgen receptor (AR) mediated effects of MEHP in contrast to the steroid receptor-binding DEHP was also indicated in an *in vivo* study. (David, 2006). The current study found no estrogenic effect of MEHP, supporting the theory that the parent compound and MEHP might have different mechanism of action with the primary metabolite leading to endocrine effects via other mechanisms than hormone receptor modulation (e.g. deregulation of steroid hormone production). It should be noted that despite different molecular mechanisms of action, both DEHP and MEHP seem to contribute to adverse effects regarding reproduction and development (Engel et al., 2017).

Adeogun et al. (2018) concludes that previous studies also show discrepancies in the estrogenic effects of the parent compound DEHP. Potential estrogenic effects have been observed in

several *in vitro* and *in vivo* experiments (Adeogun et al., 2018; Maradonna et al., 2013; Uren-Webster et al., 2010; Ye et al., 2014). In contrast, it has been reported that DEHP had no effects on *vtg* induction in goldfish, *Carassius auratus* (Golshan et al., 2015) and Harris et al. (1997) found no estrogenic activity of DEHP in an *in vitro* assay. In a previous PCLS study, Bizarro and colleagues (2016) found no significant alterations in transcription levels of *vtga* following DEHP exposure. The described inconsistencies on estrogenic effects of DEHP could be explained by the differences in exposure routes, regimes (e.g. different time span) and species as pointed out by Adeogun (2018). It should also be noted, that *in vitro* systems may not necessarily parallel *in vivo* observations since *in vitro* systems lack the absolute metabolic competence of a whole organism and may, therefore, not uncover biological processes mediated through the secondary metabolites of DEHP or other secondary pathways (Mortensen & Arukwe, 2009). This would also apply to the *ex vivo* system in the present study.

In the present study, there was no significant difference in the relative expression of the genes believed to be possible markers for lipogenic effects in the MEHP exposure group compared to solvent control (Fig. 14.D-I). Although using the metabolite and not the parent phthalate, this is in line with the observations of Bizarro et al. (2016), who found no significantly altered transcription levels of *fabp* in a PCLS study on DEHP. In a 10-days study on male zebrafish (*Danio rerio*) (Uren-Webster et al., 2010), no changes in hepatic expression of any of the peroxisome proliferator activated receptor (PPAR) responsive genes, which participate in lipid homeostasis, were found. Noteworthy, in the same study, exposure to a high concentration of DEHP increased the levels of two PPAR responsive genes in the testis. It was suggested that the adverse effects induced by DEHP exposure may occur preferentially (but not exclusively) via PPAR signalling pathways in the testis and estrogen signalling pathways in the liver. The absence of significant effect of MEHP on PPAR-related gene expression in the liver tissue in the present study, could possibly be explained by the same theory regarding the pathway for effect on PPAR signalling. However, Meng et al. (2018) found that DEHP exposure reduced lipogenesis and influenced the expression of related genes in the liver of *P. fulvidraco*. In juvenile African sharptooth catfish (*Clarias gariepinus*), a biphasic pattern of effect was observed for *ppar-α* after exposure to DEHP (Adeogun et al., 2018). In the present study, no significant effect on the expression of PPAR responsive genes was observed in the MEHP exposure, but the gene-expression was only analysed in the slices exposed to the highest concentration of MEHP. A possible biphasic pattern related to concentration might be revealed

if qPCR analysis on the lower exposures of MEHP is performed. This is further discussed in section 4.6.

No change in gene expression of *cyp1a* (Fig. 14.C) were found in this study, correlating with previous findings on DEHP exposure in PCLS study done by Bizarro and colleagues (2016). Additionally, no differences in expression of *cyp1a* were found in fathead minnow embryos exposed to phthalates DEHP, DEP, DBP and BBP) for 96 h (Mankidy et al., 2013). Summarized, this indicates that MEHP, like the parent phthalates, seems to exhibit low potency as agonists of the aryl hydrocarbon receptor (AhR).

4.4.2. BPA

According to the vtg ELISA assay, the induction of vitellogenin production was significantly higher for the BPA 22 µM and mixture x-high exposure compared to the control in the current experiment (Fig. 15, Table 8). The same effect of BPA on vitellogenin production has been shown previously in an *in vivo* experiment on Atlantic cod, where a large increase in the level of vtg following exposure to 50 µg/L BPA in the surrounding water was documented (Larsen et al., 2006). Celius et al. (1999) also found an increase in vitellogenin production in primary hepatocytes from juvenile or male Atlantic Salmon after treatment of 1, 5 and 10 µM BPA, but not until 96 hours of exposure. In addition to the increased vtg production, a significant upregulation of *vtg1* (Fig. 14A, Table 5) and *esr1* (Fig. 14B, Table 6) genes were observed in the PCLS exposed to BPA and mixture compared to the solvent control in the present experiment. A strong correlation between vtg protein and *esr1* was also found (r=0.89, Fig. 16). Altogether an estrogenic effect is observed in this study, in line with a large amount of research on the estrogenic effect of BPA, cited by e.g. Flint et al. (2012), B.S. Rubin (2011) and Vandenberg et al. (2013).

No significant difference in *cyp1a* expression was seen in this experiment (Fig. 14C), indicating no potency as agonists of the aryl hydrocarbon receptor (AhR) through *cyp1a*. Olsvik et al. (2009) found a trend towards reduction in liver *cyp1a* after BPA exposure of Atlantic cod, but it was non-significant and no conclusion towards effect could be made.

In the genes related to lipid metabolism a significant downregulation was found in *hnf4a* in the PCLS exposed to x-high mixture ($p=0.0025$) (Fig. 14H, Table 7). No significant downregulation was observed within the defined level of significance ($p<0.05$) in the BPA exposure, nevertheless a borderline significance towards downregulating *hnf4a* ($p=0.061$, Table

7) was seen and is probably the most important driver of the effect seen in the PCLS exposed to the mixture. No significant effect on regulation were seen for the remaining genes believed to be possible markers for lipogenic effects (Fig. 14D-H). The BPA has been found to be a contributing factor in promoting obesity through several genes, such as *ppars, fas, lpl,* and *hsl* (Carnevali et al., 2017). However, no significant effect was found through the *hnf4α* in an *in vivo* lipid metabolism study on glycol dibenzoate (GDB) and BPA exposure in zebrafish (Santangeli et al., 2018). Nevertheless, in a lipid metabolism study on zebrafish (Forner-Piquer et al., 2018), and rare minnow (*Gobiocypris rarus*) (Guan et al., 2016), BPA altered the expression of several hepatic genes involved in the lipid metabolism following chronic exposure of three- and four-weeks, respectively. Several studies cited by e.g. Rubin (2011) and Elobeid and Allison (2008) refers to an effect of obesity-related pathways by BPA. The trend observed in the *hnf4a* expression in the present study might support this, however not a significant finding.

4.4.3. BT

In the current experiment, no significant estrogenic or antiestrogenic effects were observed in the liver slices exposed to BT. Previous studies have investigated the endocrine disrupting effects of BT, but the existing reports demonstrating the estrogenic potential of BT *in vitro* and *in vivo* are limited and contradictory. Induction of *vtg* gene expression in the liver was found to be significant in BT-exposed male and female marine medaka *(Oryzias melastigma)* (Tangtian et al., 2012a) both after 4 and 35 days of exposure, and in adult Chinese rare minnows (*Gobiocypris rarus*) after 28 d of BT exposure (Liang et al., 2014). These exposures were of longer duration than what is feasible in a PCLS study, and the effect increased in a clear dose- and time-dependent manner in the marine medaka study. The 48-hour exposure window could influence the lack of effect in the present study. BT could also have estrogenic effects through other pathways than those investigated in this study. Contradictory to the mentioned studies, Harris et al. (2007) demonstrated a clear antiestrogenic activity of BT in an *in vitro* study , but found no evidence of antiestrogenic activity in a contemporary *in vivo* assay on fathead minnows. This lack of estrogenic and androgenic activities *in vivo*, was confirmed in a 6-days exposure of zebrafish eleuthero-embryos to BT (Fent et al., 2014). In the same study, *in vitro* analysis showed significant antiandrogenic activity at high concentrations.

No change in *cyp1a* expression (Fig. 14C) or the genes believed to be possible markers for lipogenic effects (Fig. 14D-I) were observed in the BT exposure compared to solvent control,

indicating no effect on the Ahr pathway or lipid metabolism. This is contradictory to what was found in a marine medaka experiment (Tangtian et al., 2012b), where a decrease in *cyp1a1* were observed in the livers of both males and females following BT exposure. This was found after 35 days of exposure, but not in the 4 days exposure, indicating that the duration of exposure in the present study might be too limited to detect a potential effect on *cyp1a* expression. Additionally, gene expression of *pparα* were down-regulated in the zebrafish eluthero-embryos after 6 days of exposure to BT (Fent et al., 2014), suggesting a potential interference with the fatty acid metabolism. Nonetheless, this was not confirmed in the present study.

4.5. Mixture exposure

A significant induction of vitellogenin production (Fig. 15, Table 8) and enhanced expression of the genes related to the ER pathway (Fig. 14A-B, Table 5 and 6) were observed in both the BPA exposure and the mixture. Also, a strong correlation between vtg and *vtg1*, and vtg and *esr1* were found for the x-high mixture (r=0.94, and r=0.89 respectively, Fig. 16). Although not significantly different, the effect on both vtg, *esr1* and *vtg1* transcripts were lower in the mixture compared to the BPA exposure. A possible antagonistic mixture effect could be questioned, and further assessment is warranted. A greater downregulation of *hnf4α* were found in the mixture compared to the single compounds (Fig. 14I), and although not significant in the present study, a possible synergistic or additive effect might be questioned. BPA is suspected to be the most important driver of the mixture effect, being close to significantly different from the control with p=0.061 (Table 7). However, the MEHP and BT exposures could also contribute to a possibly additive or synergistic effect with p=0.13 and p=0.12 respectively (Table 7).

Several studies have observed divergent effect of compounds in mixture compared to the individual effect of each compound. Bizarro et al. (2016) observed effects of a mixture of DEHP, perfluorooctanoic acid (PFOA) and 17α-ethinylestradiol (EE2) on PCLS, which were not detected in single exposure, suggesting interactions between the studied pollutants. Increased vtg synthesis where found in fathead minnows (FHM) (*Pimephales promelas*) when five estrogenic chemicals, including BPA, were combined at concentrations that individually did not result in any induction (Brian et al., 2005). Crago and Klaper (2012) found a reduction in plasma testosterone concentrations in male FHM exposed to a mixture containing linuron and DEHP, but not in FHM exposed individually to the compounds, indicating a potential additive or synergistic effect of the compounds. Adverse morphological changes were found in

zebrafish embryos exposed to a mixture of BPA, DEHP, nonylphenol and fucosterol, which were distinct from what was found in embryos exposed to individual contaminants. The changes were in a manner that could not be explained by simple additive effects (Kinch et al., 2016) suggesting that synergism occur when individuals are exposed to a mixture of these chemicals. Antagonism has also been found in previous experiments. Duan et al. (2017) observed that zebrafish co-exposed to BT and Cadmium (Cd) reduced the acute toxic effects induced by Cd alone. The protective mechanism of BT to Cd exposure was explained by the forming of BT-Cd complex, which might prevent the receptor binding and thus have lower toxicity than Cd ions. Cd is a metal, and a direct comparison to the present study is not feasible, but it exemplifies the complexity of mixture toxicity, where both addition/synergism and antagonism could be crucial for the downstream effect.

Predicting mixture toxicity is challenging because of the potential joint effect. Different interactions can be found for different biological endpoints and for different combinations of contaminants as well as concentrations (Li et al., 2017). A different effect of the compounds in mixture compared to their individual effect could not be confirmed in this study, but a possible trend towards antagonistic mixture effect of the selected compounds on *esr1* and *vtg1* gene-expressions and synergistic or additive mixture effect on *hnf4a* expression should be noticed and further explored.

4.6. Non-monotonic dose response (NMDR)

For EDCs, like the BPA and DEHP, several reviews (Lagarde et al., 2015; Vandenberg et al., 2012; Vom Saal & Hughes, 2005) has been done pointing at the existence of the interrelated concepts; non-monotonic dose response (NMDR), and low dose effect. The term describes a dose-response relationship characterized by a curve whose slope changes direction within the range of tested doses. NMDR curves may have an U- or inverted U-shape, and complex, multiphasic curves have also been observed (Vandenberg et al., 2012). Biological effects at or below the dose cut-offs established by an expert panel has been showed in several hundred studies (Vandenberg et al., 2013).

The vitellogenin ELISA assay was done on all concentrations of the chemicals, and there was no indication of NMDR or low-dose effect on the vtg production in the present experiment (Fig. 15). Only the highest concentration groups were chosen for the histological evaluation and the

qPCR gene-expression assay due to a restricted budget. Hence, the analyses might have missed a low-dose cytotoxic effect on a lower concentration in the present study.

5. CONCLUSION

The TEM slices in the present study were not usable for assessment of effects on ultrastructural level. There is a need for further method development before utilization, but the potential of observing the initial effects of exposure to chemical stressors makes the method relevant for the PCLS procedure and further development is warranted. No adverse effects were observed on the histology of any of the exposures in the present study.

According to the effect on gene expression (*vtg1* and *esr1*) and vitellogenin production, the BPA showed estrogenic properties, while MEHP and BT induced no estrogenic activity in this study. In the BPA exposure, the gene expression on the molecular level can be related to the protein-translation of vtg, and the MOA and first steps of an AOP can be outlined and correlates well with other studies. A significant downregulation of the *hnf4a* was observed following the mixture exposure. A tendency of downregulation of *hnf4a* was also seen in the BPA exposure although not significant. The MEHP and BT exposure displayed a borderline significance, although weaker than the BPA-exposure. The BPA is suspected to be the most important driver regarding the mixture effect, and a lipogenic effect of BPA should not be excluded.

The MEHP and BT, did not have a significant effect on the gene-expression of the selected genes. The lack of effect of MEHP in the present study is in line with the theory that the parent compound (DEHP) and its metabolites (MEHP) might have different mechanism of action with the metabolites leading to endocrine effects via indirect mechanisms.

When assessing the effects related to the mixture of the selected plastic additives in the present study, no significant difference between the mixture exposure and the individual compound exposures were found. Thus, additive, synergistic or antagonistic effect could not be confirmed regarding the mixture. Still, one might cautiously remark a tendency for antagonistic mixture effect for the variables concerning estrogenic effect (*vtg1*, *esr1* and vtg), and possibly synergistic or additive effect on the lipid metabolism related gene *hnf4a*, thus warranting further investigation. As indicated in this study, the total mixture effect cannot necessarily be predicted from the effect of the individual compounds alone.

6. REFLECTIONS

Although not within the scope of the present experiment, it is worth mentioning a second feature that makes mixture toxicity even more complex regarding plastic compounds, and that is the possible adsorption of supplementary compounds to the plastic emitted in the marine environment. As an example, DEHP has a low water solubility (0.285 mg/L) and high octanol water partition coefficient (log K_{ow} = 7.6) that defines its hydrophobic character and adsorption to suspended materials (Magdouli et al., 2013). Such adsorption could give an increase in the concentration of the already inherent compounds, or supplement other compounds that could result in a different total effect. This illustrates the complexity of mixture effects related to the plastic additive pollution in our oceans, and why this part of ecotoxicology is in urgent need of more thorough research.

Plastics and its additives are not unconditionally a threat, used in the right way they certainly have benefits also from an ecological point of view. Focus on reducing single-use plastics and general reusing/recycling could undoubtedly decrease the demand, but the benefits of the plastics make it unrealistic to assume that the usage will come to an end any time soon. Thus, further research is profoundly important in order to make right decisions in future use of plastic, with special emphasis on the effect of the mixture of compounds.

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APPENDIX 1

Vitellogenin ELISA test, raw data

Vtg conc (ng/ml media)

	DMSO
Fish 1	17,6086885
Fish 2	7,93232626
Fish 3	0,66423925
Fish 4	0,12210369
Fish 5	2,70008172
Fish 6	0,31991642

Vtg conc (ng/ml media)

	BPA_0.022uM	BPA_0.22uM	BPA_2.2uM	BPA_22uM
Fish 1	13,8578979	28,9173956	11,5144981	40,7881938
Fish 2	2,80867371	5,36036451	8,32045537	94,3575448
Fish 3	0,72262735	0,60809388	1,62608701	19,7890465
Fish 4	0,14387963	0,12773816	0,16003012	3,99660017
Fish 5	1,88996359	1,79064306	2,13179072	6,54309412
Fish 6	0,2623178	0,30062763	0,38750736	6,12977165

Vtg conc (ng/ml media)

	BT_0.042uM	BT_0.42uM	BT_4.2uM	BT_42uM
Fish 1	12,339475	2,99494301	18,5156922	8,88922734
Fish 2	5,18284431	9,11559551	6,34925768	8,29909471
Fish 3	0,60610492	0,73186584	1,08540532	1,4739906
Fish 4	0,14387963	0,15580529	0,13550023	0,10772353
Fish 5	2,09187922	2,19951057	2,45030309	1,87404732
Fish 6	0,27370848	0,33694173	0,19645336	0,33694173

Vtg conc (ng/ml media)

	MEHP_0.1uM	MEHP_1uM	MEHP_10uM	MEHP_100uM
Fish 1	13,722	13,650	13,084	7,762
Fish 2	11,922	9,931	7,927	6,699
Fish 3	0,474	0,382	0,459	0,616
Fish 4	0,109	0,108	0,164	0,209
Fish 5	1,616	1,869	1,685	2,066
Fish 6	0,247	0,337	0,226	0,178

Vtg conc (ng/ml media)

	Mix_xLow	Mix_Low	Mix_High	Mix_xHigh
Fish 1	9,32439931	18,9603237	29,5168894	11,0475724
Fish 2	6,53597292	9,15496425	38,009167	78,3159673
Fish 3	0,60526455	0,70657896	1,2631992	20,1063662
Fish 4	0,12338719	0,16003012	0,16446744	3,16319704
Fish 5	2,08617849	1,21094337	2,0131411	6,33368491
Fish 6	0,32781963	0,24373483	0,46328531	5,89572678

APPENDIX 2

Q-PCR light cykler, raw data.

F1-F6 = fish 1 - fish 6
0=DMSO
4=MEHP_100uM
8=BPA_22uM
12=BT_42uM
16=Mix_xHigh

	ACTB2+RLP22	vtg1	vtg1	esr1	esr1
	cq Mean	cq Mean	Ratio	cq Mean	Ratio
F1-0	24,0925	28,06	1,05336104	32,785	0,23379781
F2-0	24,055	33,675	0,02094138	35,37	0,03796551
F3-0	23,5075	29,555	0,24913507	32,76	0,15858556
F4-0	25,4075	37	0,00533609	35,82	0,07096903
F5-0	23,2975	31,96	0,04066693	36,065	0,01387213
F6-0	23,97	36,475	0,00283487	35,28	0,03809732
F1-4	24,185	28,88	0,63617695	34,09	0,10088848
F2-4	20,8875	29,725	0,03602145	31,835	0,04897989
F3-4	24,1525	32,06	0,0686308	35,915	0,02784058
F4-4	25,5725	37	0,00598265	36,26	0,05865237
F5-4	22,6575	31,18	0,0448111	32,255	0,12485568
F6-4	25,31	36,29	0,00815839	37,11	0,02712625
F1-8	24,6975	26,15	6,02098699	28,635	6,31303318
F2-8	23,43	25,075	5,26889343	27,615	5,31781406
F3-8	23,8725	24,45	11,0425388	28,17	4,91889184
F4-8	25,0325	31,135	0,23981603	31,235	1,31342456
F5-8	23,3075	28,13	0,58236679	28,57	2,5198421
F6-8	24,95	28,035	1,94194185	29,7	3,59460522
F1-12	23,74	28,85	0,47714511	34,15	0,07109211
F2-12	23,275	30,55	0,10639483	35,27	0,02369676
F3-12	24,535	32,2	0,08119305	34,405	0,10336598
F4-12	26,645	37	0,01258196	36,27	0,12249827
F5-12	24,81	33,4	0,0427628	35,91	0,04406672
F6-12	24,86	37	0,00365098	36,34	0,03386252
F1-16	23,45	26,32	2,25401951	27,85	4,58154228
F2-16	23,695	24,595	8,83052982	28,02	4,82601812
F3-16	25,7275	27,285	5,59834346	29,145	9,05260706
F4-16	25,935	32,215	0,21205347	32,73	0,87105356
F5-16	22,8775	28,695	0,29219431	29,305	1,12375952
F6-16	26,0375	31,415	0,39639207	31,915	1,64528023
Kalib	23,4608333	27,5033333		30,0566667	

	cyp1a	cyp1a	scd5	scd5	acly	acly
	cq Mean	Ratio	cq Mean	Ratio	cq Mean	Ratio
F1-0	30,77	2,01158588	24,8	2,0729232	31,235	1,13026939
F2-0	32,17	0,74269066	24,935	1,83931257	31,445	0,95208789
F3-0	31,69	0,70874243	25,05	1,16204559	29,35	2,78304969
F4-0	33,55	0,7286679	28,3	0,45586124	33,23	0,7054749
F5-0	32,17	0,43931673	27,56	0,17636873	32,5	0,27105672
F6-0	31,335	1,24905182	28,255	0,17363945	34,38	0,11737253
F1-4	30,83	2,05741572	25,785	1,11664195	31,8	0,81460166
F2-4	27,295	2,425586	20,835	3,510476	27,055	2,22170408
F3-4	34,305	0,18090865	26,315	0,75610928	31,245	1,17012825
F4-4	33,44	0,88168385	28,365	0,48857998	33,565	0,6270562
F5-4	32,24	0,26856316	25,97	0,34072192	31,41	0,37027439
F6-4	32,93	1,04668936	29,615	0,1712489	35,18	0,17065643
F1-8	30,75	3,10228952	25,905	1,46577805	31,725	1,22405354
F2-8	32,015	0,53619636	24,895	1,22617651	31,185	0,73926661
F3-8	32,575	0,49425701	25,765	0,91172249	30,425	1,70133432
F4-8	34,205	0,35683606	28,26	0,36139956	33,97	0,32571152
F5-8	31,35	0,78096691	27,055	0,25202988	32,64	0,24770015
F6-8	32,165	1,38590977	28,135	0,37220431	34,66	0,19067223
F1-12	30,225	2,29872412	25,335	1,12051865	31,575	0,69938883
F2-12	31,35	0,76357053	25	1,02396519	30,52	1,05275277
F3-12	32,91	0,620212	26,58	0,82026767	31,36	1,40850693
F4-12	34,315	1,01103527	29,985	0,33428874	35,335	0,38666784
F5-12	33,555	0,47990919	29,44	0,13670772	33,295	0,44570674
F6-12	32,785	0,84723455	29,15	0,1730387	35,555	0,09633252
F1-16	30,025	2,15970361	24,195	2,01973591	31,615	0,55638933
F2-16	31,23	1,11021057	25,76	0,8089748	32,14	0,45823726
F3-16	33,025	1,30888045	27,075	1,33022166	30,835	4,63210086
F4-16	33,945	0,79875963	28,97	0,41298654	35,05	0,28800518
F5-16	32,2	0,32159867	26,655	0,24684318	31,455	0,4180266
F6-16	33,06	1,58373761	29,835	0,24344481	36,175	0,14177418
Kalib	31,1466667		25,22		30,78	

	cfbp	cfbp	acox1	acox1	hnf	hnf	cebp	cebp
	cq Mean	Ratio	cq Mean	Ratio	cq mean Kor	Ratio	cq mean Kor	Ratio
F1-0	26,495	0,3745768	29,7	1,7511872	27,3705493	2,5062694	27,2518621	1,4139281
F2-0	24,82	1,1654066	30,58	0,9271234	28,6014324	1,0215214	27,5770981	1,1068442
F3-0	22,9	3,0174573	29,875	1,0340696	27,2684013	1,7961198	26,6062444	1,4554941
F4-0	25,225	2,2475190	30,885	1,9163131	28,7597617	2,3318730	28,1644645	1,9035669
F5-0	24,51	0,8546072	30,195	0,7161498	28,8261579	0,5153651	27,03342	0,9439511
F6-0	23,635	2,4981036	31,28	0,5380579	28,1366591	1,3383690	28,3586352	0,6167227
F1-4	24,935	1,1775867	30,395	1,1533524	28,7342247	1,0175324	27,7275804	1,0945637
F2-4	24,19	0,2007307	26,01	USANN	25,0824347	1,3734498	23,649995	1,7309865
F3-4	22,705	5,4013998	31,4	0,5618798	29,8476376	0,4522668	27,2712792	1,4548371
F4-4	26,385	1,1276609	31,43	1,4725671	29,3420052	1,7311603	28,174173	2,1203167
F5-4	24,955	0,4028552	28,92	1,1121361	27,0538906	1,1599012	26,0188779	1,1989370
F6-4	25,18	2,1672016	32,485	0,5908375	29,8118858	1,0347382	30,0479206	0,5009176
F1-8	25,455	1,1714808	31,035	1,0557976	29,173461	1,0635617	28,1935901	1,1411027
F2-8	23,945	1,3859098	30,145	0,8127217	28,5401436	0,6917510	27,3004048	0,8645857
F3-8	23,09	3,4066018	30,37	0,9449653	29,2041054	0,5874757	27,305259	1,1711031
F4-8	25,38	1,5565299	31,075	1,2953423	29,0253465	1,4898838	28,174173	1,4582921
F5-8	25,325	0,4891447	30,43	0,6127342	28,5503584	0,6308594	27,3246761	0,7813371
F6-8	24,125	3,5084489	31,465	0,9335721	29,071313	1,3620181	28,4411578	1,1507517
F1-12	26,08	0,3911607	30,355	0,8710536	28,4073512	0,9421113	27,630495	0,8583343
F2-12	22,805	2,7431497	30,125	0,7401211	29,4594753	0,3240405	26,7178926	1,1491881
F3-12	25,28	1,1816750	30,535	1,3340690	28,2643441	1,8088193	27,6596206	1,4603709
F4-12	27,36	1,2065045	32,565	1,4101350	31,1295947	1,0268695	29,7954987	1,4976597
F5-12	25,915	0,9207193	31,715	0,7124365	29,122387	1,1921575	28,2664041	1,1746548
F6-12	24,59	2,3880505	32,15	0,5455689	28,7137951	1,6482592	28,9023133	0,7927084
F1-16	25,79	0,3911607	29,425	1,3573882	27,3194753	1,6646427	27,1402139	0,9764396
F2-16	22,885	3,4721598	30,355	0,8443033	29,1683536	0,5327833	27,5673895	0,8680667
F3-16	27,645	0,5242524	32,115	1,0198333	29,6944157	1,5018692	28,4751377	1,9279760
F4-16	27,14	0,8590615	32,19	1,1179327	30,7669694	0,8115024	28,6062029	2,0382655
F5-16	24,04	0,8847448	29,905	0,6544402	28,315418	0,5530099	26,6305157	0,9252692
F6-16	26,43	1,5087286	33,49	0,4874524	30,710788	0,9066109	30,5236389	0,6021907
Kalib	24,4466667		29,8766667		28,0498333		27,1159426	1,0000000

APPENDIX 3

LDH assay, raw data.

F1-F6 = fish 1 - fish 6
0=DMSO

1=MEHP_0.1uM	5=BPA_0.022uM	9=BT_0.042uM	13=Mix_xLow
2=MEHP_1uM	6=BPA_0.22uM	10=BT_0.42uM	14=Mix_Low
3=MEHP_10uM	7=BPA_2.2uM	11=BT_4.2uM	15=Mix_High
4=MEHP_100uM	8=BPA_22uM	12=BT_42uM	16=Mix_xHigh

	(Abs-Bg)-med		(Abs-Bg)-med	
F1-0	0,29	F1-5	0,22333333	
F2-0	0,213	F2-5	0,43533333	
F3-0	0,17866667	F3-5	0,17966667	
F4-0	0,2565	F4-5	0,41766667	
F5-0	0,41666667	F5-5	0,41566667	
F6-0	0,222	F6-5	0,30033333	
F1-1	0,24666667	F1-6	0,186	
F2-1	0,19733333	F2-6	0,284	
F3-1	0,226	F3-6	0,15866667	
F4-1	0,40766667	F4-6	0,27133333	
F5-1	0,36	F5-6	0,27733333	
F6-1	0,19166667	F6-6	0,176	
F1-2	0,20066667	F1-7	0,25833333	
F2-2	0,19766667	F2-7	0,356	
F3-2	0,15866667	F3-7	0,193	
F4-2	0,474	F4-7	0,23133333	
F5-2	0,37633333	F5-7	0,25466667	
F6-2	0,38433333	F6-7	0,39866667	
F1-3	0,167	F1-8	0,29866667	
F2-3	0,286	F2-8	0,17566667	
F3-3	0,29533333	F3-8	0,23633333	
F4-3	0,334	F4-8	0,301	
F5-3	0,31166667	F5-8	0,25166667	
F6-3	0,33233333	F6-8	0,214	

	(Abs-Bg)-med			(Abs-Bg)-med	
F1-9	0,16633333		F1-13	0,21133333	
F2-9	0,23366667		F2-13	0,30833333	
F3-9	0,12133333		F3-13	0,21033333	
F4-9	0,29366667		F4-13	0,22733333	
F5-9	0,317		F5-13	0,04466667	
F6-9	0,19733333		F6-13	0,20866667	
F1-10	0,20566667		F1-14	0,168	
F2-10	0,29133333		F2-14	0,21333333	
F3-10	0,17933333		F3-14	0,256	
F4-10	0,29166667		F4-14	0,44733333	
F5-10	0,424		F5-14	0,329	
F6-10	0,35066667		F6-14	0,27866667	
F1-11	0,233		F1-15	0,14733333	
F2-11	0,36966667		F2-15	0,27466667	
F3-11	0,11366667		F3-15	0,26266667	
F4-11	0,369		F4-15	0,493	
F5-11	0,389		F5-15	0,312	
F6-11	0,321		F6-15	0,46866667	
F1-12	0,22133333		F1-16	0,233	
F2-12	0,19266667		F2-16	0,25733333	
F3-12	0,16766667		F3-16	0,18133333	
F4-12	0,297		F4-16	0,406	
F5-12	0,32566667		F5-16	0,23966667	
F6-12	0,267		F6-16	0,123	

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