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Abstract

Monitoring parameters for the assessment of oil and gas related contaminants and their biological effects need validation before application in the Arctic. For such monitoring purposes, we evaluated the potential use of three biomarkers (acetylcholinesterase, acyl-CoA oxidase and glutathione S-transferase) for application to an Arctic bivalve (*Astarte borealis*) and determined the body residue of pyrene and two pyrene metabolites (1-hydroxypyrene and pyrene-1-glucuronide) in Arctic benthic species (bivalve: *Macoma calcarea*; polychaete: *Nephtys ciliata*) and temperate benthic species (bivalve: *Limecola balthica*; polychaete: *Alitta virens*) in order to establish the potential of polycyclic aromatic hydrocarbons (PAHs) metabolite profiles as biomarkers of exposure in such species. Experimental PAH exposure levels were probably too low (0.2 – 1.7 mg/kg dry weight in sediment) to induce or inhibit biomarker responses in *A. borealis*. Concentrations of pyrene and pyrene metabolites varied between species, although no consistent patterns could be established among taxonomic groups and locations. Metabolites made up to 79% of the total pyrene concentrations, indicating that basal metabolic activity is affecting pyrene kinetics even at low concentrations in all species. This indicates that Arctic and temperate species could show similar metabolism patterns of PAHs, although more insight into the effects of confounding factors is needed.

Keywords: Arctic; Benthic macroinvertebrates; PAHs; Biomarkers; Biotransformation; Metabolites

1. Introduction

Current information on potential effects of oil and gas (O&G) activities on the Arctic ecosystem is still inadequate or unavailable since chronic toxicity data are largely absent for Arctic species (Camus et al. 2015; Bejarano et al. 2017). More information is needed because

the retreat of sea-ice in the Arctic will cause an increase in O&G related activities (AMAP 2010). Hence, studies on assessing the sensitivity of Arctic biota to O&G related chemicals are essential, to ensure that O&G risk assessment procedures are targeted to the native Arctic species.

Polycyclic aromatic hydrocarbons (PAHs) are the main organic pollutants related to O&G activities, and have been identified as an emerging concern in the Arctic (Laender et al. 2011). Sequestered PAHs in water or terrestrial surfaces may be mobilized into aquatic systems after melting of sea ice and thawing of permafrost with global warming (Ma et al. 2016), potentially increasing PAH exposure in aquatic organisms (Pouch et al. 2017). Once released into the marine environment, partitioning of PAHs to settling particulate organic matter generally results in contamination of sediments and chronic exposure of benthic organisms (Burkhard et al. 2005; Weisbrod et al. 2009). Accumulation of PAHs by benthic organisms depends on several factors such as the species' feeding behavior, physiological responses to PAH exposure, like detoxification and excretion of PAHs (Amiard-Triquet and Berthet 2015), as well as sediment characteristics (Selck et al. 2012). Biological responses (biomarkers) to exposure to PAHs may be used as indicators of exposure to, or sublethal ecological effects of O&G related activities, and the use of such biomarkers may provide an early warning for hazard (Esler et al. 2002) and ecological risk assessment (Eason and O'Halloran 2002; Hagger et al. 2006).

So far, studies have documented effects of oil at the cellular, individual and community level in Arctic benthic communities (Camus et al. 2002, 2003; Olsen et al. 2007a,b), but they provided little information on the specific chemicals in the complex oil matrix causing the observed effects. Although PAHs are the major group of organic contaminants in oil, there is a large knowledge gap on their specific role in causing responses of biochemical biomarkers in Arctic benthos chronically exposed to O&G derived chemicals (Jewett et al. 2002).

Furthermore, almost no information on PAH metabolites in benthic invertebrates is available, that may be used to assess oil-derived hazards and risks for Arctic benthic organisms (Carrasco-Navarro et al. 2015). In order to address these knowledge gaps the aims of the present study were to (1) identify the responsiveness of biochemical biomarkers of O&G exposure and effects in Arctic benthic species, and to (2) evaluate the use of metabolite profiles of a model PAH (i.e., pyrene) for monitoring of exposure and effects in Arctic benthic invertebrates. These aims were addressed experimentally, using representative species of the Arctic macrobenthic community (the suspension feeder *Astarte borealis* [Schumacher, 1817], the suspension/deposit feeder *Macoma calcarea* [Gmelin, 1791] and the predator/deposit feeder *Nephtys ciliata* [O.F. Müller, 1776]), which were compared to temperate counterpart species (the suspension/deposit feeder *Limecola balthica* [Linnaeus, 1758] and the omnivore/deposit feeder *Alitta virens* [Sars, 1835], formerly known as *Macoma balthica* and *Nereis virens*, respectively).

Selected biomarkers of exposure were the peroxisomal *β*-oxidation enzyme acyl-CoA oxidase (AOX, E.C.1.3.3.6) and the Phase II biotransformation enzyme glutathione S-transferase (GST, E.C.2.5.1.18). Certain organic xenobiotics such as PAHs can notably induce the activity of peroxisomal *β*-oxidation enzymes in mussels, which produce H_2O_2 during the oxidation of fatty acids. GSTs play a multiple role in the metabolism of xenobiotics by catalyzing conjugation reactions with glutathione (GSH) and by binding xenobiotics. As biomarker of neurotoxicity acetylcholinesterase (AChE, E.C.3.1.1.7) was selected. AChE terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Binding of organophosphate and carbamate pesticides to AChE results in the accumulation of acetylcholine at the synapses, which disrupts the nervous system function.

GST is widely used as a biomarker of PAH/oil exposure (Breitwieser et al. 2018) and it has shown little seasonal variation in digestive glands of Icelandic scallops (Nahrgang et al. 2013)

and of mussels (Power and Sheehan 1996). AOX shows a very rapid response to PAH exposure in mussels and, unlike 7-ethoxyresorufin O-deethylase (EROD), reproducibility of AOX results is generally achieved in digestive gland of bivalves (Orbea and Cajaraville 2006). AChE was used to dismiss toxicity by additional chemicals that may be present in sediment treatments. Additional biomarkers such as EROD were not considered as CYP induction is suggested to be AHR independent in marine invertebrates (Rewitz et al. 2006).

Biomarker responses were determined in *A. borealis*, upon exposure to PAH-contaminated sediment under laboratory conditions. In a second experiment, selected pyrene metabolites (1-hydroxypyrene [OHPyr, Phase I metabolite, Figure 1] and pyrene-1-glucuronide [GluPyr, Phase II metabolite, Figure 1]) were analyzed in Arctic (*M. calcarea*, *N. ciliata*) and temperate (*L. balthica*, *A. virens*) bivalves and polychaetes exposed to PAH-contaminated sediment under laboratory conditions.

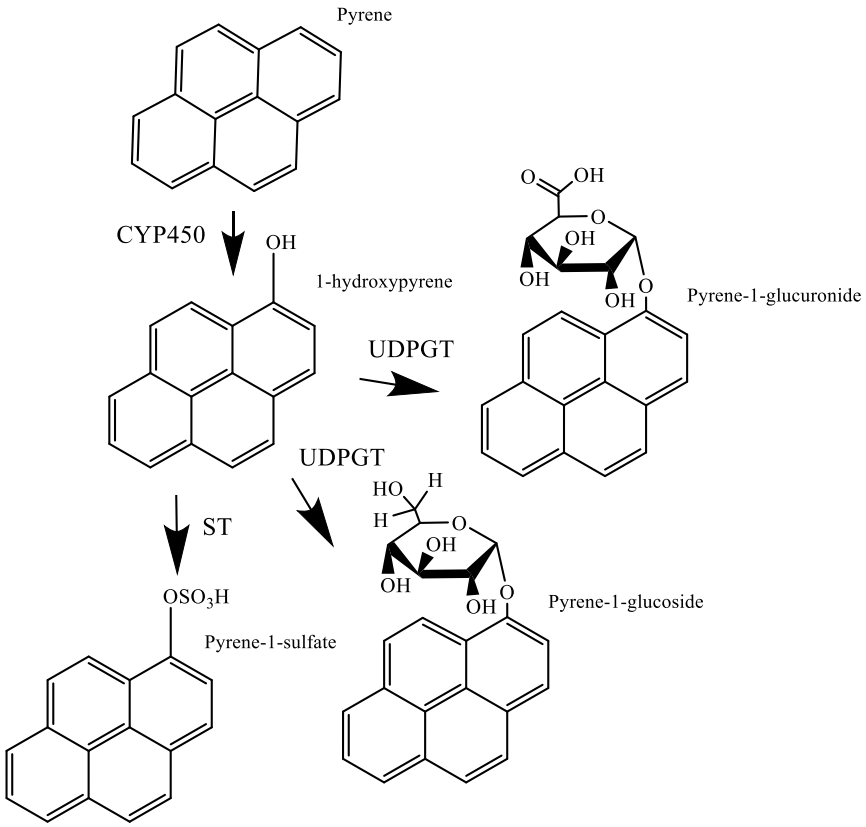

Figure 1. Biotransformation pathway for pyrene in *Alitta virens* (adapted from Jørgensen et al. [2005]). CYP450: cytochrome P450 enzymes; ST: sulfotransferase enzymes; UDPGT: glucuronosyltransferase enzymes. CYP450 forms Phase I metabolites, whereas ST and UDPGT form Phase II metabolites.

Due to limited availability of test animals it was not feasible to analyze biochemical biomarkers and metabolite profiles in the same experiments. In the first experiment, an Arctic species was selected for which ample information was available on biomarker responses in similar temperate species. As for metabolite profiles, much less information is available, so we conducted a broader experimental approach, comparing species with different feeding modes and geographical origin.

2. Material and Methods

2.1. Sediment treatments preparation

Two lots of muddy and coarse sediment were collected in the Oosterschelde estuary (The Netherlands, 51°36'13"N, 3°47'49"E) on 24 to 25 April 2014 and 2 May 2014, and a third lot

of naturally contaminated sediment was collected near Nesserdijk (Rotterdam, the Netherlands, 51°54'14"N, 4°31'17"E) by Nautisch Service Centrum in April 2014. From the first two lots of reference sediment, two different batches were prepared to ensure an optimal habitat for the test species. Since the bivalve species (*A. borealis*, *M. calcarea* and *L. balthica*) usually reside in sandy sediment, their sediment batch consisted of two thirds of coarse and one third of muddy sediment, based on volume. *N. ciliata* and *A. virens* are generally found in sandy mud, so a sediment batch consisting of two thirds of muddy and one third of coarse sediment was prepared for these species.

These two batches of reference sediment containing different proportions of muddy/coarse sediment are referred to as the 'low' (L) treatment: BSL (Bivalve Sediment Low for bivalves) and PSL (Polychaete Sediment Low for polychaetes). An additional sediment treatment was prepared with a higher amount of harbor sediment increasing the chemical concentrations, referred to as 'high' (BSH and PSH with 10% harbor sediment) treatment. These preparations thus resulted in four sediment treatments: two for bivalves (BSL and BSH) and two for polychaetes (PSL and PSH) (Figure S1 and Tables S4-S5).

All sediment treatments were thoroughly mixed before storage and transport from the Netherlands to Svalbard, before use in the exposure experiments. Due to logistics, storage time (at 3 – 7 °C) for sediment treatments was 8 and 17 weeks in the temperate and Arctic experiment, respectively. Prior to the start of exposure, sediment was allowed to settle in contact with filtered seawater in a 1:6 sediment-to-water volume ratio without aeration during 3 d and with aeration during the following 4 d. In the Arctic experiment some aquaria were aerated for 7 to 14 d because biota field sampling took longer than initially expected.

Σ_{13}PAH concentrations in the high (H) treatment were selected to be above or within average sediment Σ_{16}PAH concentration detected in Arctic oil impacted areas like Ny-Ålesund

harbour (Svalbard, Norway) (van den Heuvel-Greve et al. 2016), and the Gulf of Alaska (USA) after 4 and 13 years of the *Exxon Valdez* oil spill (Payne et al. 2008) (Table 1). PAHs were measured in sediment and biota samples (Tables S4-S7) according to methods used by Kupryianchyk et al. (2011). Briefly, samples were Soxhlet-extracted with hexane/acetone. Extracts were cleaned over Al_2O_3 and analyzed using a HPLC-UV. PAH recoveries were 85-94% and 64-75% for biota and sediment samples, respectively. Three blanks per each batch of 30 samples were used, and values were corrected for blanks.

Table 1. Total polycyclic aromatic hydrocarbon (PAHs) concentrations (min-max; µg/kg dry wt.) in sediment and biota (soft tissue) available in the literature.

			Total PAH	
Present study[a]		Analytes (# of compounds)	Sediment	Biota
Treatment	BSL	3- to 6-ring PAHs (13)	219 – 322	n.a.
	BSH		839 – 2780	446 – 1878
	PSL		1130 – 1489	249 – 264
	PSH		2125 – 2437	147 – 1183
Payne et al. (2008)[b]				
1993	Gold Creek	2- to 6-ring PAHs and selected alkylated homologues (44)	~50	~600
	Alyeska Marine Terminal		20 – 300	300 – 400
	Disk Island		1830[c]	~200
Payne et al. (2008)[b]				
1993-2002	Gold Creek	2- to 6-ring PAHs and selected alkylated homologues (44)	40 – 125	100 – 800
van den Heuvel-Greve et al. (2016)				
2012-2013	Kongsfjorden	3- to 6-ring PAHs (16)	1 – 26	n.a.
	Ny-Ålesund		476 – 2550	n.a.

[a]Sediment and *A. borealis* samples from the Arctic experiment.
[b]Sediment and *Mytilus trossulus* samples.
[c]Sediment samples from Boehm et al. (1996).
BSL: Low Bivalve Sediment; BSH: High Bivalve Sediment; PSL: Low Polychaete Sediment; PSH: High Polychaete Sediment; n.a.: not available.

2.2. Test organisms

Based on their feeding habits, sessility and relative abundance in Kongsfjorden Bay (Svalbard, Norway), three Arctic benthic species were selected (*A. borealis*, *M. calcarea* and *N. ciliata*) (Szczybelski et al. *in prep*.). Two counterpart temperate species (the suspension/deposit feeder *L. balthica* and the deposit feeder/omnivore *A. virens*) were selected based on their comparable habitat and feeding traits with Arctic species. Permission

for sampling of Arctic and temperate (*L. balthica*) species was issued by The Governor of Svalbard and the Province of Zeeland, respectively. *A. virens* was obtained from a professional bait farm, Topsy baits (Wilhelminadorp, the Netherlands). For details of the sampling and acclimatization of test species and sediment collection, the reader is referred to the Supporting Information (SI).

2.3. Experimental design

Two 28-d exposure experiments were conducted: one with Arctic species and conditions at Kings Bay Marine Laboratory (Ny-Ålesund, Svalbard) (September – October 2014); and another with temperate species and conditions at Wageningen Marine Research (Yerseke, the Netherlands) (July – August 2014). The Arctic experiment was performed in a temperature controlled room (3±1 °C) with a photoperiod of 12 h light: 12 h dark, whereas the temperate experiment was performed in a temperature controlled room (18±1 °C) with a photoperiod of 18 h light: 6 h dark.

Exposure treatments were prepared in quadruplicate or more for *A. borealis* (BSL and BSH), whereas for the other test species, these were prepared at least in duplicate. For *A. borealis*, each aquarium contained 25 individuals of which 18 individuals were classified as 'small' (< 2 cm) and 7 individuals were classified as 'large' (2 – 4 cm), based on shell length. For *M. calcarea*, *L. balthica*, *N. ciliata* and *A. virens*, each aquarium contained 40 to 50, 50, 20 and 16 individuals, respectively (Table 2). Individuals were not fed during exposure.

Table 2. Overview of the number of experimental aquaria per species and sediment treatment.

			Treatment (n)[b]			
Experiment	Species	Ind./aq.[a]	BSL	BSH	PSL	PSH
Arctic	*Macoma calcarea*	40 – 50	2	2		
	Astarte borealis	25	4	6		
	Nephtys ciliata	20			2	3
Temperate	*Limecola balthica*	50	2	3		
	Alitta virens	16			3	4

[a]Ind./aq.: number of organisms per aquarium.
[b]n: number of replicates per sediment treatment.
n.a.: not available. Additional abbreviations are defined in Table 1.

No mortality was observed for any species in any treatment. The experiment included bioaccumulation analyses as well, which were not addressed by this paper. In order to eliminate any likely sediment particles from biota samples that could interfere with bioaccumulation analyses, organisms were allowed to depurate their guts for 24 h in filtered seawater at the end of the experiments. Animals were weighed, measured for body or shell length, dissected (e.g., bivalves), snap-frozen and stored at -80 °C for the determination of enzymatic activities in *A. borealis* samples or at -20 °C for the analysis of pyrene and pyrene metabolites in Arctic (*M. calcarea*, *N. ciliata*) and temperate (*L. balthica*, *A. virens*) species.

A. borealis samples were shipped in a dry-shipper in liquid nitrogen (Arctic Express 20, Thermo Fisher Scientific) to Plentzia Marine Station (University of the Basque Country) at Plentzia (Biscay, Spain), while the remaining samples were shipped in dry-ice to the Department of Analytical Chemistry (University of the Basque Country) at Leioa (Biscay, Spain).

2.4. Chemicals

2′,7′-dichlorofluorescin diacetate (DCF), N,N-dimethylformamide (DMF), palmitoyl coenzyme A lithium salt, peroxidase from horseradish, sodium azide, Triton™ X-100 and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-N_2) (Sigma-Aldrich) were used for the determination of AOX activity. Butylated hydroxytoluene (BHT),

acetylthiocholine iodide (ATC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich) and sodium bicarbonate (VWR) were used for the determination of AChE activity. 1-chloro-2,4-dinitrobenzene (CDNB) and L-glutathione reduced (GSH) (Sigma-Aldrich) were used for the determination of GST activity. Quick Start™ Bradford Kit 4 (Bio-Rad) was used for protein determination.

Pyrene (98%), 1-hydroxypyrene (98%) (Sigma-Aldrich), pyrenyl-1-O-glucuronide (98.2%) (Isostandards Material S.L., Madrid, Spain), acetone, methanol, acetonitrile (HPLC grade) (Macrom Fine Chemicals), 0.45 µm polyamide filters (Macherey-Nagel) and Oasis HLB (200 mg) cartridges (Waters) were used for the determination of pyrene metabolites.

2.5. Enzymatic activities

Pooled digestive glands of either 9 'small' or 3 'large' *A. borealis* individuals were homogenized in 1:5 volumes of 100 mM potassium phosphate buffer (pH 7.4) in a tissue homogenizer (Precellys®24, Bertin Technologies) at 6,000 *rpm* for 30 seconds (5 °C). Homogenates for AOX determination were obtained after centrifugating the homogenate at 500 *g* for 15 min (4 °C) (Allegra® 25R Benchtop Centrifuge, Beckman Coulter). 100 µL of supernatant was used for AOX determination, whereas the remaining volume was centrifuged at 13,280 *g* for 20 min (4 °C) (Microfuge® 22R Microcentrifuge, Beckman Coulter) to obtain the post-mitochondrial fraction (S12) in the supernatant for further biochemical determinations of AChE and GST activities. Homogenates were stored at -80 ºC if not directly analyzed. All assays were carried out in quadruplicate per homogenate at 22 °C (AChE, GST) or in duplicate at 25 °C (AOX).

AOX activity was analyzed in *A. borealis* 1:4 500 *g* homogenates following Small et al. (1985). AChE activity was analyzed in *A. borealis* S12 homogenates following Guilhermino et al. (1996). GST activity was analyzed in S12 homogenates following Habig et al. (1974)

with some modifications (i.e., reaction medium is 100 mM potassium phosphate buffer pH 7.4). AChE and GST activities were recorded in 96-well Sterilin™ Clear Microtiter™ plates (Thermo Fisher Scientific) at 412 and 340 nm every 60 s during 5 and 3 min, respectively (PowerWave HT Microplate Spectrophotometer, BioTek). Total cytosolic protein was measured in the same homogenate fraction where enzymatic activities were analyzed following Lowry et al. (1951).

2.6. Pyrene metabolites

Samples were frozen and freeze-dried prior to the extraction step. Each sample was weighed and extracted in 10 mL of acetone in a 40 mL polypropylene vessel. Focused ultrasound solid-liquid extraction (FUSLE) was performed in the pulsed mode for 2 min, with a pulsed time 'on' of 0.8 s and pulsed time 'off' of 0.2 s, at 20% of irradiation power (Zabaleta et al. 2015). During FUSLE ultrasound waves coming from the micro-tip made of titanium cause cavitation bubbles that implode in the solvent and sample, resulting in a highly efficient extraction. This procedure allows using little amounts of organic solvents and short extraction times. Extractions were performed at 0 °C in an ice-water bath (Errekatxo et al. 2008). After the extraction step, the supernatant was filtered through 0.45 μm poliamide filters and evaporated to 500 μL under a nitrogen stream at 35 °C using a Turbovap LV evaporator (Zymark).

200-mg Oasis HLB cartridges were conditioned with 10 mL of Milli-Q: acetonitrile (1:9, v/v). Then, the sample was loaded and 1 mL of Milli-Q water was added and cartridges were dried for an hour under vacuum. The analytes were eluted using 10 mL of acetonitrile and collected in a test tube. The eluate was evaporated until dryness under a gentle stream of nitrogen at 35 °C and reconstituted in 150 μL of methanol. Finally, the analyses of pyrene metabolites were performed by high-performance liquid chromatograph with fluorescence detector (HPLC-

FLD). QC standard solutions and solvent blanks were measured every 10 samples, and values were corrected for recoveries. For further details on the extraction, clean-up and instrumental analysis see the SI.

2.7. Data analyses

Data were checked for normality with Q-Q plots and the Shapiro-Wilk test and for equality of variances with Levene's test. Differences among treatment groups were assessed either by an independent *t* test in case of normally distributed data, or by the Mann-Whitney *U* test non-parametric rank test for each of the biomarkers in *A. borealis*. For each taxonomic group (i.e., bivalves and polychaetes), differences in the content of GluPyr among L and H treatment groups and among climatic groups were analyzed by either ANCOVA, or multiple linear regression, respectively. For the ANCOVA test the content of pyrene (Pyr) was used as the covariate and for multiple linear regression the treatment group and source region of the species were used as predictors. A simple linear regression between Pyr and GluPyr concentration was also calculated for each of the species. The simple linear regression was used to analyze any likely difference in GluPyr/Pyr ratios among species. Significance level of an overall statistical comparison was set at $p \leq 0.05$, while for pairwise comparisons a Bonferroni correction was applied. All statistical calculations were performed using SPSS version 22.

3. Results and Discussion

3.1. Enzymatic activities

Exposure to the selected Σ_{13}PAH concentration range (i.e., 287 – 1710 µg/kg dry weight sediment; Table S4) had no significant effect on any biomarker response in *A. borealis* digestive gland (Tables 3, S10-S12). Nevertheless, AChE activity in BSH exposed *A. borealis*

was relatively low and within the range as detected in mussels from either historically polluted areas (Leiniö and Lehtonen 2005) or transplanted to harbor areas (Vidal-Liñán et al. 2014), indicating a potential neurotoxic response. Examples of classical AChE inhibitors are organophosphate and carbamate pesticides, although PAHs may also cause AChE inhibition (Leiniö and Lehtonen 2005; Froment et al. 2016). However, low AChE activity could also be due to either a low AChE substrate specificity in digestive gland with respect to gills as observed in scallops (Bonacci et al. 2009) or a stressed physiological status in *A. borealis* as observed in caged mussels (Lehtonen et al. 2016).

Table 3. Acyl-CoA oxidase, acetylcholinesterase and glutathione S-transferase activities (mean ± SD) in *Astarte borealis* digestive gland.

Treatment	EA (*n*)	AOX	AChE	GST
BSL	4	2.1 ± 0.4	27.9 ± 8.4	49.8 ± 13.4
BSH	6	2.3 ± 0.6	30.7 ± 14.4	46.7 ± 11.2

EA: experimental aquarium; AOX: Acyl-CoA oxidase (mU AOX/mg prot.); AChE: Acetylcholinesterase (nmol/min/mg prot.); GST: Glutathione S-transferase (nmol/min/mg prot.). Additional abbreviations are defined in Table 1.

The absence of AChE inhibition in *A. borealis* was in line with the lack of GST and AOX induction (Table 3). GST activity in *A. borealis* was below baseline levels as detected in digestive gland of scallops (*Chlamys islandica*) (Baussant et al. 2009; Nahrgang et al. 2013) and slightly lower than those in mussels (*Mytilus galloprovincialis*) experimentally exposed to similar PAH concentrations (Giuliani et al. 2013) (Table 4). GST activity is mainly correlated to the concentration of 5- to 6-ring PAHs in mussels (Gowland et al. 2002). Yet, higher concentrations of high-molecular-weight PAHs in BSH exposed *A. borealis* compared to sediment PAH exposed *M. galloprovincialis* yielded lower GST activity values in *A. borealis* than in mussels (Giuliani et al. 2013). Furthermore, a negative correlation was observed between GST activity rates in *M. galloprovincialis* and sediment PAH exposure (Table 4; [Giuliani et al. 2013]). This indicates that even if lower-molecular-weight PAHs such as phenanthrene, anthracene, fluoranthene and pyrene were mainly accumulated by *A. borealis*

during BSH exposure (Szczybelski et al., *under revision*), pointing to an absence of GST induction, likely confounding factors such as the nutritional and reproductive state of *A. borealis* could have masked the bivalves' response to organic pollution (González-Fernández et al. 2017).

Table 4. Total polycyclic aromatic hydrocarbon (PAH) concentrations in whole body soft tissue and glutathione S-transferase activity in digestive gland of exposed bivalve species.

Source	Species (type of exposure)	Total PAH		GST
		Analytes (# of compounds)	Whole body soft tissue (weight units)	
Present study	*Astarte borealis*[a] (sediment)	3- to 6-ring PAHs (13)	170 (wet wt.) 34830 (lipid wt.)	34 – 62
Baussant et al. (2009)	*Chlamys islandica* (dispersed oil)	2- to 6-ring PAHs (16)	0 (wet wt.) 6 (wet wt.) 8 (wet wt.)	475 355 425
	Mytilus edulis (dispersed oil)		0 (wet wt.) 3 (wet wt.) 10 (wet wt.)	71 62 85
Nahrgang et al. (2013)	*Chlamys islandica*[b] (bakground)	2- to 6-ring PAHs (16)	<5 – 5.7 (wet wt.)	500 – 800
	Mytilus edulis[b] (background)		5.7 – 6.3 (wet wt.)	0 – 100
Giuliani et al. (2013)	*Mytilus galloprovincialis* (sediment)	2- to 6-ring PAHs (15)	140 (dry wt.) 300 (dry wt.) 600 (dry wt.)	120 110 90

[a]*A. borealis* exposed to BSH treatment.
[b]Biota samples collected from the field in September 2010.
Whole body soft tissue (µg/kg); GST: Glutathione S-transferase (nmol/min/mg prot.).

AOX activity in all *A. borealis* pooled samples was above levels as detected in digestive glands of mussels from or transplanted to harbour areas (Orbea and Cajaraville 2006) and at O&G produced water discharge points (Brooks et al. 2012). This indicates that AOX levels in the present study were high, even in the controls (i.e., BSL exposed *A. borealis*). Relatively high AOX activity in *A. borealis* may be related to non-toxic factors like low food availability or changing metabolic status under post bloom conditions (Renaud et al. 2011). Although AOX induction has been described as a rapid and reversible response to PAH and PCB exposure (Cajaraville et al. 2003; Orbea and Cajaraville 2006), this process can also be affected by seasonal changes (Cajaraville et al. 2003). AOX activity in mussels (*M. galloprovincialis*) is usually induced during late winter and spring, while AOX levels are

negatively correlated with lipid content in digestive tubules of mussels during phytoplankton blooms (Cancio et al. 1999). AOX may be further induced in *A. borealis* due to the increased synthesis of prostaglandins during the species spawning in early autumn (Von Oertzen 1972; Cancio et al. 1999). Hence, nutritional and reproductive status impacts on peroxisome proliferation should be considered as likely confounding factors when interpreting AOX as a biomarker for exposure to environmental contaminants (Cajaraville et al. 2003).

In summary, we observed a general lack of dose related responses of the selected biomarkers in digestive glands of *A. borealis*. This may indicate that exposure levels were too low to induce such changes. However, within treatment variation in catalytic activity was rather large, which prevented a sensitive analysis of dose response relationships, but for which knowledge of possible confounding factors was lacking. Therefore, in order to interpret variation in biomarker responses in Arctic marine invertebrates, relevant confounding parameters should be identified such as time scales for enzymatic induction, conditions of the assays, and maturation and nutritional status for field individuals.

3.2. Pyrene metabolites

Phase I biotransformation of PAHs is mostly mediated by CYP1A enzymes in vertebrates, whereas other CYP enzymes than the CYP1 subfamily seem to be involved in PAH biotransformation in marine invertebrates (Livingstone 1993). Only a few studies showed the existence of the aryl hydrocarbon receptor (AHR) transcription factor in marine invertebrates and suggested that the expression of Phase I enzymes through AHR in response to hydrocarbon exposure might be tissue-specific in bivalves (Skarphéðinsdóttir et al. 2003; Jenny et al. 2016). Additionally, relatively large differences in dominating Phase II biotransformation pathways between invertebrates could yield different PAH metabolite patterns. In the present study, GluPyr was selected as the major Phase II metabolite, because

glucuronosyltransferases (UDPGTs) have a higher activity rate than sulfotransferases (STs), moreover, glucuronidation is the main conjugation pathway in *A. virens* (Figure 1). Consequently, this selection presents some limitations for an interspecies comparison since UDPGTs and STs may show different inter- and intraspecies activation patterns during exposure time (Guo et al. 2017). .

3.2.1. Pyrene hydroxylation

OHPyr concentrations were often below the limit of detection among all analyzed individuals (Tables 5, S8-S9). This agrees with typically low OHPyr/Pyr ratios measured in other freshwater (Carrasco Navarro et al. 2011) and marine worms (Christensen et al. 2002; Jørgensen et al. 2005), as well as in marine clams (Simpson et al. 2002). This is likely due to generally high Phase II biotransformation levels and/or low induction capacity of Phase I biotransformation pathways in polychaetes and bivalves, respectively. Based on the species-specific sediment threshold for *A. virens* (i.e., 10 µg pyrene/g dry weight [Jørgensen et al. 2005]), an absence of CYP induction and therefore low OHPyr concentrations, was expected in *A. virens* exposed to PSH (i.e., 0.5 µg pyrene/g dry weight). This was also expected for *L. balthica*, since its PAH biotransformation capacity is generally lower than that of *A. virens* (Rust et al. 2004). However, sediment treatments in our study contained 5-10% of naturally contaminated sediment in which different inducers (e.g., PCBs; Table S3) were present, which means both Phase I and Phase II biotransformation activities were probably not restricted in polychaetes (Jørgensen et al. 2008).

Table 5. Pyrene, 1-hydroxypyrene and pyrene-1-glucuronide concentrations (geomean [min-max]; ng/g wet wt.) in Arctic (*Macoma calcarea*, *Nephtys ciliata*) and temperate species (*Limecola balthica*, *Alitta virens*).

Species	Treatment	Pyr	OHPyr	GluPyr
Limecola balthica	BSL	11.6 (<LOQ – 36.4)	0.4 (<LOD – 2.98)	17.4 (<LOD – 74.0)
	BSH	11.8 (<LOQ – 26.7)	0.4 (<LOD – 2.22)	12.1 (<LOQ – 50.7)
Macoma calcarea	BSL	3.6 (0.7 – 12.3)	0.5 (<LOD – 1.5)	7.8 (<LOD – 34.7)
	BSH	5.8 (1.1 – 21.3)	0.6 (<LOD – 3.1)	7.1 (<LOQ – 41.6)
Alitta virens	PSL	1.7 (0.5 – 5.4)	0.1 (<LOD – 1.5)	3.9 (2.2 – 10.0)
	PSH	1.5 (0.5 – 4.3)	<LOD	6.7 (4.5 – 13.7)
Nephtys ciliata	PSL	5.9 (2.0 – 15.5)	0.2 (<LOD – 0.9)	8.2 (4.1 – 20.9)
	PSH	9.2 (3.6 – 28.5)	0.3 (<LOD – 3.7)	11.4 (6.4 – 22.2)

Pyr: Pyrene; OHPyr: 1-hydroxypyrene; GluPyr: pyrene-1-glucuronide; LOD: limit of detection; LOQ: limit of quantification. Additional abbreviations are defined in Table 1.

3.2.2. 1-hydroxypyrene glucuronidation

A significant effect of the high treatment (PSH) compared to the low sediment treatment (PSL) was observed for the concentration of GluPyr in polychaetes (p-value = 0.005, Table S13). In contrast, such difference was not detected between BSH and BSL exposed bivalves (Table S14). Similarly, a multiple linear regression on the concentration of GluPyr did generate a significant model for polychaetes when both treatment and climatic groups were included as predictors ($R^2 = 0.466$, p-value < 0.001, Table S15). This was not the case for bivalves (Table S16).

3.2.3. Biotransformation of pyrene among climatic groups

For polychaetes and bivalves different GluPyr/Pyr ratios were observed when comparing Arctic and temperate species (Figures 2 and 3). GluPyr concentrations in the Arctic *N. ciliata* were on average twice as high as in the temperate *A. virens* in both L and H treatments. This agrees with higher pyrene concentrations in *N. ciliata* (Figure 2) and a positive correlation between pyrene and GluPyr concentrations in this species ($R^2 = 0.252$, $p = 0.012$), which was not the case for *A. virens* (Tables S17-S18). In H exposed *A. virens*, low GluPyr concentrations may be due to pyrene concentrations in *A. virens* that were too low to lead to

significant induction of Phase I (e.g., CYP) and thus, consecutive Phase II (UDPGT) biotransformation (Christensen et al. 2002). However, GluPyr/Pyr ratios in H exposed *A. virens* were on average 3.5 times higher than in *N. ciliata*. This shows that biotransformation rates (estimated as GluPyr/[GluPyr + Pyr]) were limited among polychaete species at the end of experiments (Table 5), although a higher induction of CYP450 enzymes may be expected in the broad omnivore *A. virens* than in *N. ciliata* (Kędra et al. 2012; Jumars et al. 2015) possibly due to a stronger bioactivity of PAHs and additional inducer chemicals, such as PBDEs (Table S3; [Díaz-Jaramillo et al. 2016]).

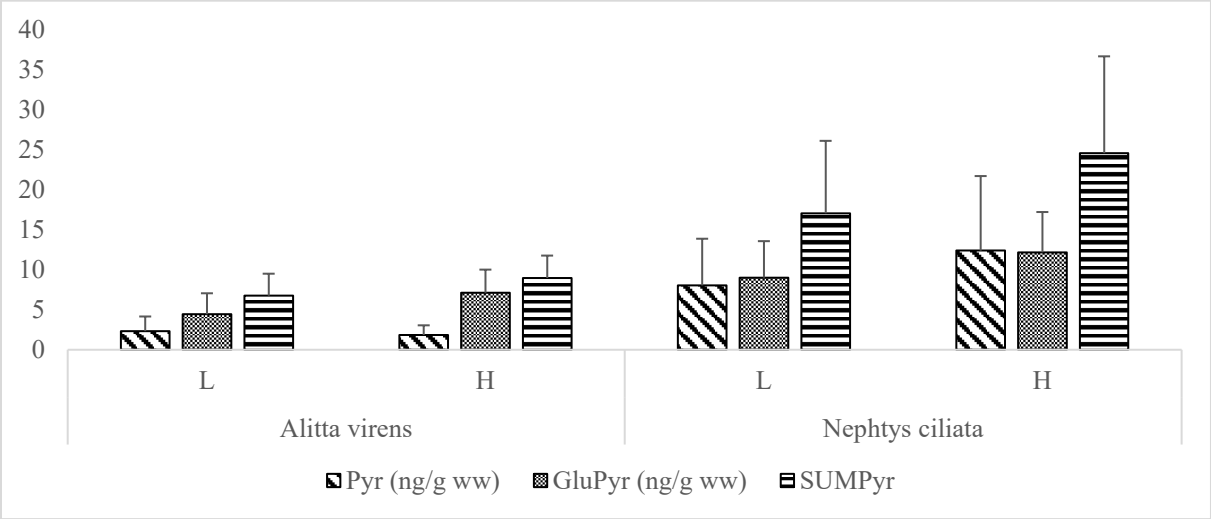

Figure 2. Concentrations of pyrene (Pyr; ng/g wet wt.), pyrene-1-glucuronide (GluPyr; ng/g wet wt.) and the sum of both compounds (SUMPyr) in whole body tissue of temperate (*Alitta virens*) and Arctic (*Nephtys ciliata*) polychaete species exposed to the low (L) and high (H) sediment treatments.

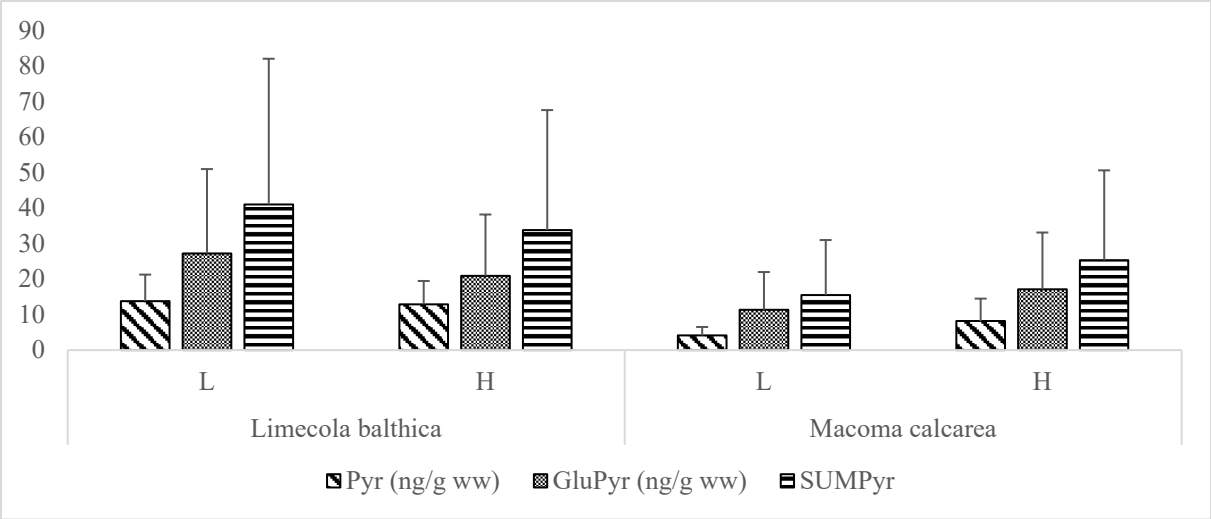

Figure 3. Concentrations of pyrene (Pyr; ng/g wet wt.), pyrene-1-glucuronide (GluPyr; ng/g wet wt.) and the sum of both compounds (SUMPyr) in whole body soft tissue of temperate (*Limecola balthica*) and Arctic (*Macoma calcarea*) bivalve species exposed to the low (L) and high (H) sediment treatments.

Mean GluPyr concentrations in the temperate *L. balthica* were higher than in the Arctic *M. calcarea*. This complies with higher pyrene concentrations in *L. balthica* than in *M. calcarea* (Figure 3). In H exposed bivalves, pyrene concentrations were within the same range as observed concentrations of another 4-ring PAH, chrysene, in scallops (*Chlamys farreri*) after exposure to 0.1 µg/L (i.e., 35-45 ng chrysene/g dry weight [Guo et al. 2017]). Within this range, concentrations of chrysene in scallops did not reveal a significant correlation with ethoxyresorufin-O-deethylase activity, which is the catalytic measurement of CYP450 induction. Absence of CYP induction would explain similar biotransformation rates between *L. balthica* and *M. calcarea* (Figure 3), but it would not account for a seemingly decreasing trend in GluPyr concentration with PAH exposure (Table 5). This stresses the importance of considering different Phase II biotransformation pathways for the characterization and comparison of pyrene metabolite patterns among bivalves (Simpson et al. 2002; Beach et al. 2010).

In summary, pyrene metabolite patterns appeared to be similar among all target species after exposure to different concentrations of PAH-contaminated sediments at the different sediment

treatment levels. However, GluPyr yielded different concentration profiles between Arctic (*N. ciliata*) and temperate polychaetes (*A. virens*) exposed to the high treatment, possibly related to a higher induction of CYP450 enzymes in *A. virens*. The suitability of this metabolite as biomarker of exposure was confirmed for *N. ciliata* according to its positive correlation with pyrene exposure. In bivalves, both temperate (*L. balthica*) and Arctic species (*M. calcarea*) showed similar GluPyr concentration profiles, although lower GluPyr concentrations with PAH exposure pointed towards the activation of alternative Phase II biotransformation pathways in *L. balthica*.

4. Conclusions

No effects of environmentally relevant PAH concentrations in sediment were found on the activities of the biomarkers AChE, GST and AOX in digestive glands of *A. borealis*. Exposure levels were probably too low to induce detectable biomarker responses in *A. borealis* digestive glands, while relatively low AChE levels and high baseline AOX levels potentially indicated a stressed physiological status of *A. borealis*. A further identification of crucial confounding parameters in both enzymatic induction and inhibition, as well as optimizing the biomarker assays for Arctic species is therefore needed for an appropriate evaluation.

In Arctic and temperate benthic invertebrates similarly exposed to PAHs, the concentrations of Phase I and Phase II biotransformation metabolites of pyrene did not increase with pyrene exposure. Biotransformation rates appeared to be limited by low exposure to sediment-bound chemicals among bivalve and polychaete species, although glucuronide conjugates represented up to 74% and 79% of the total pyrene concentrations in Arctic bivalves and temperate polychaetes, respectively. This shows that in order to characterize and quantify

PAH exposure among invertebrate species, the metabolic products of Phase I and Phase II biotransformation should be equally considered.

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