Phthalate metabolites in harbor porpoises (*Phocoena phocoena*) from Norwegian coastal waters

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ABSTRACT

The exposure of marine mammals to phthalates has received considerable attention due to the ubiquitous occurrence of these pollutants in the marine environment and their potential adverse health effects. The occurrence of phthalate metabolites is well established in human populations, but data is scarce for marine mammals. In this study, concentrations of 17 phthalate metabolites were determined in liver samples collected from one hundred ($n = 100$) by-caught harbor porpoises (*Phocoena phocoena*) along the coast of Norway. Overall, thirteen phthalate metabolites were detected in the samples. Monoethyl phthalate (mEP), mono-iso-butyl phthalate (mIBP), mono-n-butyl phthalate (mBP) and phthalic acid (PA) were the most abundant metabolites, accounting for detection rates $\geq$ 85%. The highest median concentrations were found for mIBP (30.6 ng/g wet weight [w.w.]) and mBP (25.2 ng/g w.w.) followed by PA (7.75 ng/g w.w.) and mEP (5.67 ng/g w.w.). The sum of the median phthalate metabolites concentrations that were found in the majority of samples (detection rates > 50%) indicated that concentrations were lower for porpoises collected along the coastal area of Bodø (Nordland), Lebesby (Finnmark) and Varangerfjord (as compared to other coastal areas); these areas are among the least populated coastal areas but also the most distant (> 700 km) from offshore active oil and gas fields. The monomethyl phthalate metabolite (mMP) was detected in 69% of the samples, and to our knowledge, alongside with PA, this is the first report of their occurrence in marine mammals. PA, as the non-specific marker of phthalate exposures, showed a statistically significant negative association with the body mass and length of the harbor porpoises. Among the phthalate metabolites, statistically significant positive associations were found between mBP and mIBP, mMP and mEP, PA and mEP, mIBP and mono(2-ethyl-5-oxohexyl) phthalate (mEOHP), mIBP and mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), mBP and mEHHP, mono-n-nonyl phthalate (mNP) and PA, and between monobenzyl phthalate (mBzP) and mNP. To our knowledge, this is the first study on the biomonitoring of 17 phthalate metabolites in harbor porpoises.

1. Introduction

Phthalates have received considerable public and scientific attention due to their widespread occurrence in the environment and their associations to adverse health effects (e.g., reproductive impairment) in organisms (AMAP, 2017; Casarett et al., 2013; Fourgous et al., 2016; Oehlmann et al., 2009). Phthalates are synthetic organic chemicals that impart flexibility and elasticity to plastics (also known as "plasticizers"), and thus used in numerous applications, including polyvinyl chloride (PVC) plastics, building materials, oil and gas drilling operations, and personal care products (Hart et al., 2018; Kassotis et al., 2016; Rocha et al., 2017; Tsochatzis et al., 2017). Phthalates are not chemically bound to the plastic product matrix, and therefore demonstrate a high leaching potential to abiotic and biotic environmental compartments (AMAP, 2017; Fourgous et al., 2016; Hu et al., 2016; Vorkamp et al., 2004).

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Table 1
Mean concentrations of reported phthalate metabolites in different species.

Species (n = samples)	Matrix	Concentration unit#	mMP	mEP	mIBP	mBP	mBzP	mEHP	mOP	mEOHP	mEHHP	Location	Study
Humans(n = 300)	Urine	ng/mL	10.4	278	73.8	113	3.83	36.1	1.66	36.6	53.8	Brazil	Rocha et al. (2017)
Humans(n = 130)	Urine	ng/mL	14.4	261	46.0	66.5	4.96	133	495	42.8	29.9	Saudi Arabia	Asimakopoulos et al. (2016)
Humans(n = 145)	Urine	ng/mL	-*	74.0	48.0	26.0	6.10	4.70	n.d.	10.0	21.0	Denmark	Frederiksen et al. (2013)
European eel(Anguilla anguilla)(n = 117)	Muscle	ng/g (d.w.)	5.70	33.0	206	174	2.00	282	82.0	34.0	94.0	France	Fourgous et al. (2016)
Wild marine fish(18 species; n = 69)	Whole fish	ng/g (w.w.)	n.d.**-2.59*#	0.06-4.70*#	-	n.d.-49.0*#	-	n.d.-24.8*#	n.d.-8.60*#	-	-	China	Hu et al. (2016)
Prawn(2 species; n = 20*##)	Whole prawns	ng/g (w.w.)	0.07-5.02*#	n.d.-6.82*#	-	2.81-37.5*#	-	n.d.-61.6*#	n.d.-1.13*#	-	-	China	Hu et al. (2016)
Mollusc(3 species; n = 6*##)	Whole molluscs	ng/g (w.w.)	0.02-1.71*#	0.42-3.31*#	-	5.31-34.5*#	-	7.50-11.6*#	0.41-1.19*#	-	-	China	Hu et al. (2016)
Roach(Rutilus rutilus)(n = 4)	Liver	ng/g (d.w.)	18.9	263	1610	1500	19.7	237	17.7	7.10	163	France	Valton et al. (2014)
American alligator (Alligator mississippiensis)(n = 9)	Urine	ng/mL	-	-	-	22.0	6.34	4540	-	-	-	U.S.	Brock et al. (2016)
Basking shark(Cetorhinus maximus)(n = 6)	Muscle	ng/g (l.b.)	-	-	-	-	-	84.2	-	-	-	Italia	Fossi et al. (2014)
Fin whale(Balaenoptera physalus)(n = 5)	Blubber	ng/g (l.b.)	-	-	-	-	-	177	-	-	-	Italy	Fossi et al. (2014)
Fin whale(Balaenoptera physalus)(n = 3)	Skin##	ng/g (d.w.)	-	-	-	984	32.1	n.d.	-	-	-	Mediterranean Sea	Baini et al. (2017)
Striped dolphin(Stenella coeruleoalba)(n = 2)	Skin##	ng/g (d.w.)	-	-	-	n.d.	n.d.	1720	-	-	-	Mediterranean Sea	Baini et al. (2017)
Rissó's dolphin(Grampus griseus)(n = 1)	Skin##	ng/g (d.w.)	-	-	-	n.d.	n.d.	464	-	-	-	Mediterranean Sea	Baini et al. (2017)
Bottlenose dolphin(Tursiops truncatus)(n = 1)	Skin##	ng/g (d.w.)	-	-	-	780	n.d.	1770	-	-	-	Mediterranean Sea	Baini et al. (2017)
Bottlenose dolphin(Tursiops truncatus)(n = 17)	Urine	ng/mL	-	11.0	n.d.	n.d.	n.d.	2.30	-	0.30	n.d.	U.S.	Hart et al. (2018)

Expressed in ng/mL, ng/g dry weight (d.w.), ng/g wet weight (w.w.), and ng/g lipid basis (l.b.).
* Not reported.
** n.d.: not detected.
*# Range of means.
*## Pool samples.
Samples included blubber tissue.

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Currently, phthalate metabolites are used as biomarkers to assess phthalate exposure in humans (Asimakopoulos et al., 2016; Frederiksen et al., 2007, 2013; Rocha et al., 2017) since their precursors (phthalates) are not always deemed reliable for this purpose (AMAP, 2017). The determination of precursor phthalates in biological samples is challenging due to the ubiquitous background occurrence of these chemicals, which substantially increases the contamination risk of samples during handling, sample preparation and instrumental analysis (AMAP, 2017; Tsochatzis et al., 2017). However, even though phthalates demonstrate a relatively rapid metabolism in marine mammals (Hu et al., 2016), reports of phthalate metabolites in marine mammals remain scarce. The toxicokinetics of phthalates are largely understudied among species, except for humans where the formation of specific metabolites is well-established (Rocha et al., 2017). Moreover, phthalate metabolites are considered widespread environmental pollutants (as standalones apart from their precursor phthalates) that are currently found in a variety of marine compartments (Blair et al., 2009; González-Mariño et al., 2017). It is noteworthy that the actual sample sizes in the marine mammal studies are limited ($n = 1$–17) compared to the larger sample sizes investigated in human studies (Table 1). Thus, these low sample sizes have tempered the efforts towards studying the toxicokinetics of phthalates in marine mammals, and particularly in relation to sex differences, tissue distribution and animal body size.

Harbor porpoises (*Phocoena phocoena*) are among the smallest marine mammals (Bjørge and Tolley, 2018; Rojano-Doñate et al., 2018) that inhabit Norwegian waters. They are top predator species that are exposed to a variety of human derived stressors, including chemical pollution (Bjørge and Tolley, 2018; Kleivane et al., 1995). Therefore, they are commonly used as a model to study pollutants in marine mammals (Norman et al., 2017). Earlier studies have reported the occurrence of persistent organic pollutants (POPs) in tissues of harbor porpoises, indicating that concentrations of selected POPs may potentially cause exposure-related health effects (Huber et al., 2012; Skaare, 1996; Weijs et al., 2010).

With this background, the present study aimed to establish liver concentrations of phthalate metabolites in a large harbor porpoise population ($n = 100$) that was sampled along the coastal waters of Norway. The objectives were to: (1) investigate the occurrence of phthalate metabolites; (2) assess the geographic patterns of the major phthalate metabolites in porpoises along the Norwegian coast; (3) establish correlations among the target metabolites; and (4) establish baseline concentrations needed for determining future trends in exposures. Sex differences and the relationship between liver concentrations and body mass and length were also investigated. To our knowledge, this is the first study on the occurrence of 17 phthalate metabolites in harbor porpoises.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards of phthalate metabolites, namely monomethyl (mMP), monoethyl (mEP), mono-n-butyl (mBP), mono-iso-butyl (mIBP), mono-n-pentyl (mPeP), mono-iso-pentyl (mIPeP), mono-n-hexyl (mHxP), monocyclohexyl (mCHP), mono-n-heptyl (mHpP), monobenzyl (mBzP), mono-n-octyl (mOP), mono(2-ethyl-1-hexyl) (mEHP), mono(2-ethyl-5-oxohexyl) (mEOHP), mono(2-ethyl-5-hydroxyhexyl) (mEHHP), mono-n-decyl (mDP), mono-n-nonyl phthalate (mNP) and phthalic acid (PA), and the deuterated internal standards, namely monoethyl-3,4,5,6-d$_4$ (mEP-d$_4$), mono-n-butyl-3,4,5,6-d$_4$ (mBP-d$_4$) and mono-n-nonyl phthalate-3,4,5,6-d$_4$ (mNP-d$_4$) were purchased from Chiron AS (Trondheim, Norway). Specific information regarding the target phthalate metabolites and their respective precursors is presented in Table S1. Individual stock solutions of each target analyte and internal standard were prepared by dissolution in acetonitrile and storage in the dark at -20 °C. β-Glucuronidase from *Helix pomatia* (type

HP-2, aqueous solution, $\geq 100,000$ units/mL) was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile of LC-MS grade were purchased from Merck (Darmstadt, Germany). Ethyl acetate, formic acid (98% v/v), hydrochloric acid (HCl), acetic acid ($\geq 99.0\%$) and ammonium acetate were purchased from Sigma-Aldrich (Steinheim, Germany). Orthophosphoric acid (85%) and sodium phosphate monobasic dihydrate ($\geq 99.0\%$) were purchased from VWR Chemicals (Trondheim, Norway). Water was purified with a Milli-Q grade water purification system (Q-option, Elga Labwater, Veolia Water Systems LTD, U.K.). The SPE cartridges, ABS Elut-NEXUS 60 mg/3 cc, were purchased from Agilent Technologies, Inc (Folsom, CA, U.S.).

2.2. Study population and sample collection

Liver samples were collected from 100 harbor porpoises by-caught in gillnets along the Norwegian coast during autumn (September-October) 2016 ($n = 55$) and winter (February-April) 2017 ($n = 45$). The samples were grouped into nine (9) major coastal areas: Hordaland/Rogaland ($n = 4$; samples obtained from 4 locations); Møre og Romsdal/Trøndelag ($n = 9$; 5 locations), Trøndelag/Nordland ($n = 5$; 5 locations), Bodø (Nordland) ($n = 3$; 3 locations), Lofoten (Nordland) ($n = 22$; 7 locations), Troms ($n = 33$; 15 locations), Troms/Finnmark ($n = 9$; 7 locations), Lebesby (Finnmark) ($n = 6$; 4 locations) and Varangerfjord (Finnmark) ($n = 9$; 1 location); the sampling locations are shown in Fig. 1. The latitude and longitude of the sampling locations are provided in Table S2. The sample population demonstrated a sex distribution of 54 females and 46 males, a body mass ranging from 17 to 74 kg (females: 17–74 kg; males: 19–59 kg), and a body length ranging from 101 to 173 cm (females 106–173 cm; males: 101–158 cm). In addition, blubber and muscle matrix from one animal was used to investigate the bioanalytical suitability of those compared to liver matrix. All weighed tissues were homogenized, and transferred to clean polypropylene (PP) tubes and stored in the dark at -20 °C.

2.3. Sample preparation

Extraction and isolation of the target analytes were performed according to Asimakopoulos et al. (2016) with minor modifications. A portion of 100 mg ($\pm$ 25 mg) of each tissue sample was transferred into a 15 mL PP tube, and 600 μL 1.0 M ammonium acetate (aqueous solution) were added, followed by 45 min ultrasonication. Thereafter, the samples were buffered with 600 μL of 1.0 M ammonium acetate (aqueous solution) that contained 22 units of β-glucuronidase (prepared by spiking 50 μL of β-glucuronidase into 100 mL of 1.0 M ammonium acetate solution) and were digested at 37° C for 12 h in an incubator shaker, to establish total concentrations (free and conjugated species). The samples were further centrifuged for 5 min, and the obtained supernatants were transferred into new 15 mL PP tubes, where they were diluted with 2 mL phosphate buffer (2 g sodium phosphate monobasic dihydrate dissolved in 100 mL milli-Q water and 1 mL orthophosphoric acid 85% v/v) and loaded onto the ABS Elut-NEXUS cartridges. Prior to the loading step, the SPE cartridges were conditioned with 1.5 mL acetonitrile and equilibrated with 1.2 mL phosphate buffer. After the loading step, the cartridges were washed with 2 mL aqueous formic acid (1% v/v) followed by 1.2 mL milli-Q water. The cartridges were then dried under vacuum for 5 min. Elution was performed with 1.2 mL acetonitrile followed by 1.2 mL ethyl acetate. Then, the eluates were collected into 15 mL PP tubes and concentrated to near dryness under a gentle nitrogen stream. Finally, the eluents were diluted to 500 μL with acetonitrile:milli-Q water (1:9 v/v), centrifuged for 5 min, and the supernatants were transferred into a glass vial for UPLC-MS/MS analysis.

2.4. UPLC-MS/MS analysis

The chromatographic separation was carried out using an Acquity UPLC I-Class system (Waters, Milford, U.S.) coupled to a triple
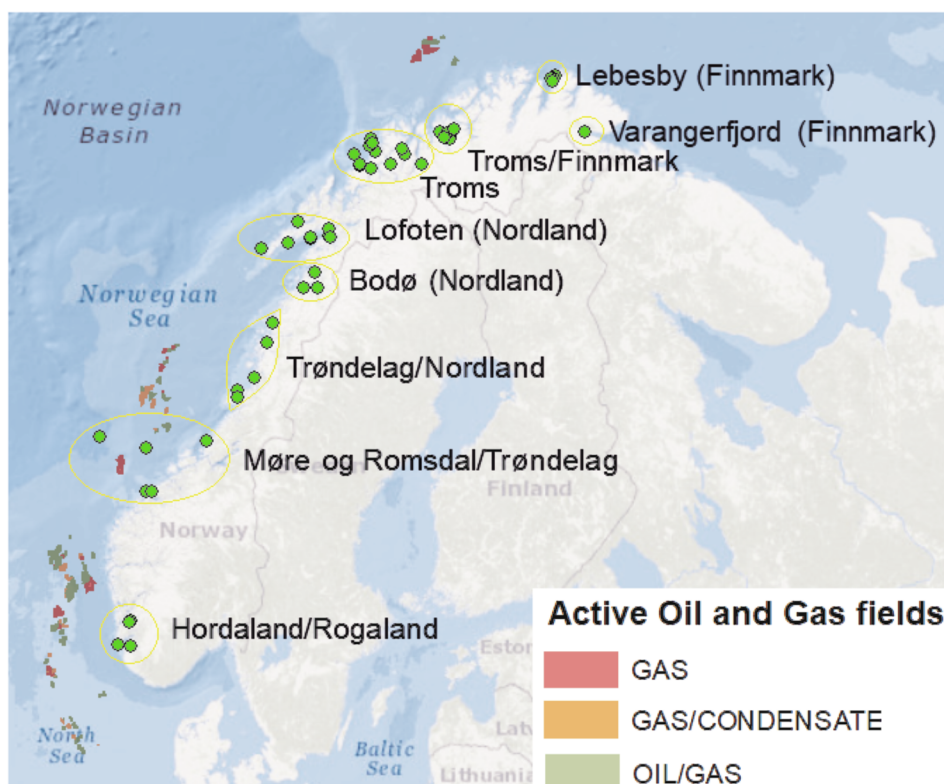
Fig. 1. Distribution of the sampling locations of the harbor porpoises that were by-caught along the coast of Norway during September-October 2016 and February-April 2017.

quadrupole mass analyser (QqQ; Xevo TQ-S) with a *Z*Spray ESI ion source (Waters, Milford, U.S.). The used LC column was a Kinetex C18 (50 × 2.1 mm, 1.3 μm) connected to a Phenomenex C18 guard column (2.0 × 2.1 mm). The column temperature was set at 30 °C. The chromatographic separation was carried out using a gradient elution program with an aquatic (milli-Q water with 0.1% v/v acetic acid) and an organic phase (acetonitrile with 0.1% v/v acetic acid) as binary mobile phase at a flow rate of 0.40 mL/min. The electrospray ionisation (ESI) was applied at a potential of −3.0 kV. The cone and source offset voltages were set at 20 and 50 V, respectively. The desolvation and cone gas flow rates were set at 1000 and 150 L/h, respectively. The collision gas flow was set at 0.15 mL/min, and the nebulizer gas pressure was set at 87 psi. The source and desolvation temperatures were set at 150 and 350 °C, respectively. The method limits of detection (LODs) and quantification (LOQs) of the target analytes are presented in Table S3. Quantification of the target analytes was accomplished based on the internal standard method and with matrix-matched standard addition calibration standards prepared by spiking target analytes into the specified matrices prior to extraction (Asheim et al., 2019; Asimakopoulos et al., 2016). More details concerning the UPLC-MS/MS analysis are available in Supplementary data (Tables S4–S7).

2.5. Quality assurance and quality control (QA/QC)

Procedure blanks were analysed to monitor and control background contamination arising from laboratory materials and solvents. A 10-point calibration curve in concentrations ranging from 0.10 to 50.0 ng/mL (0.10, 0.20, 0.50, 1.00, 2.00, 5.00, 10.0, 20.0, 25.0, and 50.0 ng/mL) was prepared, and demonstrated a satisfactory regression coefficient for every phthalate metabolite ($R^2 > 0.998$). Pre- and post- extraction spiked matrix samples were used as QA/QC samples and were prepared by spiking known concentrations of the target analytes and internal standards prior and post to the sample preparation (extraction and clean-up). To monitor the drift in instrumental sensitivity and

carry-over effects during analysis, a calibration check standard and a methanol solvent blank solution was injected, respectively, after the analysis of 25 consecutive samples.

2.6. Data analysis and statistical treatment

UPLC-MS/MS data was acquired with the MassLynx v4.1 software, while quantification processing was performed with TargetLynx (Waters, Milford, U.S.). Excel (Microsoft, 2018) was used for general descriptive statistics. SPSS Statistics (IBM, version 25) was used for Pearson correlation analysis and for the statistical tests of Shapiro-Wilk, Mann Whitney U, and Kruskal Wallis H. Since the data (concentrations) were not normally distributed (even when log-transformed; confirmed in both cases by the Shapiro-Wilk test), statistical differences were assessed by the non-parametric tests: the Mann Whitney *U* test, when comparing differences between two independent groups of data; and the Kruskal Wallis H-test, when comparing differences between three or more independent groups of data. Data analysis did not include censored data (i.e., non-detects; NDs). Concentrations were reported as ng/g wet weight (w.w.). The statistical significance was set at p < 0.01 (unless stated otherwise). Geographic information for sampling locations was managed by ArcMap 10.7 (Esri, California, U.S.) using the exprodat ArcGIS platform that comprised publically available petroleum data (map released 23.11.2018).

3. Results and discussion

3.1. Preliminary screening for the bioanalytical suitability of liver

To obtain insight on differences in tissue bioanalysis of phthalate metabolites, the methodology that was developed for biomonitoring in liver was also applied to blubber and muscle. All three sample matrices were obtained and analysed in triplicates (*n* = 3) and the results are shown in Table S8. Overall, 10 metabolites were detected in liver (Fig.

S1), followed by 9 and 6 metabolites that were detected in muscle (Fig. S2) and blubber (Fig. S3), respectively. In addition, it was observed that the more polar metabolites (short-chained, e.g., mEP and PA; carbon chain of metabolite containing up to 4 carbons) were found mostly in liver and muscle. The mEP concentrations were significantly higher (~6-fold) in the liver than the muscle matrix, while mEP was not detected in blubber. The mMP concentrations were ~1.5-fold higher in the liver than those determined in both muscle and blubber. The isomers, mBP and mIBP, were found in similar concentrations in muscle and liver, but in the blubber, mBP was not detected and mIBP was determined in trace concentrations. mHxP and PA were determined in trace concentrations in all three matrices, although the concentration of the latter, being the most polar metabolite from the phthalate metabolites class, was found in higher (~2-fold) concentrations in the muscle than the blubber. In contrast to PA (no carbon chain on its structure), mOP contains an octane carbon chain and was found in higher concentrations (~2-fold) in the blubber than both the muscle and liver. mBzP and mEOHP were only detected in the liver, while mEHHP was only detected in the muscle. Although the liver provided higher analytical uncertainties potentially due to its higher metabolic activity compared to muscle and blubber (Ashrap et al., 2017; Dudda and Kürzel, 2006), most metabolites were detected in liver, and consequently, this matrix was selected for biomonitoring phthalate metabolites.

3.2. Liver concentrations of phthalate metabolites

The liver concentrations of phthalate metabolites determined in harbor porpoises are shown in Table 2 (and in Table S9 the concentrations are shown per coastal area). Four metabolites, namely mEP, mIBP, mBP and PA, showed detection rates (DRs) ≥ 85%. The highest median concentrations were found for mIBP (30.6 ng/g w.w.) and mBP (25.2 ng/g w.w.), followed by PA (7.75 ng/g w.w.) and mEP (5.67 ng/g w.w.). To our knowledge, this is the first report of PA in marine mammals. PA is a hydrolysis product of phthalates and can be used as a non-specific biomarker that can indicate overall exposure to phthalates (Kluwe, 1982). The low molecular weight metabolites (mEP, mMP, mIBP and mBP) were determined in abundance (higher concentrations and DRs) in liver compared to those of high molecular weight (mHxP, mEHHP, mNP, mOP, mEOHP, mHpP, mBzP and mDP). Studies have shown that phthalates with shorter ester chains are more susceptible to metabolic breakdown than those with longer ester chains (Chang et al., 2004; Jianlong et al., 2000). The rank order of DR for the remaining metabolites in the liver was: mMP (69%) > mHxP (45) > mEHHP (27) > mNP (23) > mOP (21) > mEOHP (17) > mHpP (11) > mBzP (10) > mDP (2). Three metabolites, namely mPeP, mIPeP and mCHP were not detected. Although the metabolites, mEP, mBP and mBzP were previously reported in tissues of marine mammals, including blubber, skin and urine, they were only investigated in a few animals in those studies (Table 1).

Table 2
Occurrence of phthalate metabolites in liver samples (*n* = 100).

Target analytes	Detection rate (%)	Mean(ng/ g w.w.)	Median(ng/ g w.w.)	Min(ng/g w.w.)	Max(ng/g w.w.)
mEP	100	5.99	5.67	2.62	17.4
mIBP	99	41.1	30.6	3.78	419
mBP	97	37.2	25.2	1.73	299
PA	85	9.22	7.75	0.21	42.0
mMP	69	2.24	1.72	0.34	8.72
mHxP	45	0.98	0.63	0.17	4.64
mEHHP	27	1.39	1.02	0.17	5.93
mNP	23	48.1	48.2	24.0	98.9
mOP	21	1.19	0.63	0.19	5.53
mEOHP	17	0.61	0.36	0.20	2.12
mHpP	11	0.68	0.45	0.21	1.87
mBzP	10	3.02	2.08	0.69	7.29
mDP	2	10.1	12.4	0.25	17.6

The mEHP metabolite was found in high concentrations (median: 39.9 ng/g w.w.; max.: 331) in all samples but was also found in reagent blanks. Thus, this metabolite was not used here for further assessment since it was not considered a reliable biomarker for di(2-ethyl-1-hexyl) phthalate (DEHP) exposure, even though its concentrations were found similar to previous studies (Table 1). Exposure to DEHP is often estimated based on the sum concentration of its metabolites in biological matrices (Rocha et al., 2017), which in the present study consisted of three metabolites, namely mEHP, mEHHP and mEOHP. Asimakopoulos et al. (2016) and Rocha et al. (2017) previously excluded mEHP from the estimation of DEHP exposure in humans since the former can be formed by abiotic processes (Heudorf et al., 2007), which can occur spontaneously (hydrolysis) in the environment. In contrast to mEHP, the remaining DEHP metabolites, mEHHP and mEOHP, are secondary oxidative metabolites that are produced only *in vivo* (Asimakopoulos et al., 2016), and the risk of background contamination for those during sample handling, preparation and instrumental analysis is limited. Even so, when the sum concentration of the DEHP metabolites was calculated, without excluding mEHP concentrations, the results were comparable with previously reported DEHP concentrations in marine mammals (Table S10). In relation to this, an important challenge associated with the estimation of DEHP exposure using its metabolites as biomarkers is that DEHP can demonstrate significantly different metabolic profiles among species (Albro et al., 1982; Frederiksen et al., 2013). For instance, a previous study on the DEHP metabolite distribution showed that mEHHP accounts for 38.2% of the urinary metabolites in green monkey (*Chlorocebus sabaeus*), but only 3.40% of those in guinea pig (*Cavia porcellus*) (Albro et al., 1982). Therefore, with no species-specific information on the metabolic products of DEHP in harbor porpoises, an actual estimation of DEHP exposure is tempered. The secondary metabolites of DEHP, namely mEHHP and mEOHP, were detected in 27 and 17% of samples, respectively. The mEHHP metabolite demonstrated higher concentrations (median: 1.02 ng/g w.w.) than those of mEOHP (median: 0.36 ng/g w.w.), suggesting the predominance of the former in the liver. On the contrary, mEOHP was detected (mean: 0.30 ng/mL), while mEHHP was not detected in urine from bottlenose dolphins (*Tursiops truncates*) (Hart et al., 2018).

In the present study, the isomers mBP and mIBP were detected in more than (≥) 97% of harbor porpoise liver samples. The isomers, mIBP and mBP, were previously reported with DRs of 23 and 19%, respectively, in muscle media from the European eel (*Anguilla anguilla*) (Fourgous et al., 2016), and mBP was reported in 42.1% of skin samples from four marine mammal species [fin whale (*Balaenoptera physalus*), bottlenose dolphin (*Tursiops truncatus*), Risso's dolphin (*Grampus griseus*) and striped dolphin (*Stenella coeruleoalba*)] (Baini et al., 2017). It is noteworthy that the skin samples obtained by Baini et al. (2017) included blubber tissue. The concentrations of di-n-butyl phthalate (DBP) measured in marine mammals from Greenland and Faroe Islands (Table S10) were within the same order of magnitude as the mBP concentrations measured in the present study.

For mEP, the results shown here were similar to those of Hart et al. (2018) who reported mEP being abundant (high concentrations and DR) in bottlenose dolphin urine. The precursor diethyl phthalate (DEP) was previously reported in concentrations ranging from 15.1 to 31.6 ng/g w.w. in livers from four species (sampled in Greenland and the Faroe Islands), namely polar bear (*Ursus maritimus*), minke whale (*Balaenoptera acutorostrata*), pilot whale (*Globicephala melas*) and ringed seal (*Phoca hispida*) (Vorkamp et al., 2004). The order of magnitude of DEP concentrations reported in that study corresponds to those of mEP in the present harbor porpoises.

The mMP metabolite was detected in concentrations reaching up to 8.72 ng/g w.w. in the liver of harbor porpoises, and to our knowledge, this is the first report of its occurrence in marine mammals. However, its precursor dimethyl phthalate (DMP) was previously reported in concentrations ranging from 2.50 to 8.40 ng/g w.w. in livers from marine mammal species (e.g., seals and whales) (Vorkamp et al., 2004).

The order of magnitude of DMP concentrations reported in those studies corresponds to those of mMP in this study (similarly to DEP and mEP as mentioned above).

In human biomonitoring studies, mHxP was detected in < 13% of the populations with median concentrations < 1.00 ng/mL (Asimakopoulos et al., 2016; Rocha et al., 2017). It is challenging to compare marine mammal liver with human urine concentrations, but relatively low concentrations of mHxP were determined in both matrices. Nonetheless, the detection rate in the livers of the harbor porpoises was higher than human urine, reaching up to 45%. The precursor di-n-hexyl phthalate (DHxP) was previously determined in marine mammal livers (Vorkamp et al., 2004), while it was not detected in skin samples (Baini et al., 2017). Moreover, the mBzP concentrations found in the muscle tissue of European eel (from the French Mediterranean coast) were similar to those reported here but accounted only for 0.2% of the total measured phthalate metabolite content (Fourgous et al. 2016). mBzP was found with a DR of 57.9% in the skin of fin whale (Balaenoptera physalus) that was also sampled in the Mediterranean Sea (Baini et al., 2017). Thus, it is noteworthy that relatively high concentrations of selected phthalate metabolites (Table 1) and precursors (Table S10) in the skin or muscle samples of species from the Mediterranean Sea can be possibly attributed to the increased pollution pressures of the specific locations (Baini et al., 2017; Fourgous et al. 2016) rather than the species themselves.

The mOP concentrations in the harbor porpoise liver samples ranged from 0.19 to 5.53 ng/g w.w. and were found within the same order of magnitude as the di-n-octyl phthalate (DnOP; precursor) concentrations determined in skin samples from marine mammals (Baini et al., 2017). DnOP is reported to demonstrate extensive metabolism to mOP and other oxidation species in Sprague–Dawley rat liver (Silva et al., 2005), while in roach (Rutilus rutilus) liver it was shown that mOP was found in higher concentrations compared to other investigated biological matrices (Valton et al., 2014).

3.3. Exposure profiles and correlations

The mEP concentrations were significantly higher ($p < 0.05$) in 2016 (median: 6.43 ng/g w.w.) than in 2017 (median: 4.95 ng/g w.w.), while for the other assessed metabolites there was no statistical difference between the sampling years. Statistically significant sex differences in the profiles of phthalate metabolites were also not identified. The sum of the median phthalate metabolites concentrations (Σ_5PhMet: sum of 5 metabolites; median) that were found in the majority of samples (DR > 50%; mEP, mMP, mIBP, mBP and PA, except for mEHP) was calculated (Fig. 2) and their geographic distribution along the Norwegian coast was assessed. The Σ_5PhMet concentrations were lower for porpoises collected along the coastal area of Bodø (Nordland), Lebesby (Finnmark) and Varangerfjord. These areas are among the least populated coastal areas (Fig. S4), but also the most distant (> 700 km) from the active oil and gas fields of the North, Norwegian and Barents Sea (Fig. 1), indicating potential concentration dependence to human coastal development and offshore activities. The Σ_5PhMet concentrations in Troms/Finnmark, Lebesby (Finnmark) and Varangerfjord (Finnmark) demonstrated a significant association ($r = 0.87$) to the log-transformed human population sizes in these areas (Fig. S4). On the contrary, an increasing concentration trend was observed from Hordaland/Rogaland (South Norway) to Trøndelag/Nordland (Mid-Norway), even though the populations in each area were within the same order of magnitude (Fig. S4). In northern Norway, the highest Σ_5PhMet concentration was found in Troms followed by those concentrations found in Lofoten (Nordland) and Troms/Finnmark (Fig. 2). It is noteworthy that Lofoten (Nordland) maintains significant fishing, aquaculture and tourism industry activity, and recently it was documented that plastic pollution has significantly impacted its marine ecosystem (Bråte et al., 2017; Sundt et al., 2015).

A strong significant correlation was observed between mIBP and mBP metabolites ($r = 0.87$), suggesting similar sources and concomitant exposure routes. The correlations between these two isomers were also reported in earlier studies in humans but also in the European eel (Fourgous et al., 2016; Rocha et al., 2017). Their precursors, DIBP and DBP, demonstrate similar application properties, and can therefore be used as an isomer mixture in industrial applications (Maag et al., 2010). Other significant correlations observed were between: mMP and mEP ($r = 0.37$); PA and mEP ($r = 0.41$); mIBP and mEOHP ($r = 0.53$, $p < 0.05$); mIBP and mEHHP ($r = 0.43$, $p < 0.05$); mBP and mEHHP ($r = 0.47$, $p < 0.05$); mNP and PA ($r = 0.49$, $p < 0.05$); and between mBzP and mNP ($r = 0.89$, $p < 0.05$).

Body mass and length of the animals may influence accumulation and biotransformation processes. For instance, in harbor porpoises, the hepatic concentrations of per- and poly-fluoroalkyl substances (PFASs) were shown to be negatively associated with body mass (Huber et al., 2012), while legacy POPs (PCBs and DDT) concentrations in blubber appeared to increase with body mass (Kleivane et al., 1995). In the present study, there was a significant negative correlation between PA and the body mass and length ($r = -0.29$ and -0.32, respectively), indicating that the biotransformation process may be more efficient in larger animals. Nevertheless, for the other metabolites there were no correlations with the body mass and length of the porpoises. In general, the results indicated that there is a balance between uptake and biotransformation of phthalates in harbor porpoises. However, it is also possible that the negative correlation identified for PA is related to dietary differences, and thus exposure, among animals with smaller and larger body mass and length. Recent studies have shown that newborn harbor porpoises (smaller body mass and length) stranded in the Netherlands and the Aegean Sea in Greece did not have any plastic debris in their stomach (Alexiadou et al., 2019; van Franeker et al., 2018). However, van Franeker et al. (2018) found no differences between juveniles and adults, nor between the sexes with respect to frequencies of occurrence of plastic litter in their stomachs (and thus potentially plasticizers such as phthalates). In addition, van Franeker et al. (2018) indicated that harbor porpoises that ingested benthic fishes had higher frequencies of ingesting plastic litter than those that had ingested pelagic fish. Thus, it is possible that prey differences could indirectly influence exposure to phthalates in porpoises since benthic feeders could ingest more plastic litter when feeding as compared to pelagic feeders. Nonetheless, it must be highlighted that ingestion of plastic debris is not the sole source of potential phthalate exposures.

The occurrence profile of phthalate metabolites found in liver from harbor porpoises demonstrated some similarities to those found in human urine (Asimakopoulos et al., 2016). The metabolites, mMP, mEP, mBP, mIBP and PA are found frequently in both human urine and harbor porpoise liver. Similarly, low DRs were found in both matrices for mHxP, mOP and mHpP. The major differences between the matrices were observed for mEHHP and mEOHP, which were frequently found in human urine, but not in the liver of harbor porpoises. This indicates that mEHHP and mEOHP may be more readily excreted in urine as final excretion products than in liver where metabolism is considered ongoing.

4. Conclusions

To conclude, this is the first study to document phthalate metabolites concentrations in harbor porpoises, suggesting a novel approach in assessing phthalate exposures in marine mammals by measuring their metabolites, and not their respective precursors, as performed extensively up to now. Higher concentrations of phthalate metabolites were measured in animals inhabiting waters adjacent to areas with higher human activity and populations, while sex differences were not identified. Furthermore, the concentration profiles along the coast of Norway indicated that the harbor porpoises can be potentially used as tracers of phthalate (plasticizers) pollution in the marine environment.
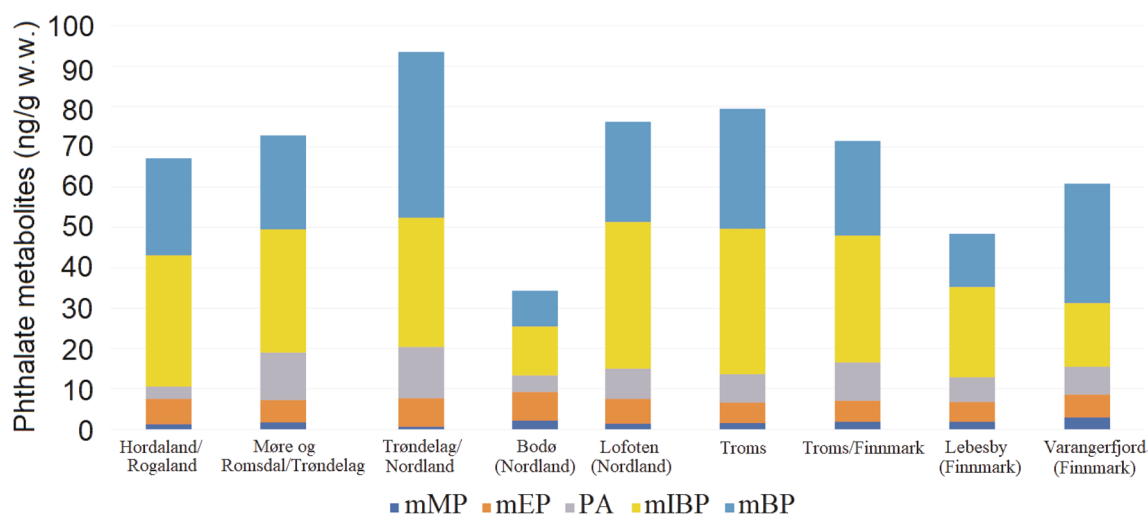
Fig. 2. Concentration profiles of the sum concentrations (Σ_5PhMet) of the major phthalate metabolites in liver samples of harbor porpoises collected along nine major coastal areas of Norway [Hordaland/Rogaland ($n = 4$); Møre og Romsdal/Trøndelag ($n = 9$), Trøndelag/Nordland ($n = 5$), Bodø (Nordland) ($n = 3$), Lofoten (Nordland) ($n = 22$), Troms ($n = 33$), Troms/Finnmark ($n = 9$), Lebesby (Finnmark) ($n = 6$), Varangerfjord (Finnmark) ($n = 9$)]; the median concentration values for each metabolite were used to generate the profiles in the chart.

5. Animal ethics permit

All animals were unintended by-catches in legal coastal fisheries and the animals were deceased. Thus, no handling or sampling were performed on live animals.

CRediT authorship contribution statement

May Britt Rian: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft. **Kristine Vike-Jonas:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing - review & editing. **Susana Villa Gonzalez:** Investigation, Methodology, Resources, Supervision, Writing - review & editing. **Tomasz Maciej Ciesielski:** Investigation, Methodology, Resources, Supervision, Writing - review & editing. **Vishwesh Venkatraman:** Data curation, Formal analysis, Writing - review & editing. **Ulf Lindstrøm:** Funding acquisition, Investigation, Project administration, Resources, Writing - review & editing. **Bjørn Munro Jenssen:** Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Alexandros G. Asimakopoulos:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105525.

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